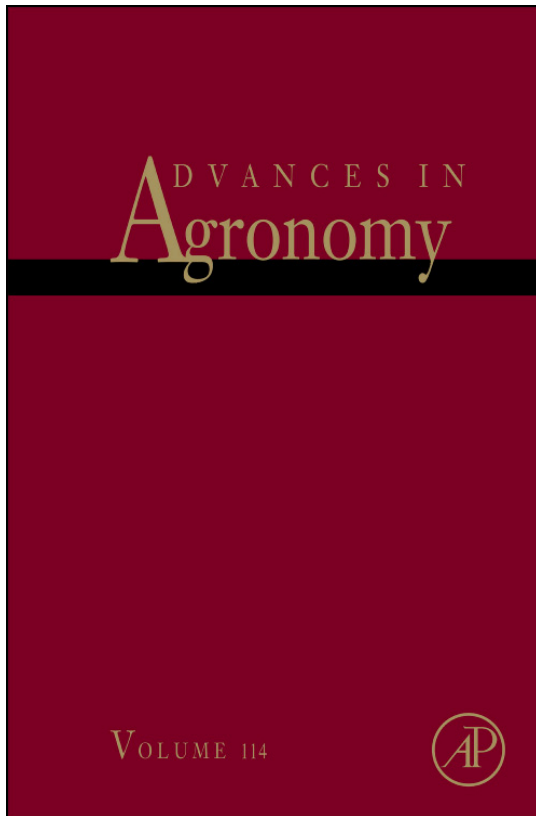


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BIOLOGICAL NITRIFICATION INHIBITION—A NOVEL STRATEGY TO REGULATE NITRIFICATION IN AGRICULTURAL SYSTEMS

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Abstract

Human activity has had the single largest influence on the global nitrogen (N) cycle by introducing unprecedented amounts of reactive-N into ecosystems. A major portion of this reactive-N, applied as fertilizer to crops, leaks into the environment with cascading negative effects on ecosystem functions and contributes to global warming. Natural ecosystems use multiple pathways of the N-cycle to regulate the flow of this element. By contrast, the large amounts of N currently applied in agricultural systems cycle primarily through the nitrification process, a single inefficient route that allows much of the reactive-N to leak into the environment. The fact that present agricultural systems do not channel this reactive-N through alternate pathways is largely due to uncontrolled soil nitrifier activity, creating a rapid nitrifying soil environment. Regulating nitrification is therefore central to any strategy for improving nitrogen-use efficiency. Biological nitrification inhibition (BNI) is an active plant-mediated natural function, where nitrification inhibitors released from plant roots suppress soil-nitrifying activity, thereby forcing N into other pathways. This review illustrates the presence of detection methods for variation in physiological regulation of BNI-function in field crops and pasture grasses and analyzes the potential for its genetic manipulation. We present a conceptual framework utilizing a BNI-platform that integrates diverse crop science disciplines with ecological principles. Sustainable agriculture will require development of production systems that include new crop cultivars capable of controlling nitrification (i.e., high BNI-capacity) and improved agronomic practices to minimize leakage of reactive-N during the N-cycle, a critical requirement for increasing food production while avoiding environmental damage.

1. INTRODUCTION

Agriculture is the single largest human activity altering the global N-cycle (Liu *et al.*, 2010; Smil, 1999). Organic-N mineralization, nitrification, and denitrification are important components of soil-N-cycle (Fig. 1). Plants have the ability to utilize various forms of N: organic-N, ammonium (NH_4^+) -N, and nitrate (NO_3^-) -N (Kielland, 2001; Nasholm *et al.*, 1998; Nishizawa and Mori, 2001; Northup *et al.*, 1995; Salsac *et al.*, 1987; Yamagata *et al.*, 2001). However, NH_4^+ and NO_3^- are the primary N

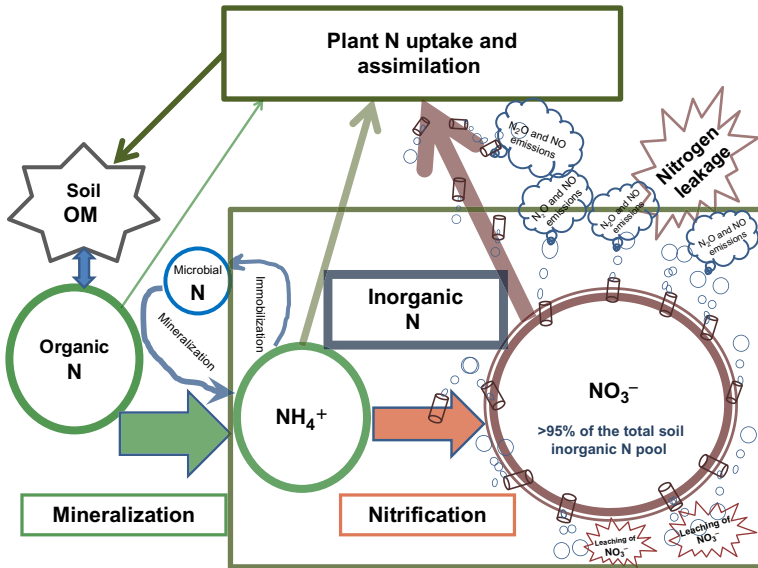


Figure 1 Nitrogen cycle in typical agricultural systems (i.e., neutral upland aerobic soils) dominated by nitrification pathway where >95% of the N flows through and NO_3^- remains the dominant inorganic form absorbed and assimilated.

forms utilized by field crops (Haynes and Goh, 1978). In agricultural systems, nitrification is the dominant pathway for N flow (Fig. 1). This is reflected in typical production systems (i.e., neutral upland aerobic soils) with NO_3^- accounting for >95% of the total N uptake. This makes N-cycle prone to loss to the greater environment of reactive-N, making agricultural systems the greatest source of environmental N pollution (Galloway *et al.*, 2008; Schlesinger, 2009).

The biological oxidation of NH_4^+ to NO_3^- via nitrite is termed “nitrification.” It is carried out primarily by two groups of chemo-lithotrophic bacteria, ammonia-oxidizing bacteria (AOB) (*Nitrosomonas* sp. and *Nitrobacter* spp.), which are ubiquitous components of soil microbial population (Norton *et al.*, 2002). In addition, the ammonia-oxidizing archaea (AOA) group of soil bacteria is believed to carry out nitrification as they possess the same ammonia monooxygenase (*AMO*) gene as do *Nitrosomonas* spp. The presence of archaea has been reported in most soils, but their relative contribution to nitrification is unknown or uncertain (Leninger *et al.*, 2006; Taylor *et al.*, 2010). Recent reports suggest that soil pH may be a critical factor controlling the relative abundances of AOA and AOB communities; for example, in some acidic soils AOA, not AOB, is the major contributor to nitrification (Gubry-Rangin *et al.*, 2010). Nitrification and

denitrification are components of the N-cycle critical to the removal of N from organic waste systems (e.g., sewage treatment). However, in agricultural systems, rapid and unchecked nitrification results in inefficient N-use by crops, leading to N-leakage and environmental pollution (Clark, 1962; Schlesinger, 2009; Subbarao *et al.*, 2006a, 2009a). Most plants have the ability to utilize either NH_4^+ or NO_3^- as a N source, and thus are not solely dependent on NO_3^- (Haynes and Goh, 1978). Reducing nitrification rates in agricultural systems will not alter the availability of N to plants, but will retain N in the root zone for an extended period due to the lower mobility of NH_4^+ , providing more time for plants to absorb soil-N, thus reducing the amount of N liable to loss via leaching and denitrification.

1.1. Why regulate nitrification in agricultural systems?

Nearly 90% of the N-fertilizer applied worldwide is in the NH_4^+ form (or is converted into NH_4^+ from urea hydrolysis), which is rapidly oxidized to NO_3^- by soil nitrifier bacteria (Mason, 1992; Sahrawat, 1980a; Strong and Cooper, 1992). Being a cation, NH_4^+ is held electrostatically by the negatively charged clay surfaces and functional groups of soil organic matter (SOM) (Sahrawat, 1989). This binding is sufficiently strong to limit NH_4^+ -N loss by leaching. In contrast, NO_3^- , with its negative charge, does not bind to the soil, and is liable to be leached out of the root zone. Several heterotrophic soil bacteria denitrify NO_3^- [i.e., convert NO_3^- into gaseous N forms: N_2O (a potent greenhouse gas), NO , and N_2] under anaerobic or partially anaerobic conditions. This often coincides with temporary water-logging after heavy rainfall or irrigation, and/or improper drainage of fields (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 can cause serious N pollution (Clark, 1962; Jarvis, 1996). In alkaline soils, NH_4^+ can be lost via volatilization, thus reducing somewhat the advantage of nitrification inhibition (Rodgers, 1983; Sahrawat, 1989).

Rapid conversion of NH_4^+ to NO_3^- in the soil results in the inefficient use of both soil-N and applied N. Soil organic-N is also subject to nitrification, making it liable to N loss by the same pathways as fertilizer-N (Dinnes *et al.*, 2002; Subbarao *et al.*, 2006a, 2009a,b). Nitrification is the single most important process in the N-cycle that leads to N losses (Barker and Mills, 1980; Clark, 1962) (Fig. 1). In addition, the assimilation of NO_3^- by plants requires more metabolic energy than is required for the assimilation of NH_4^+ (20 mol of ATP per mole of NO_3^- vs. 5 mol of ATP per mole of NH_4^+) (Salsac *et al.*, 1987); thus NH_4^+ assimilation is energetically more efficient than NO_3^- for plants. In addition, the assimilation of NO_3^- , but not NH_4^+ , results in the direct emission of N_2O from crop canopies, further reducing nitrogen-use efficiency (NUE) (Smart and Bloom, 2001). Consequently, maintaining N in NH_4^+ form is advantageous even after taking

into consideration the potential negative effects on rhizosphere acidification from NH_4^+ uptake and metabolism (caused by H^+ excretion). Relatively better utilization of NH_4^+ also depends on N-preference of plant species or cultivars and their plasticity. Many of these advantages especially enhanced crop yield and quality and improved environmental quality have been demonstrated using various chemical inhibitors of nitrification (Huber *et al.*, 1977; Sahrawat, 1989; Sahrawat and Keeney, 1984; Slangen and Kerkhoff, 1984; Subbarao *et al.*, 2006a; Wolt, 2004).

1.2. Is modern agriculture moving toward high-nitrifying systems?

Nitrification plays a relatively minor role in many natural climax plant communities where only a small portion of the N follows the nitrification pathway. In contrast, nitrification is the major pathway in most agricultural systems (Fig. 1) (Nasholm *et al.*, 1998; Smolander *et al.*, 1998; Subbarao *et al.*, 2006a; Vitousek *et al.*, 1997). Most modern agricultural systems rely primarily on large inputs of external N (through chemical N-fertilizer) to maintain their high productivity, as naturally fixed-N is seldom adequate for optimum productivity (Dinnes *et al.*, 2002). During the 20th century, several changes took place in agricultural management practices that led to high-nitrifying soil environments (Poudel *et al.*, 2002; Rabalais *et al.*, 1996). These include (a) decreased use of diversified crop rotations; (b) separation of crop production from livestock production; (c) increased soil tillage; (d) irrigation and drainage of agricultural fields; and (e) increased use of N-fertilizers.

Alkalization and salinization of soils, often associated with irrigated agriculture in the semi-arid tropics (SAT), can greatly influence soil nitrification potential. For example, there has been an increase in soil pH from 5.5 in the mid-1970s to about 8.5 at present in Alfisols intensively cultivated with full irrigation and fertilization at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) research farm in Patancheru, near Hyderabad, India. This is largely due to the accumulation of salts from the irrigation water because of high evaporative demand in this SAT environment (K.L. Sahrawat, unpublished data). Soil pH greatly influences nitrification; greatest nitrifier activity ($15\text{--}20 \text{ mg N kg}^{-1} \text{ soil day}^{-1}$) is generally at a pH in the range of 8.0–9.0 (Sahrawat, 2008).

Current production systems that depend heavily on industrially produced inorganic N have replaced earlier production systems that relied primarily on legumes and/or animal wastes for their N inputs (Dinnes *et al.*, 2002). The separation of crops from animal production has led to an even greater dependence on mineral N-fertilizers, bypassing agricultural systems for organic matter recycling. This has also resulted in the reduction of SOM levels in croplands worldwide (Bruce *et al.*, 1999; Celik, 2005;

Elliott, 1986; Neff *et al.*, 2002; Ross, 1993; Tiessen *et al.*, 1994; van Wesemael *et al.*, 2010). The heavy dependence on mineral N-fertilizers has contributed to the stimulation of nitrifier activity and the subsequent development of high-nitrifying soil environments (Bellamy *et al.*, 2005; Lal, 2003; McGill *et al.*, 1981; Poudel *et al.*, 2002). In addition, installation of sub-surface drainage systems has further accelerated NO_3^- leaching and denitrification, leading to reduced NUE (Clark, 1962; Dinnes *et al.*, 2002; Ju *et al.*, 2009; Pratt and Adriano, 1973; Sahrawat, 1989).

1.3. Consequences of high-nitrifying systems on the global environment

The Green Revolution, largely based on the application of the industrially fixed-N to semi-dwarf rice and wheat cultivars, doubled global food grain production, but at a large environmental cost (Hungate *et al.*, 2003; Matson *et al.*, 1999; Ross, 1993; Tilman *et al.*, 2001). The rapid and unrestricted nitrification found in these intensive production systems, results in the loss of up to 70% of N-fertilizer inputs (Peterjohn and Schlesinger, 1990; Raun and Johnson, 1999; Vitousek and Howarth, 1991). With the worldwide N-fertilizer application reaching 150 Tg year^{-1} (Galloway *et al.*, 2008) and the cost of urea-N reaching US\$ $0.45 \text{ kg}^{-1} \text{ N}$, the direct annual economic loss is estimated at nearly US\$ 81 billion. Moreover, other costs such as damage to the environment are difficult to quantify in economic terms and have not yet been adequately addressed (Ryden *et al.*, 1984; Schlesinger, 2009; Tilman *et al.*, 2001; Viets, 1975).

Fertilizer-N use is expected to double by 2050 from the $150 \text{ TgN year}^{-1}$ currently used in agricultural systems (Galloway *et al.*, 2008; Schlesinger, 2009). This will further increase N-leakage from agricultural systems, placing an even greater pollution load on the environment (IFA, 2005; Ju *et al.*, 2009; Schlesinger, 2009; Tilman *et al.*, 2001; Vitousek *et al.*, 1997). 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 (Galloway *et al.*, 2008; Schlesinger, 2009; Tilman *et al.*, 2001). Moreover, close links among N-fertilizer usage, increased groundwater NO_3^- levels, and human health and environmental problems (e.g., severe eutrophication) have been shown in several studies (Broadbent and Rauschkolb, 1977; Schlesinger, 2009; Subbarao *et al.*, 2006a; Vitousek *et al.*, 1997). Current estimates indicate that N lost by NO_3^- leaching from agricultural systems could reach $61.5 \text{ TgN year}^{-1}$ by 2050 (Schlesinger, 2009).

Globally, agricultural systems contribute nearly 30% of nitric oxide (NO) and 70% of N_2O emissions to the atmosphere (Bremner and Blackmer, 1978; Hofstra and Bouwman, 2005; Smith *et al.*, 1997); N_2O is a powerful greenhouse gas having a global warming potential 300 times

greater than that of CO₂ (IPCC, 2001; Kroeze, 1994), while the Earth's protective ozone layer is damaged by NO_x that reach the stratosphere (Crutzen and Ehhalt, 1977). Current estimates indicate that nearly 17 TgN year⁻¹ is emitted to the atmosphere as N₂O (Galloway *et al.*, 2008; Schlesinger, 2009). By 2100, the global N₂O emissions are projected to be four times greater than the current emissions, due largely to an increase in the use of N-fertilizers (Burney *et al.*, 2010; Galloway *et al.*, 2008; Hofstra and Bouwman, 2005; Kahrl, *et al.*, 2010).

1.4. Options for regulating nitrification in agricultural systems

Several N-management strategies that utilize rate and/or timing of fertilizer application such as fall versus spring, basal versus split, band versus broadcast, deep versus surface application, point-injection placement of solutions, and foliar applications of urea have been used to enhance the NUE of applied fertilizer. Various strategies have been developed to synchronize fertilizer application with crop N requirements to facilitate rapid uptake and reduce N residence time in soil, thereby limiting losses due to denitrification and/or NO₃⁻ leaching (Dinnes *et al.*, 2002; Newbould, 1989). Often, these agronomic strategies have limitations associated with additional labor costs and other practical difficulties (Dinnes *et al.*, 2002).

1.4.1. Synthetic chemical inhibitors

Nitrification inhibitors (NIs) are compounds that delay the bacterial oxidation of NH₄⁺ by depressing the activities of soil-nitrifying bacteria. In theory, reducing nitrification under conditions, where there is a high risk of N loss by NO₃⁻ leaching or denitrification, should improve NUE (Bremner *et al.*, 1981; Hendrickson *et al.*, 1978; Hughes and Welch, 1970; Ranney, 1978; Rodgers, 1986). Reducing nitrification rates until the primary crop is in its establishment phase would provide plants a better opportunity to absorb the N that remains in the root zone. In addition, rapidly growing crops absorb more water from the soil, which would lower the risk of NO₃⁻ being leached out of the root zone (Dinnes *et al.*, 2002; Liao *et al.*, 2004).

Numerous compounds have been proposed and patented as NIs (Malzer, 1979; McCarty, 1999; Subbarao *et al.*, 2006a). Only a few selected NIs, for example, nitrapyrin, DCD (dicyandiamide), and DMPP (3, 4-dimethyl pyrazole phosphate), have been thoroughly evaluated under field conditions (Di and Cameron, 2002; Goring, 1962; Guthrie and Bomke, 1980; Subbarao *et al.*, 2006a; Weiske *et al.*, 2001; Zerulla *et al.*, 2001). Soil factors, in particular, soil type can have a major influence on the persistence and effectiveness of NIs. For example, certain NIs may persist in the soil for a long time but are not bioactive because they are adsorbed on soil colloids. NIs such as nitrapyrin have high bioactivity for a short period of time but

the inhibitor is rapidly degraded into 6-chloropicolinic acid, which is less effective as a NI. Also, nitrapyrin is lost from the soil through volatilization. Environmental factors supporting adsorption, hydrolysis, and volatilization of NIs may largely modulate their effectiveness in production agriculture. In addition, factors such as soil pH, temperature, and the level of soil-nitrifying activity further limit the effectiveness of synthetic NIs. Synthetic NIs are not widely used in production agriculture due to their inconsistent performance across diverse agro-climatic and soil environments (Gomes and Loynachan, 1984; McCall and Swann, 1978; Subbarao *et al.*, 2006a). Despite a great deal of interest and research effort invested during the past 50 years, only a few compounds have been adopted for practical agriculture in certain niche production systems, such as nitrapyrin application for winter wheat production systems in North America. Moreover, the major challenge for the development of next-generation NIs remains the high costs of development of NIs that are economical and have a stable performance in tropical and sub-tropical production environments (Sahrawat, 1996, 2003; Sahrawat and Keeney, 1985).

1.4.2. Slow and controlled-release nitrogen fertilizers

Slow and controlled-release (SCR) fertilizers are forms of N-fertilizers that extend the time of N availability for plant uptake (Shaviv and Mikkelsen, 1993). The SCR fertilizers slow the release of N into the soil solution by special chemical and physical characteristics. SCR fertilizers are produced by providing a protective coating (water-insoluble, semi-permeable, or impermeable with pores) or encapsulating the conventional soluble fertilizer materials to control water entry and rate of dissolution, therefore, synchronizing nutrient release and N availability with the plant N requirements (Fujita *et al.*, 1992). Due to the slow release of N, the availability of NH_4^+ to the nitrifiers is limited, thus N loss during and following nitrification is reduced. Field evaluations with polyolefin-coated urea (POCU) indicate that N losses associated with nitrification can be substantially reduced with improvement in N recovery by the crop (Shoji and Kanno, 1994). Due to reduction of N loss, the N application rates for POCU are often 40% less than those for normal N-fertilizers (Zvomuya *et al.*, 2003). However, POCU is about four to eight times more expensive than normal urea and hence is not cost-effective, limiting its use to niche areas, such as high-value horticultural and floricultural systems (Detrick, 1996).

1.5. Learning from natural ecosystems to regulate nitrification in agricultural systems

Natural ecosystems have evolved a range of mechanisms allowing multiple pathways for N uptake and conservation (“closing the cycle”) including direct uptake of organic-N by plants, bypassing the mineralization process,

thus minimizing N losses from the system. For example, in certain pine forest systems, polyphenols released from litter form a complex with dissolved organic-N, making it resistant to mineralization. Moreover, direct uptake of N from this polyphenolic organic-N is facilitated through association with certain mycorrhizae, bypassing the mineralization process and several pathways of the N-cycle that are associated with N-leakage, resulting in tighter N-cycling in these pine forest ecosystems (Northup *et al.*, 1995).

Several studies indicate that soil nitrification potential differs among ecosystems. These differences in nitrification potential do not seem to be associated directly with soil-physical or -chemical characteristics (Clark *et al.*, 1960; Hattenschwiler and Vitousek, 2000; Lata *et al.*, 2004; Laverman *et al.*, 2000; Lovett *et al.*, 2004; Montagnini *et al.*, 1989; Northup *et al.*, 1995; Robertson, 1982a,b; Schimel *et al.*, 1998). Often in these cases, NH_4^+ levels exceed NO_3^- concentrations by a factor of 10, indicating that the availability of NH_4^+ is not the limiting factor for nitrification. The influence of vegetation in inhibiting nitrification has long been speculated and suspected, but not directly proven (Christ *et al.*, 2002; Donaldson and Henderson, 1990a,b; Lewis and Likens, 2000; Lovett *et al.*, 2004; Smits *et al.*, 2010a,b; Steltzer and Bowman, 1998). Certain forest trees, such as *Arbutus unedo*, are reported to suppress soil nitrification and N_2O emission, which is hypothesized to be due to the release of gallicocatechin and catechin from the litter (Castaldi *et al.*, 2009). Several researchers have observed a slow rate of nitrification in soils under certain tropical pasture grasses and forests (Christ *et al.*, 2002; Cooper, 1986; Li *et al.*, 2001; Sylvester-Bradley *et al.*, 1988). This led to the hypothesis that plant roots may influence nitrification by releasing certain phytochemicals that affect soil nitrifier activity (Fillery, 2007; Jones *et al.*, 1994; Subbarao *et al.*, 2006a).

It has been suggested that selected mature (e.g., climax stage) grassland ecosystems have the ability to inhibit soil nitrification (Boughey *et al.*, 1964; Lata *et al.*, 1999; Smits *et al.*, 2010a,b). In the natural grasslands dominated by *Andropogon* spp., *Brachiaria humidicola*, and *Hyparrhenia diplandra*, most of the inorganic soil-N is in NH_4^+ form, and this is considered to be an indicator of the ecosystem's maturity (Castaldi *et al.*, 2009; Lata *et al.*, 1999; Lodhi, 1979; Meiklejohn, 1968; Subbarao *et al.*, 2006a; Sylvester-Bradley *et al.*, 1988). There have been several attempts to test the hypothesis of nitrification inhibition, but with little success, due to the lack of a suitable methodology to collect, detect, and quantify the amount and type of inhibitor(s) released from roots (Arslan *et al.*, 2010; Moore and Waid, 1971; Munro, 1966a,b; Purchase, 1974; Rice and Panchoy, 1974; Robinson, 1963).

Unlike most agricultural systems, some natural climax ecosystems are known to retain large amounts of N through its incorporation into the

SOM; but the underlying mechanisms remain poorly understood (Magill *et al.*, 2000). The hypothesis that plants can suppress or stimulate nitrification has been debated for a long time, but with limited evidence from *in situ* studies (Fillery, 2007; Ishikawa *et al.*, 2003; Knops *et al.*, 2002; Lata *et al.*, 1999; 2004; Lovett *et al.*, 2004; Smits *et al.*, 2010a,b; Stienstra *et al.*, 1994; Sylvester-Bradley *et al.*, 1988).

Plant species that dominate some of these climax ecosystems with relatively low nitrification were shown to produce organic compounds that inhibit nitrifier activity (Basaraba, 1964; Courtney *et al.*, 1991; Donaldson and Henderson, 1990a,b; Jordan *et al.*, 1979; Likens *et al.*, 1969). These inhibitory compounds, when added to the soil, suppressed nitrification in the rhizosphere (Jordan *et al.*, 1979). The degree of nitrification inhibition appears to increase with the ecosystem's maturity (Baldwin *et al.*, 1983; Cooper, 1986; Erickson *et al.*, 2000; Howard and Howard, 1991; Lata *et al.*, 1999; Lodhi, 1982; Northup *et al.*, 1995; Paavolainen *et al.*, 1998; Rice and Panchoy, 1972–1974; Schimel *et al.*, 1996; Ste Marie and Pare, 1999; Thibault *et al.*, 1982; White, 1991).

1.5.1. Is there ecological advantage for plants that control nitrification?

Since NO_3^- assimilation by plants requires four times more metabolic energy than that needed for NH_4^+ , it is hypothesized that inhibition of nitrification could be an ecological driving force for the development of low NO_3^- climax ecosystems (Lata *et al.*, 2004; Rice and Panchoy, 1972; Salsac *et al.*, 1987). However, it is difficult to predict what impact such a plant-mediated inhibitory function has on the competitiveness of a species or plant community. Part of the answer lies in the primary productivity measurements made in natural ecosystems deprived of nitrification by plant action; for example, West African savannas known to have very low rates of soil nitrification (Robertson, 1989). These systems have high primary production ($20\text{--}30 \text{ Mg ha}^{-1} \text{ year}^{-1}$) under relatively low soil mineral N and C (Boudsocq *et al.*, 2009; Lata *et al.*, 2004). This kind of ecosystem is dominated by perennial grasses with high longevity (several decades), and often show some of the highest plant productivity ever recorded (Bate, 1981; Menaut and Cesar, 1979).

It can be hypothesized that by controlling nitrification, plants increase the availability of N for their own survival and growth in an environment where N is limiting. From an evolutionary viewpoint, a major question remains regarding the conditions or perturbations that allow BNI-plants to outcompete non-inhibiting plants. The hypothesis would be that the ability to depress nitrification should provide a competitive advantage for N acquisition. Moreover, the success of African grasses in invading the South American and Australian Tropics suggests that BNI-function may provide a competitive advantage in N acquisition to the nitrification inhibiting

populations of grasses, and that this attribute may contribute to their out-competing native grasses without the BNI-function (Barot *et al.*, 2007).

Recent modeling studies (Boudsocq *et al.*, 2011a,b) on the role of BNI in controlling nitrification in temperate and tropical grasslands show that contrasting preferences for NH_4^+ or NO_3^- between two plant species can facilitate their coexistence. The ability of one species to control nitrification (i.e., to stimulate or inhibit) could enhance their ability to compete for mineral N with other species. In particular, a species' ability to inhibit nitrification allows it to outcompete other species without this ability. This is consistent with the results of the studies suggesting that BNI strongly affects plant invasions (Hawkes *et al.*, 2005; Lata *et al.*, 2004; Rossiter-Rachor *et al.*, 2009). The next step is to understand how the control of nitrification plays a key role in the dynamics of plant communities.

2. BIOLOGICAL NITRIFICATION INHIBITION

2.1. The concept of BNI-function and its potential impacts on NUE

BNI is an active plant-mediated rhizosphere process where NIs are exuded/released from plant roots that suppress soil-nitrifying bacteria (Subbarao *et al.*, 2006a,b, 2009a,b). A schematic representation of the BNI-concept with various processes of the soil-N-cycle that are potentially influenced by this plant function is presented in Fig. 2.

Nitrogen-use efficiency ($\text{NUE}_{\text{agronomic}} = \text{yield per unit of applied N}$) is a function of both intrinsic N-use efficiency ($\text{NUE}_{\text{intrinsic}} = \text{dry matter produced per unit of N uptake}$) and total N uptake. $\text{NUE}_{\text{intrinsic}}$ of a plant is a physiologically conserved function (Glass, 2003), thus may not be easy to manipulate genetically. Improvements in $\text{NUE}_{\text{agronomic}}$ mostly come from improvement in crop N uptake (Finzi *et al.*, 2007) or greater recovery of applied N-fertilizer. As discussed earlier, the BNI-function can improve N uptake due to its inhibitory effects on nitrification (Subbarao *et al.*, 2006a).

The results of recent modeling studies indicate that by inhibiting nitrification, N recovery can be improved. A general theoretical ecosystem model that considers both NO_3^- and NH_4^+ as N sources was used to investigate the general conditions under which nitrification inhibition enhances primary productivity and its quantitative impact on N dynamics and utilization. Primary productivity is positively impacted in the tropical savannas dominated by native African grasses such as *H. diplandra* which appear to have a significant ability to suppress nitrification (Boudsocq *et al.*, 2009). For natural and agro-ecosystems, which are subject to high-nitrifying and denitrifying activities, this model predicts that nitrification inhibition by plants is a process that can lead to better N conservation, and thus increase

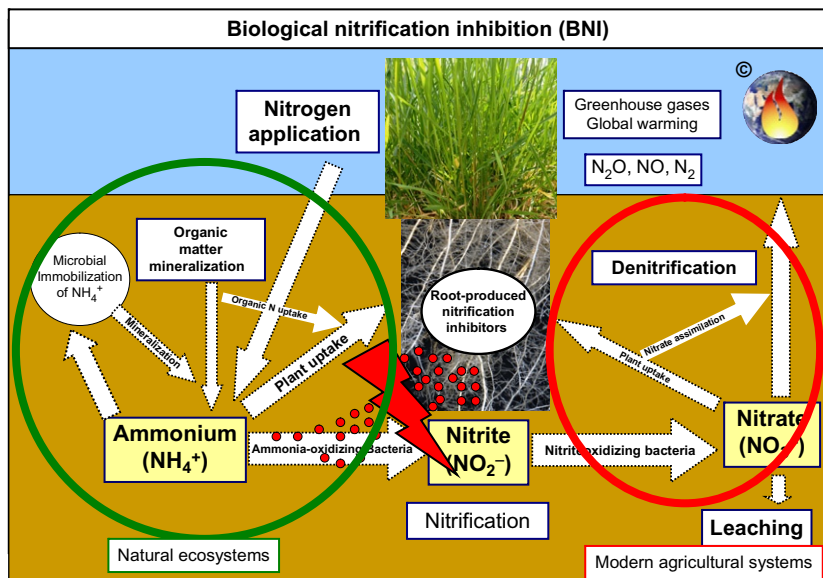


Figure 2 A schematic representation of the biological nitrification inhibition (BNI) interfaces with the nitrogen cycle. The BNI produced by the root inhibits the process that converts ammonium to nitrate. In ecosystems with large amounts of BNI such as *Brachiaria* pastures, the flow of nitrogen from ammonium to nitrate is restricted and ammonium accumulates in soil and root systems. In systems with little or no BNI such as modern agricultural systems, nitrification occurs at a rapid rate and ammonium is converted to nitrate-N, which is highly susceptible to loss from the soil and root systems (source: Subbarao *et al.*, 2009b).

primary productivity as the NH_4^+ pathway is more N efficient (i.e., more conservative) than the NO_3^- pathway. This would be the case if the considered ecosystem is subjected to higher losses under NO_3^- (leaching and denitrification) than under NH_4^+ (volatilization). Moreover, this model supports previous *in situ* measurements in savanna systems (Lata, 1999), which showed that grasses that inhibit nitrification exhibit a twofold greater productivity in above-ground biomass than those that lack this ability (see Section 2.8 for further discussion).

2.2. Methodology for the detection of BNIs in plant–soil systems

Lack of a suitable methodology and experimental system to detect the release of NIs in the rhizosphere, that is, BNI-activity, has been a major hurdle for characterizing the BNI-function in plants (Subbarao *et al.*,

2006a). However, recently a bioluminescence assay that uses a recombinant strain of *Nitrosomonas europaea* has been developed that can detect and quantify BNI-activity released from roots, a plant function termed BNI-capacity (Iizumi *et al.*, 1998; Subbarao *et al.*, 2006b). The recombinant strain of *N. europaea* carries an expression vector for the *Vibrio harveyi luxAB* genes (Fig. 3) and produces a distinct two-peak luminescence pattern during a 30-s analysis period (Subbarao *et al.*, 2006b). The functional relationship between bioluminescence emission and nitrite production in the assay has been shown to be linear using the synthetic NI, allylthiourea (AT) (Subbarao *et al.*, 2006b). The inhibition caused by 0.22 μM AT in assay (about 80% inhibition in bioluminescence and NO_2^- production) is defined as one allylthiourea unit (ATU) (Subbarao *et al.*, 2006b). Using the response to a concentration gradient of AT (i.e., a standard dose–response curve), the inhibitory effects of test samples, for example, root exudates, soil, or plant extracts, are determined and expressed in ATU. These recently developed research tools facilitate the characterization of plant BNI-capacity (Subbarao *et al.*, 2006b).

Determining the BNI-activity release from roots is, however, only the first step toward the characterization of a plant species for BNI-capacity. Moreover, the effectiveness of the released BNI-activity in suppressing soil nitrification needs to be confirmed. There are a number of reasons why inhibitors released from roots (i.e., BNIs; estimated as BNI-activity) may be ineffective in certain soil environments. For example, the variability among soils in indigenous populations of AOB (Matsuba *et al.*, 2003) can make BNI-activity, which is determined using a single strain of *N. europaea* in the bioassay, functionally ineffective in certain soils. In addition, the soil chemical and physical properties could impair the BNI-activity from functioning

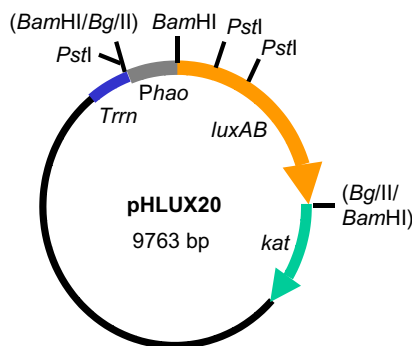


Figure 3 Physical map of recombinant luminous *Nitrosomonas europaea* (pHLUX20) developed to detect and quantify nitrification inhibitors in the plant–soil system (source: Iizumi *et al.*, 1998).

in some agro-ecosystems. Nitrapyrin, for example, is ineffective in some soils due to the formation of biofilms around nitrifier populations, adsorption to the SOM, and chemical hydrolysis (Powell and Prosser, 1991, 1992). Complementary evaluation of the BNI-function using soil-based assays, that is, using soil from the target environment where the crop is to be grown, is thus necessary to assess the BNI-capacity of a particular plant genotype under investigation. Potential soil nitrification can be rapidly and reliably determined by measuring ammonia-oxidizing activity (Belser and Mays, 1980; Berg and Rosswall, 1987; Hansson *et al.*, 1991; Hart *et al.*, 1994; Neufeld and Knowles, 1999) and has been successfully deployed to assess the BNI-capacity of *Brachiaria* sp. and matgrass swards in the field (Smits *et al.*, 2010a,b; Subbarao *et al.*, 2009a).

2.3. Variation in the BNI-capacity of major crops and forage grasses

An evaluation of a range of tropical forage grasses, cereal, and legume crops have indicated a wide range in the BNI-capacity (Table 1) (Subbarao *et al.*, 2007b). The highest BNI-capacity was found in *Brachiaria* spp., and substantial genotypic variation was detected in BNI-capacity within *B. humidicola* (Table 2). Forage grasses of *B. humidicola* and *B. decumbens*, which are highly adapted to the low-N production environments of South American savannas (Miles *et al.*, 2004; Rao *et al.*, 1996), showed the greatest BNI-capacity among the tropical grasses tested (Subbarao *et al.*, 2007b). In contrast, *Panicum maximum*, which is adapted to high N availability environments, showed the least BNI-capacity (Rao *et al.*, 1996; Subbarao *et al.*, 2007b). Among the cereal crops evaluated, only sorghum [*Sorghum bicolor* (L.) Moench] showed significant BNI-capacity. Other cereal crops including rice, maize (*Zea mays* L.), wheat, and barley (*Hordeum vulgare* L.) did not show any detectable BNI-capacity in these initial studies (Subbarao *et al.*, 2007b; Zakir *et al.*, 2008).

Inhibition of nitrification (i.e., BNI-capacity) is most likely part of an adaptation mechanism for the conservation and efficient use of N in natural systems having low-N availability (Lata *et al.*, 2004; Subbarao *et al.*, 2006a). Thus, N stress (i.e., sub-optimum levels to support normal growth) is likely to be a driving force for the evolution of the BNI-function (Lata *et al.*, 2004; Rice and Pancholy, 1972). It is, therefore, not surprising that legumes do not show appreciable BNI-capacity. In the case of legumes, it is likely that BNI-capacity would have little or no adaptive value due to their ability to fix N symbiotically. Conserving N may not offer much of a comparative advantage for legumes to coexist with grasses since grasses tend to have more abundant root systems than legumes (Rao *et al.*, 1995). Our preliminary studies indicate that soybean [*Glycine max* (L.) Merr.] root exudates stimulated nitrification in the laboratory soil incubation tests (Subbarao *et al.*,

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

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

Note: “0” activity indicates that the inhibitory effect is possibly below the detection limit of the assay system used.

Source: Subbarao *et al.* (2007b).

Table 2 Genotypic variation in the BNI-function from roots of seven *Brachiaria humidicola* germplasm accessions and one genotype of *Panicum maximum*

Serial no.	Accession no.	Total BNI released from four plants (ATU day ⁻¹)	Specific BNI (ATU g ⁻¹ root dry wt. day ⁻¹)
1.	CIAT 26159	126.2	46.3
2.	CIAT 26427	118.5	31.6
3.	CIAT 26430	151.0	24.1
4.	CIAT 679	68.8	17.5
5.	CIAT 26438	93.5	6.5
6.	CIAT 26149	22.3	7.1
7.	CIAT 682	53.4	7.5
8.	<i>P. maximum</i>	0.6	0.1
	LSD (0.05)	21.7	6.0

Four plants per pot were grown for 180 days before collecting the root exudates.

Source: Subbarao *et al.* (2007b).

2007c). Several forest systems dominated by leguminous trees (*Acacia mangium* Willd. and *A. auriculiformis* A. Cunn. ex Benth.) are on soils that did not inhibit or even stimulated nitrification. In contrast, forests dominated by non-legume trees such as *Eucalyptus citriodora* Hook., *Pinus elliottii* Engelm., and *Schima superba* Gardner & Champ. showed low-nitrification rates (Li *et al.*, 2001). Recent studies indicate that a wild relative of wheat, *Leymus racemosus* (Lam.) Tzvelev, possesses BNI-capacity similar to that of *Brachiaria* spp., with BNI-activity release rates reaching close to 30 ATU g⁻¹ root dry wt. day⁻¹ (Subbarao *et al.*, 2007c).

2.4. Regulatory nature of BNI-function

The synthesis and release of BNIs is a regulated attribute in *B. humidicola* (Subbarao *et al.*, 2007a). To some extent, the release of BNIs from roots is related to the plant N status (Subbarao *et al.*, 2006b). In addition, the N-form applied (i.e., NH₄⁺ or NO₃⁻) has a major influence on the synthesis and release of BNIs from roots in *B. humidicola* and in wild wheat, *L. racemosus* (Subbarao *et al.*, 2007a,c). Plants grown with NO₃⁻ as their N source did not release BNIs from roots, whereas BNIs were released from plants grown with NH₄⁺ as their N source (Subbarao *et al.*, 2007a,c, 2009a,b). Even for plants grown with NH₄⁺, the presence of NH₄⁺ in the rhizosphere was critical for the synthesis and release of BNIs from their roots (Subbarao *et al.*, 2007a,c). Despite high levels of BNIs detected in the root tissues of NH₄⁺-grown plants, the release of BNIs was observed only when the roots were directly exposed to NH₄⁺ (Subbarao *et al.*, 2007a,c, 2009a,b).

In addition to the presence of NH_4^+ in the medium, the rhizosphere pH may also influence the release of BNIs from roots. Recent results indicated that sorghum plants do not release BNIs from roots in the presence of NH_4^+ , when the rhizosphere pH was 7 or higher. If the pH of the solution used for collecting root exudate (1 mM NH_4Cl) was not controlled and allowed to drop to 4, sorghum released substantial amounts of BNI-activity from roots (about 15 ATU g^{-1} root dry wt. day^{-1}) (G.V. Subbarao, unpublished data). Moreover, it is likely that BNI-function is better expressed in plants when grown on light-textured (e.g., sandy or sandy-loam) soils with a pH 6.0 or lower. Such pH effects on the BNI release from roots are based on solution culture studies and have not yet been evaluated using soil systems.

Further, the release of BNIs from plant roots appears to be a highly regulated physiological function. The presence of NH_4^+ in the root environment is necessary not only for an accelerated synthesis of BNIs and/or precursors of BNI compounds in roots, but also for their release (Subbarao *et al.*, 2007a, 2009a). The physiological consequences associated with the uptake of NH_4^+ , such as activation of H^+ pumps in the plasmalemma and acidification of the rhizosphere, appear to facilitate BNI release from sorghum roots (Zhu *et al.*, 2010). Further, the release of BNIs from roots is a localized phenomenon (Subbarao *et al.*, 2009a). The release of BNIs appears to be confined to only part of the roots exposed to NH_4^+ in the rhizosphere and is not extended to the entire root system. Moreover, such localized release of BNIs from roots ensures high concentrations of BNIs in the soil pockets where nitrifiers are active, which is often associated with the presence of NH_4^+ (Subbarao *et al.*, 2009a). The availability of NH_4^+ in the soil either from soil organic-N mineralization or through the application of N-fertilizers such as urea or ammonium sulfate can 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 adaptation mechanism (Subbarao *et al.*, 2007a).

2.5. Stability of BNIs in soil systems

The BNI-activity released from roots is quantified by an assay of their inhibitory effects on the biological activity of a recombinant luminescent *Nitrosomonas* sp. during a 30-min incubation period (Subbarao *et al.*, 2006b). However, nitrification in soil occurs over a much longer period of time, often taking several weeks for the oxidation of soil- NH_4^+ ; the persistence of the inhibitory compounds released from roots for several weeks may be a requirement to ensure a stable inhibitory effect on soil nitrification. This hypothesis was tested by adding extracted BNI-activity (from root exudates of *B. humificicola*) to soil at different levels (0–20 ATU g^{-1} soil) along with an NH_4^+ source (200 mg N kg^{-1}) and incubating for 55 days at 20 °C.

These studies indicated that for the inhibitory activity to be effective in reducing soil nitrification, 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 (Fig. 4) (Gopalakrishnan *et al.*, 2009; Subbarao *et al.*, 2006b). Further, it was shown that certain BNIs (such as linoleic acid and linolenic acid) partially lose their effectiveness in soil after 80 days, and their inhibitory effect was completely lost after 100 days (Subbarao *et al.*, 2008). In addition, preliminary measurements on mixed tropical savanna soils showed that this inhibitory effect can resist natural air drying and storage in the dark (Lata, 1999).

The effectiveness of various synthetic NIs and natural BNIs can be influenced by several soil and environmental factors (Gopalakrishnan *et al.*, 2009; Sahrawat, 1980b, 1996; Slangen and Kerkhoff, 1984; Subbarao *et al.*, 2006a; Wolt, 2004). The most important among these include: (a) the nature and characteristics of the inhibitory compound (especially its structure, C chain length and whether C is attached to aryl or alkyl moiety, and easily degradable functional groups in the structure), water solubility, mobility, volatility, degradation into biologically active products or not, sorption of inhibitors on soil minerals and organic matter; (b) the soil-physical (clay-content, soil-type, and -texture), -chemical (pH, chemical immobilization, and chemical degradation), and -biological (microbial activity and diversity) properties (Gopalakrishnan *et al.*, 2009; Raynaud,

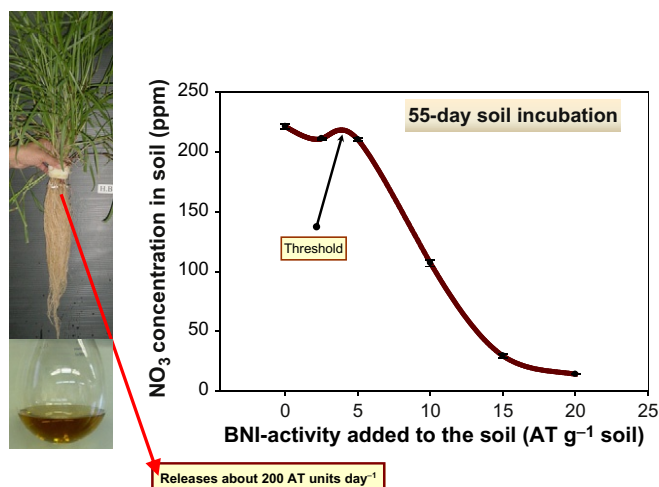


Figure 4 Effectiveness of the BNI-activity released from roots of *Brachiaria humidicola* in inhibiting nitrate formation in the soil (during 55 days of incubation at 20°C) (source: Subbarao *et al.*, 2006b).

2010; Sahrawat, 1980b, 1996; Slangen and Kerkhoff, 1984; Wolt, 2004); and (c) environmental factors, including temperature and soil moisture regimes, can greatly influence the persistence, degradation, and effectiveness of inhibitors. For example, the high temperatures of tropical soils not only increase the rate of soil nitrification (Sahrawat, 2008), but also enhance the degradation of NIs (Sahrawat, 1980b; Slangen and Kerkhoff, 1984). In addition, the soil water regime via redox-modulated effects differentially influences degradation and effectiveness of NIs (Fiedler *et al.*, 2007; Laskowski *et al.*, 1974). There is a paucity of information on the fate and efficacy of BNIs in soil–plant systems. Thus, intensification of research is justified to generate information on the behavior of plant products, especially the 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; Subbarao *et al.*, 2006a; Wolt, 2004).

2.6. Biological molecules with BNI potential and their mode of inhibitory action

Plants are known to release a wide range of substances with biological activity (Bending and Lincoln, 2000; Bremner and McCarty, 1988; Raaijmakers *et al.*, 2009; Subbarao *et al.*, 2006a). These include molecules that belong to phenolic, alkaloid, fatty acid, isothiocyanate, and terpene groups (Bending and Lincoln, 2000; Bennett and Wallsgrove, 1994; Bertin *et al.*, 2003; Choesin and Boerner, 1991; Flores *et al.*, 1999; Gopalakrishnan *et al.*, 2007; Kraus *et al.*, 2003; Langenheim, 1994; Lewis and Papavizas, 1970; Putnam, 1988; Subbarao *et al.*, 2006a, 2008, 2009a,b; Walker *et al.*, 2003; Zakir *et al.*, 2008; Zucker, 1983). The compounds responsible for the BNI-activity were only recently elucidated, despite the fact that the phenomenon was first proposed in the early 1960s, from empirical field studies (for review see Subbarao *et al.*, 2006a). Several BNIs belonging to different chemical groups have been successfully isolated and identified from plant tissue or root exudates using bioassay-guided purification approaches (Fig. 5) (Gopalakrishnan *et al.*, 2007; Subbarao *et al.*, 2006b, 2008, 2009a; Zakir *et al.*, 2008).

The compounds with BNI-activity in the aerial parts of *B. humidicola* are the unsaturated free fatty acids, linoleic acid, and α -linolenic acid (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 NI 1-allyl-2-thiourea is 1×10^{-7} M. However, other free fatty acids having different chain lengths or number of double bonds, for example, stearic, oleic, arachidonic, and *cis*-vaccenic acid, did not show inhibitory activity, indicating the requirement of specific chemical structure to inhibit *Nitrosomonas* sp.

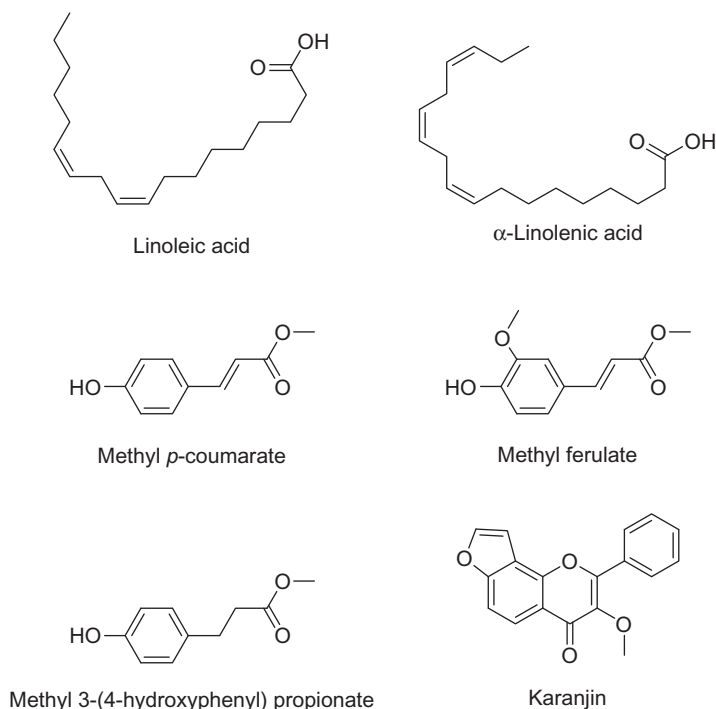


Figure 5 Chemical structures of compounds reported to show BNI-activity in plants.

function (Subbarao *et al.*, 2008). BNI compounds such as linoleic acid and α -linolenic acid apparently possess the structure and chain length needed to inhibit nitrification. These two BNI compounds released from plant tissues possibly inhibit both AMO and hydroxylamine oxidoreductase (HAO) enzymatic pathways, which catalyze essential reactions of the ammonia oxidation process in *Nitrosomonas* sp. (Subbarao *et al.*, 2008). When linoleic acid and α -linolenic acid were added to soil, nitrification rates were suppressed for several months (Subbarao *et al.*, 2008). The BNI-activity from crude extracts of root exudates of *B. humidicola* and *L. racemosus* appears to block both AMO and HAO enzymatic pathways with similar effectiveness (Subbarao *et al.*, 2007a,c). Moreover, the BNIs could also disrupt the electron transfer pathway(s) from HAO to ubiquinone and cytochrome (which needs to be maintained to generate reducing power, i.e., NADPH) that is critical to the metabolic functions of *Nitrosomonas* sp. (Fig. 6); further research is needed to elucidate the mechanisms involved (Subbarao *et al.*, 2009b). In contrast, synthetic NIs such as AT, nitrapyrin, and DCD inhibit nitrification by suppressing only the AMO enzymatic pathway in *Nitrosomonas* (Subbarao *et al.*, 2007a,c) (Fig. 6).

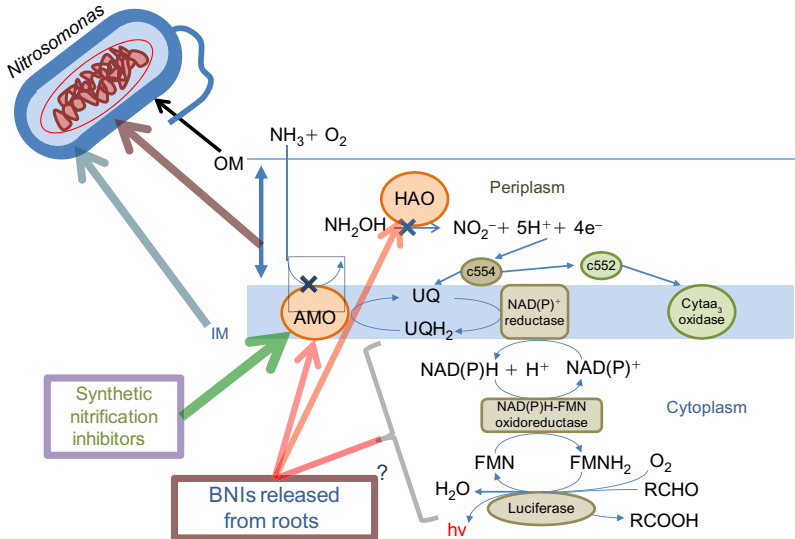


Figure 6 The mechanisms involved in the inhibitory effects of selected synthetic nitrification inhibitors and of the BNIs released from the roots of *Brachiaria humidicola* (based on [Iizumi et al., 1998](#); [Subbarao et al., 2007a](#)).

From root exudates of hydroponically grown sorghum, a phenyl-propanoid, methyl 3-(4-hydroxyphenyl) propionate (MHPP) has been identified as the BNI ([Fig. 5](#)), and this compound partially accounted for the inhibitory activity released from roots ([Zakir et al., 2008](#)). The IC_{50} value for MHPP is approximately 9×10^{-6} M ([Zakir et al., 2008](#)). In root tissues of *B. humidicola*, two phenyl propanoids, methyl-*p*-coumarate and methyl ferulate ([Fig. 5](#)), were identified as major BNIs ([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 of $<1 \times 10^{-2}$ M ([Gopalakrishnan et al., 2007](#)). It is hypothesized that *B. humidicola* releases methyl-*p*-coumarate and methyl ferulate, or simple metabolites derived from these BNI compounds into the soil environment via the turnover of root tissues in pasture systems ([Gopalakrishnan et al., 2007](#)), because these nitrification inhibitory compounds were not detected in root exudates.

Karanjin (3-methoxy furano-2,3,7,8-flavone or 3-methoxy-2-phenyl furo-[2,3-*h*]chromen-4-one) ([Fig. 5](#)) isolated from *Pongamia glabra* Vent. seeds showed a strong inhibitory effect on soil nitrifier activity, and is reported to be as effective as some of the most commonly used synthetic

NIs, nitrapyrin, DCD, and AM (Sahrawat, 1981; Sahrawat and Mukerjee, 1977). By altering the chemical structure of the karanjin molecule, it was shown that the furan ring present in the molecule is critical for the biological activity (i.e., nitrification inhibition); this hypothesis was confirmed with the synthesis of furfuraldehyde and furfuryl alcohol-based compounds that showed varying levels of inhibitory effects on soil nitrification (Sahrawat and Mukerjee, 1977; Sahrawat *et al.*, 1977).

2.6.1. Discovery of brachialactone

The major NI released from roots of *B. humidicola* has been discovered and named “brachialactone,” a cyclic diterpene. 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.*, 2009a) (Fig. 7). Similarly, 5–8–5 tricyclic terpenoids (ophiobolanes and fusicocanes) 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 is novel to the nitrification inhibitory groups. Fusicoccane-type cyclic diterpenes are biologically synthesized from geranylgeranyl diphosphate by a two-step cyclization catalyzed by terpene cyclases (Toyomasu *et al.*, 2007) (Fig. 7).

The inhibition of nitrification in an *in vitro* assay with pure cultures of *N. europaea* was linearly related to the brachialactone concentration in the

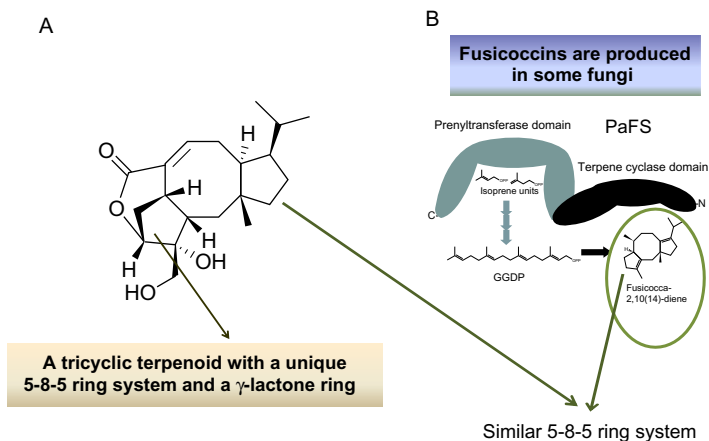


Figure 7 (A) The chemical structure of brachialactone, the major nitrification inhibitor isolated from the root exudates of *Brachiaria humidicola* (source: Subbarao *et al.*, 2009a). (B) Synthesis of fusicocca-2,10(14)-diene from isoprene units by PaFS. This enzyme is a diterpene hydrocarbon synthase possessing both prenyltransferase and terpene cyclase activity (source: Toyomasu *et al.*, 2007).

range of 1.3–13.3 μM (Fig. 8). Moreover, brachialactone with an ED_{80} (effective dose for 80% reduction) of 10.6 μM should be considered a potent NI when compared with nitrapyrin or dicyandiamide, two of the most widely used synthetic NIs (ED_{80} of 5.8 μM for nitrapyrin and 2200 μM for dicyandiamide). Brachialactone inhibits *Nitrosomonas* sp. by

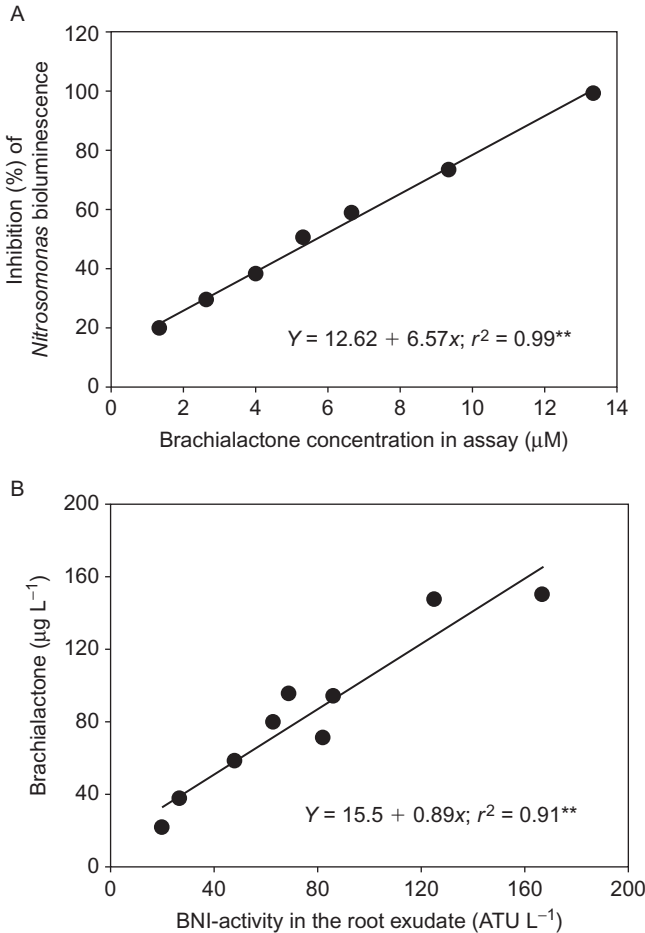


Figure 8 Inhibition of nitrification by brachialactone and the contribution of brachialactone to the BNI-activity released from roots. (A) Inhibitory effects of brachialactone on *Nitrosomonas europaea* in an *in vitro* assay. (B) Contribution of brachialactone to the BNI-activity released from roots (i.e., root exudates) of *Brachiaria humidicola*. Root exudates were collected from intact plants using 1L of aerated solution of 1mM NH_4Cl with 200 μM CaCl_2 over 24h. Each data point represents the effects of the root exudates collected from hydroponically grown plants in a glasshouse from March to May of 2007 and of 2008 (source: Subbarao *et al.*, 2009a).

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.*, 2009a). Some 60–90% of the inhibitory activity released from roots of *B. humidicola* is due to brachialactone (Fig. 8).

2.7. Potential for genetic manipulation in cereals and pasture grasses

Cereals are known to release phenolic acids (*p*-hydroxybenzoic, syringic, vanillic, ferulic, *p*-coumaric, chlorogenic, caffeic, *p*-hydroxybenzaldehyde, gallic, and protocatechuic), hydroxamic acids, alkaloids (hordenine and gramine), and quinines (sorgoleone and *p*-benzoquinones) (Walker *et al.*, 2003). These biologically active molecules have a diverse range of chemical structures and have been identified in a number of cereal crops and their wild relatives (Bennett and Wallsgrove, 1994). Moreover, these compounds when released from roots are involved in a wide range of functions including nutrient acquisition and pest and pathogen defense (Rengel and Marschner, 2005; Walker *et al.*, 2003). Some of these compounds have BNI properties and are therefore important targets for characterization; such knowledge is critical to the development of genetic strategies to enhance BNI-capacity in economically important cereals such as maize, wheat, sorghum, barley, and rye.

2.7.1. Extent of genetic variability in BNI-capacity

The existence of genotypic variability is a prerequisite for the genetic improvement of any plant trait using a conventional and/or molecular breeding program. Significant genetic variability exists for the BNI-capacity in *B. humidicola* (Table 2). Specific BNI-activity (ATU g⁻¹ root dry wt. day⁻¹) 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.*, 2007a). The on-going *Brachiaria* breeding program at the International Center for Tropical Agriculture (CIAT) in collaboration with Japan International Research Center for Agricultural Sciences (JIRCAS) plans to identify genetic markers associated with BNI-function, using a mapping population derived from crosses between apomictic and sexual germplasm accessions of *B. humidicola* that have contrasting BNI-capacity. The same mapping population will be useful to analyze the trade-offs between BNI-function and other plant attributes such as forage productivity and quality in terms of digestibility and protein content.

Using two ecotypes of the tropical grass *H. 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 by these tropical grasses could be a genetic attribute (Fig. 9) (Lata *et al.*, 2004). Such abilities of grasses to regulate soil nitrification are

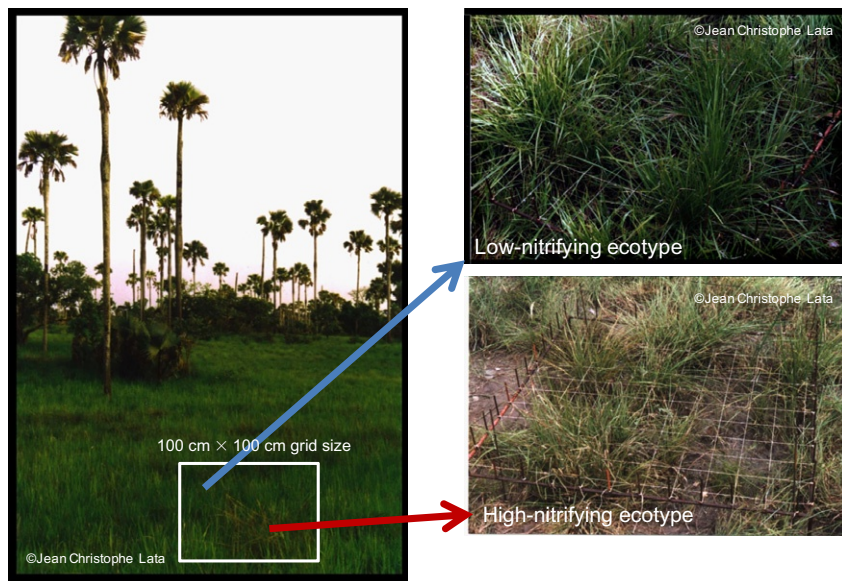


Figure 9 The experimental site in Lamto Savanna in Cote d'Ivoire, with the ecotypes of *Hypparrhenia diplandra*, a tropical grass, differing in their ability to influence soil nitrification. Photographs were taken in April 1995. The sites are under similar climatic and pedologic conditions, but exhibit different above-ground biomass: $270 \pm 55 \text{ g m}^{-2}$ in the low-nitrifying site or $130 \pm 30 \text{ g m}^{-2}$ in the high-nitrifying site (source: Lata, 1999).

reflected in their growth and biomass production (Lata *et al.*, 2000); they are also variable in population at the individual level. An attempt to correlate this variability to markers such as plant microsatellites showed no clear pattern (J.C. Lata, unpublished data). In addition, preliminary results have indicated significant genotypic variability for the BNI-capacity in barley germplasm (T.S. George and G.V. Subbarao, unpublished data). Sorghum, which is one of the most promising field crops producing BNI-activity, showed significant genotypic variation for the BNI-capacity in roots (Fig. 10), suggesting opportunity to understand the genetic control of this trait (Subbarao *et al.*, 2009b).

2.7.2. Improvement of BNI-capacity in wheat and barley

Traditional varieties/landraces and wild progenitors of crop species often have traits that do not exist in 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



Figure 10 The system used to collect root exudates from sorghum plants; methanol extracts of root exudates from three sorghum genotypes that differ in BNI-capacity (i.e., the capacity to release inhibitors from roots) (G.V. Subbarao and N. Kudo, unpublished data).

relatives and elite germplasm is often attributed to the impact of decades of breeding and selection under favorable agronomic conditions (Buso and Bliss, 1988). In cultivated wheat, preliminary results suggested a lack of significant BNI-capacity (Subbarao *et al.*, 2006b). However, the research with wild wheat relatives indicated that roots of *L. racemosus* possess high BNI-capacity (Fig. 11) (Subbarao *et al.*, 2007c). Inhibitors released from the roots of *L. racemosus* effectively suppressed soil nitrification for more than 60 days (Subbarao *et al.*, 2007c). 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 chromosome Lr#n and could be successfully introduced into and expressed in cultivated wheat (Fig. 12) (Subbarao *et al.*, 2007c). 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.*, 2007c; Zahn, 2007).

As wheat utilizes nearly a third of the global N-fertilizer output (Raun and Johnson, 1999), introducing high BNI-capacity into cultivated wheat could have a large impact on reducing N-leakage from wheat production. However, the alien chromosome of this chromosome addition line may also carry many undesirable traits that could reduce the potential grain yield. For

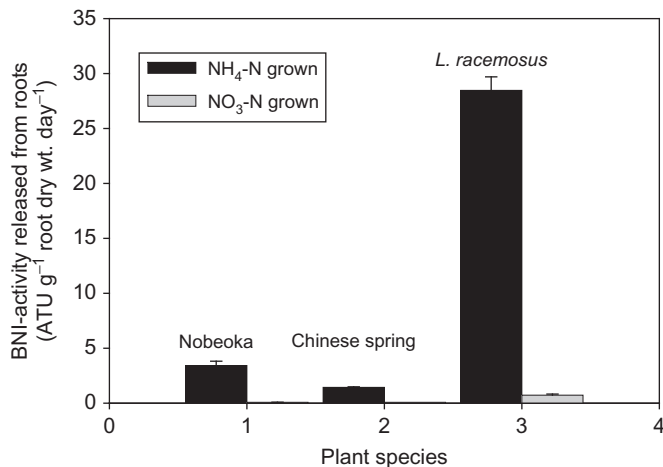


Figure 11 BNIs released from roots (i.e., root exudates) of two cultivars of wheat and their wild relative *Leymus racemosus*; plants were grown with either NH_4^+ or NO_3^- as the nitrogen source; root exudates were collected from intact roots in aerated collecting solutions over a 24-h period (source: Subbarao *et al.*, 2007c).

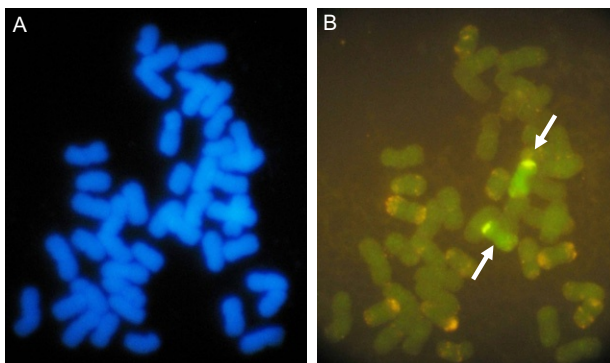


Figure 12 Karyotype analysis of DALr#n, a chromosome addition line derived from *Leymus racemosus* × *Triticum aestivum*. (A) DAPI staining revealed 44 chromosomes. (B) The probe of *L. racemosus* genomic DNA (green) and Tail and Afa family repetitive sequences showed the presence of two Lr#n chromosomes; arrows indicate Lr#n chromosomes conferring high BNI-capacity which was successfully expressed in cultivated wheat addition lines (source: Subbarao *et al.*, 2007c).

example, Preliminary field evaluations indicate that introduction of the Lr#n chromosome into Chinese Spring (i.e., DALr#n) made them susceptible to rust disease (K. Masahiro, unpublished data). It will be necessary,

therefore, to transfer to wheat only a small segment of this *L. racemosus* chromosome containing favorable 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 manipulation methods 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, 1993). Reciprocal exchange of alien chromosome segments with the corresponding wheat chromosomes (maintaining homoeology) without disrupting the genetic balance would be preferred. 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), 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 3B and 7B chromosome monosomic lines of wheat, in which one of 3B or 7B chromosomes is missing, to produce an F1 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; Kishii *et al.*, 2008).

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 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 poor agronomic background of this line, these translocations are being transferred into elite CIMMYT bread wheats. This should allow a realistic evaluation of BNI potential to reduce N-leakage from wheat systems and increase grain yields at lower N-fertilizer application. 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.1RS translocation involving the short arm of chromosome 1R from rye (*Secale cereale*), which is present in most wheat cultivars in the Middle East and West Asia (most of these CIMMYT derived), and in a significant proportion of cultivars in China, USA, and East Europe (Stokstad, 2007). The wide distribution of these wheats can be attributed to their high yield in diverse environments, despite

all 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 (Lukaszewski, 2000; Sears, 1977). 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. The material currently under development in elite backgrounds could be utilized to rapidly develop new cultivars if field trials indicate that translocations show high BNI-capacity.

Introduction of BNI-function from *L. racemosus* to barley would be problematic following this strategy, because diploid barley is very sensitive to chromosome manipulation (compared to tetraploid durum wheat [*Triticum turgidum* L.] or hexaploid bread wheat). Also, a gene to induce homoeologous recombination like that found in wheat has not been reported for barley. One possible method to introduce *L. racemosus* chromosome to barley could be through the use of a tetraploid barley line, which has its chromosome number doubled with colchicine as this would be more tolerant to the addition of alien chromosomes. 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 and generating the required centromeric translocation) and then transferring the translocation into barley by crossing the tetraploid barley chromosome substitution line with cultivated diploid barley. The selected case studies presented above suggest that the BNI-function merits exploitation as a trait to introduce/improve/strengthen the BNI-capacity of major food and feed crops (Subbarao *et al.*, 2009b).

2.7.3. Sorgoleone as a BNI and its potential role in genetic improvement of BNI-capacity in sorghum

It was recently discovered that sorgoleone, a *p*-benzoquinone exuded from sorghum roots, has a strong inhibitory effect on *Nitrosomonas* sp. and contributes significantly to BNI-capacity in sorghum (Subbarao *et al.*, 2009c). Sorgoleone is a major root exudate component in sorghum, and preliminary investigations indicate variability among sorghum genotypes in sorgoleone exudation (Czarnota *et al.*, 2003; Nimbale *et al.*, 1996; Subbarao *et al.*, 2009c). Sorgoleone is reported to have bio-herbicidal properties (Czarnota *et al.*, 2001; Einhellig and Souza, 1992; Einhellig *et al.*, 1993; Netzly and Butler, 1986; Netzly *et al.*, 1988). Factors that regulate sorgoleone exudation (Czarnota *et al.*, 2001, 2003; Dayan, 2006), its biosynthetic

pathway (Dayan *et al.*, 2003), and mode of herbicidal action (Gonzalez *et al.*, 1997; Rimando *et al.*, 1998) are known. Sorgoleone is also reported to function as a germination stimulant for *Striga* sp. seeds (Hauck *et al.*, 1992); although sorgolactones (Sugimoto *et al.*, 1998) appear to be the most important and effective *Striga* sp. germination stimulant exuded by sorghum roots. Inheritance of sorgoleone production has been reported (Yang *et al.*, 2004). Several genes controlling the biosynthetic pathway of sorgoleone are known (Baerson *et al.*, 2007; Pan *et al.*, 2007), and their positions on the aligned genomic sequences of sorghum chromosomes SBI-04, SBI-05, SBI-06, and SBI-08 are determined (Ramu *et al.*, 2010) (Table 3). Genomic

Table 3 Sorghum genes involved in the sorgoleone biosynthetic pathway, and QTLs for the *Striga* seed germination stimulant and their approximate genomic positions

Gene symbol	Gene description	Genomic position: Chromosome and location (bp) on aligned sequence	Reference
<i>lgs</i>	Low germination stimulant	SBI-06: long arm	Haussmann <i>et al.</i>, 2004
<i>SbDES1</i>	FAD3-type plant fatty acid desaturatase	SBI-08: 310,759	Pan <i>et al.</i>, 2007
<i>SbDES2</i>	FAD2-type desaturatase associated with sorgoleone biosynthesis; catalyzes conversion of 16:1 Δ^9 to 16:2 $\Delta^{9,12}$	SBI-04: 59,930,467	Pan <i>et al.</i>, 2007
<i>SbDES3</i>	FAD3-type desaturatase associated with sorgoleone biosynthesis; catalyzes conversion of 16:2 $\Delta^{9,12}$ to the unusual 16:3 $\Delta^{9,12,15}$ fatty acid, which possesses a terminal double bond	SBI-05: 216,583	Pan <i>et al.</i>, 2007
<i>SbOMT3</i>	O-methyl transferase associated with sorgoleone biosynthesis	SBI-06: short arm	Baerson <i>et al.</i>, 2007
<i>SbSOR1</i>	Omega-3 fatty acid desaturase expressed in sorghum root hairs and associated with sorgoleone production; shows homology with <i>SbDES3</i>		Yang <i>et al.</i>, 2004

Source: [Ramu *et al.* \(2010\)](#).

regions associated with production of sorgolactone may also be involved in regulating sorgoleone production (Ejeta, 2007; Haussmann *et al.*, 2004). Further research is needed to unravel the inter-connectivity in the biosynthetic pathways and regulation of sorgoleone and sorgolactone exudation (Akiyama and Hayashi, 2006; Gomez-Roldan *et al.*, 2008) and their functional relationship to 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. The ICRISAT has recently developed several populations of random inbred lines based on sorghum parental lines that differ in sorgoleone exudation (G.V. Subbarao and C.T. Hash, unpublished data). Since these populations are generally based on elite germplasm, this approach has the advantage of easy deployment of traits into relevant high-yielding cultivars of sorghum. Association mapping approaches could also be explored by evaluating the mini-core subset (10% of the core collection and 1% of the entire collection, which amounts to 242 accessions) of ICRISAT's global sorghum germplasm collection, the recently developed reference germplasm set of 384 wild and cultivated accessions, for 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 have been identified, they can be rapidly transferred to genetic backgrounds of elite sorghum hybrid parental lines and/or open-pollinated varieties by backcrossing. With the introgression of favorable alleles of one or two major genes (to accelerate exudation of sorgoleone) into elite genetic backgrounds, it should be possible to improve the BNI-capacity in sorghum.

2.7.4. Deployable genetic tools and population approaches for the introduction of high BNI-capacity into common crop species

With the plethora of forward and reverse genetic techniques at our disposal to characterize genotypic variation and generate isogenic lines [transgenics, mutants, RNA interference (RNAi), synthetic microRNA (smiRNA)] and near-isogenic lines (NILs) differing in their capacity to modify the rhizosphere environment (Caldwell *et al.*, 2004; Hash *et al.*, 2002; Neumann *et al.*, 2009), it should be possible to analyze the genetic control of BNI-function and deploy it as a trait into elite germplasm of a range of crops. This will require (a) the availability of sufficient genetic variation in the BNI-capacity of germplasm, (b) optional identification of candidate genes controlling the trait and heterologous expression to verify their roles in BNI-function, and (c) introgression of genes controlling the BNI-trait into elite germplasm.

The availability of the entire genome sequences of *Arabidopsis* and a range of crop plants [rice, sorghum, potato (*Solanum tuberosum* L.), barley,

tomato (*Solanum lycopersicum* L.), and maize] could facilitate the use of tools such as “genome-wide expression profiling” to identify candidate genes controlling BNI (Caldwell *et al.*, 2004; Vij and Tyagi, 2007). Once verified, the expression of the candidate genes in elite germplasm following marker-assisted breeding or transgenic approaches may lead to crop varieties that can reduce nitrification. While this process may seem straightforward, it is important to note that despite a large number of transcriptomic studies on interactions between plants and soils, to date, only a handful have identified genes with functions that have been successfully deployed in elite germplasm (Oh *et al.*, 2007). With the rapid advances in sequencing and data analysis capability (Varshney *et al.* 2009), transcriptomic approach could lead to identification of candidate genes associated with the BNI-function. In addition, integrated map-based approaches can be adopted for traits where high-throughput phenotyping systems are available (Magalhaes *et al.*, 2007; Raman *et al.*, 2006; Sasaki *et al.*, 2004).

Another possibility for understanding the genetic control of BNI-trait in crops would be to use association mapping population approach, which would allow screening of genetically diverse elite cultivars for specific BNI-traits and association of variation in these traits with chromosome maps of cultivars annotated with several thousand single-nucleotide polymorphism (SNP) and/or diversity array technology (DArT) markers. As the genomic regions associated with BNI variation are likely to differ from cultivated wheat for DArT, SNP, indel (insertion–deletion), and/or STMS (sequence-tagged microsatellite) markers, high-throughput genotyping and marker-assisted selection to transfer the trait should be highly efficient. This could facilitate rapid identification of quantitative trait loci (QTLs) and specific markers for genes. However, this is contingent upon the availability of (a) a trait-phenotyping protocol that can handle a large number of cultivars (e.g., >100), (b) significant genetic variation for the trait, and (c) polymorphic marker density (across the entire genome or at least within specific candidate genes and their regulatory regions) that is good enough to detect linkage genetic disequilibrium between cultivars having high and low values for the target trait. Use of association mapping populations has previously elucidated potential QTLs for unknown NUE and P-use efficiency mechanisms in wheat (Liao *et al.*, 2008) and for resistance or tolerance to yellow dwarf virus in barley (Kraakman *et al.*, 2006).

A complimentary approach would be to use populations saturated with mutations. Such mutant populations exist in *Arabidopsis*, wheat, barley, and sorghum, where their genomes are saturated with either mutations “knocking-out” genes or up- or downregulating genes downstream of the mutation. Currently, the available BNI phenotyping protocol, however, is adequate only to evaluate a modest number of mutants (a few hundred at most) in a particular candidate gene that might be identified from such populations. Such an approach could be relevant in barley, as the extremes

of an initial screen (G.V. Subbarao and T. George, unpublished data) for BNI-capacity were in genotypes that have pre-existing mutant populations (Caldwell *et al.*, 2004; Kojima *et al.*, 2006; Yuan *et al.*, 2007). Genes identified using these population screening approaches can then be validated by overexpression using transgenic approaches, by coupling the gene with specific promoters, or by monitoring their loss of function after down-regulating the gene of interest by RNAi or with the use of smiRNA technologies (Alvarez *et al.*, 2006; Delhaize *et al.*, 1993; Magalhaes *et al.*, 2007; Miki and Shimamoto, 2004; Raman *et al.*, 2006; Sasaki *et al.*, 2004). If genetic control of BNI proves to be simple, then such transgenic, RNAi, or smiRNA approaches would be appropriate for candidate gene validation.

2.8. Evidence for BNI-function in the field

Grass-alone pastures planted with *B. humidicola* in the acid soils (Oxisol) of the Colombian Eastern Plains (Llanos Orientales) showed reduced nitrification rates compared to legume-alone pastures or bare soil (Sylvester-Bradley *et al.*, 1988). *B. humidicola* pastures develop abundant and highly vigorous root systems that explore deep soil layers and sequester large amounts of C in soil (Fig. 13) (Fisher *et al.*, 1994; Rao, 1998). A conservative estimate of the live root biomass from a long-term grass pasture was 1.5 Mg ha⁻¹ (Fisher *et al.*, 1994), with a BNI-capacity of 17–50 ATU g⁻¹ root dry wt.day⁻¹ (Subbarao *et al.*, 2007a). We thus estimate that BNI-activity of 2.6×10^6 to 7.5×10^6 ATU ha⁻¹ day⁻¹ could potentially be released from *B. humidicola* roots, which amounts to an inhibitory



Figure 13 *Brachiaria humidicola* cv. Llanero with abundant root system grown in a low fertility acid soil of the Llanos in Colombia (source: I.M. Rao, CIAT, Cali, Colombia).

potential equivalent to the application of 6.2–18 kg of nitrapyrin $\text{ha}^{-1} \text{year}^{-1}$ (based on 1 ATU being equal to 0.6 μg of nitrapyrin); this inhibitory effect would be strong enough to have a significant influence on the function of soil nitrifier populations and nitrification rates (Subbarao *et al.*, 2009a).

Field studies at the CIAT (Palmira, Colombia) (Mollisol) (Fig. 14) indicated a 90% decline in soil- NH_4^+ oxidation rates (Fig. 15), largely due to low nitrifier populations [AO bacteria and AO archaea; determined as *amoA* genes] in *B. humidicola* plots within 3 years of establishment (Subbarao *et al.*, 2009a). Two other pasture grasses, *P. maximum* and *Brachiaria* hybrid cv. Mulato, that have a low to moderate level of BNI-capacity (3–10 ATU g^{-1} root dry wt. day^{-1}) showed only an intermediate level of inhibitory effect on soil- NH_4^+ oxidation rates (Fig. 15). The inhibitory function of roots of these tropical pasture grasses appears to be primarily targeted at reducing soil nitrifier activity rather than the general soil microbial activity. Moreover, soil nitrifier activity as estimated from AOB and AOA populations indicated a 90% decline in field plots planted with *B. humidicola* within 3 years, but with no significant effect on the total soil bacterial population (Subbarao *et al.*, 2009a). Nitrous oxide emission was also suppressed by >90% in field plots planted to *B. humidicola* CIAT

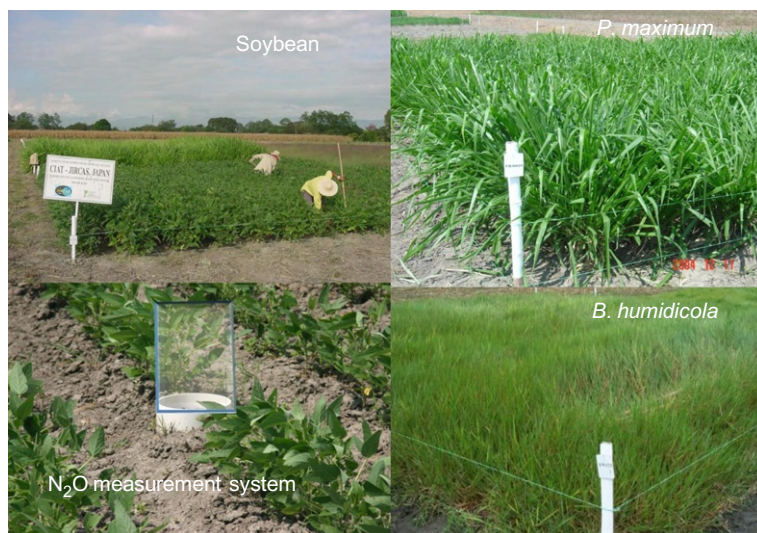


Figure 14 Testing the proof-of-concept for BNI-function in the field. The tropical pasture grasses [*Brachiaria humidicola* (CIAT 679; CIAT 16888)], *Brachiaria* hybrid cv. Mulato and *Panicum maximum*, and soybean were grown in the field for 3 years to monitor the changes in soil nitrification potentials by the BNI-function and its effects on nitrous oxide emissions.

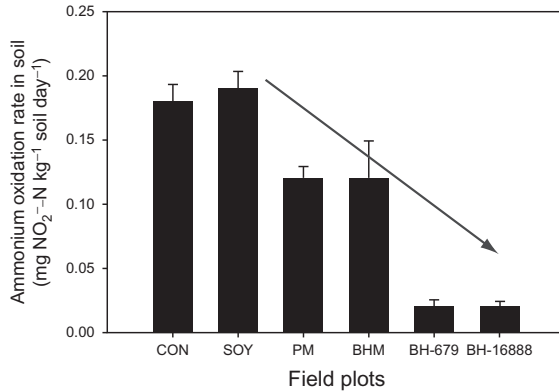


Figure 15 Soil ammonium oxidation rates ($\text{mg NO}_2^- \text{kg}^{-1}$ of soil day^{-1}) in field plots planted to tropical pasture grasses (differing in BNI-capacity) and soybean (lacking BNI-capacity in roots) [over 3 years from establishment of pastures (September 2004 to November 2007); for soybean, during planting seasons every year and after six seasons of cultivation]. CON, control (plant-free) plots; SOY, soybean; PM, *Panicum maximum*; BHM, *Brachiaria* hybrid cv. Mulato; BH-679, *B. humidicola* CIAT 679 (standard cultivar); BH-16888, *B. humidicola* CIAT 16888 (a germplasm accession). Values are means \pm SE from three replications (source: Subbarao *et al.*, 2009a).

16888, compared to the emission from plots of soybean, which lack BNI-capacity (Fig. 16). There appears to be a negative relationship 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 their roots (Fig. 16).

Field studies made at Lamto Reserve (Ivory Coast) showed that savanna cover of the African grass *H. diplandra* and some other species induced a 240-fold lower nitrification potential in their root zone (Lata, 1999; Lata *et al.*, 2004). This effect can be considered as permanent in this ecosystem as the basal nitrate-reductase activities in plants grown in greenhouse are linked to this capacity. Decimetric-scale experiments (Lata *et al.*, 2000) demonstrated a close negative relationship between the roots and nitrification (in the 0–10-cm soil layer), showing an unexpectedly high sensitivity of nitrification process to root density. This correlation between the roots and nitrification decreased with depth and nearly disappeared in the 20–30-cm soil layer. Finally, *in situ* experimental transplantations (Lata, 1999; Lata *et al.*, 2004) of individual grass plant (whether seedlings or vegetative propagules) showed that grasses significantly modified nitrification rates similar to those at their respective control sites. The inhibitory effect on soil nitrification was stable (during the 3-year experimental period) and enhanced biomass production. Moreover, low-nitrifying sites covered by inhibiting grasses showed a 10-fold lower denitrification potential than

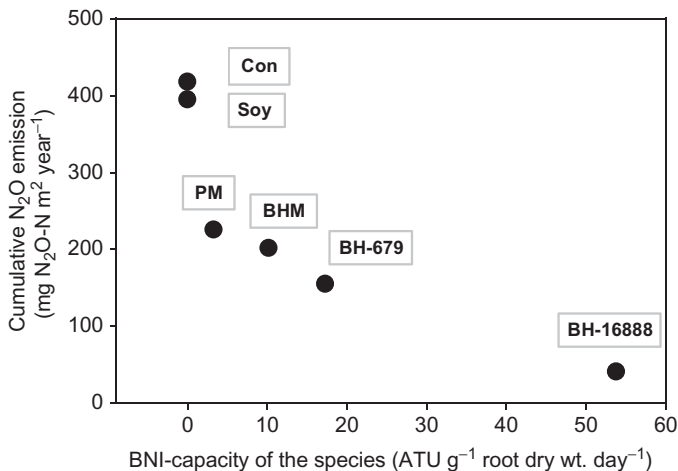


Figure 16 The relationship between the BNI-capacity of plant species and the N₂O emissions from field plots. The N₂O emissions were monitored over a period of 3 years, September 2004 to November 2007 (adapted from Subbarao *et al.*, 2009a).

high-nitrifying sites. This better conservation of the N-resource resulted in doubling of the above-ground biomass of these grasses compared to grasses of high-nitrifying sites (Fig. 9). This grass species impact must be highlighted by comparing it to the effect of trees present in this ecosystem that stimulate nitrification under their canopy, by 6- to 100-fold (Lata, 1999) compared to a grass cover. These opposite effects influence tree-grass competition for resources in this type of highly constrained ecosystems.

2.9. Deploying the BNI-function in agro-ecosystems—A systems approach to reducing nitrification in agriculture

Agro-climatic factors need to be considered for using the BNI-function as a strategy to control nitrification. For example, alkaline soil pH limits the expression and stability of the BNI-function. Heavy clay soils such as Vertisols that are alkaline may not be suitable for the expression of BNI-function. Also, a high bacterial activity in soils with relatively high organic matter might enhance the degradation of BNIs. The influence of soil-physical, -chemical, and -biological properties on the expression and stability of the BNI-function is not adequately understood at present and requires further research. Also, little information is available on the effects of soil environmental factors, especially temperature and soil water status (linked to inter- and intraseasonal variability or to stresses due to excess or insufficient

moisture), in modulating the BNI-function. For instance, when modeling the rhizosphere and associated exudate gradients, it was shown that adsorption properties, solute lifetime, and soil water content 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).

In production systems, where the targeted crop's BNI-capacity is limited, the BNIs may not reach the critical threshold levels to reduce soil nitrification. Tropical pastures with high BNI-capacity coupled with a perennial growth habit favor the accumulation of BNIs to a threshold level sufficient to suppress soil nitrifier activity. The pasture component could provide the required BNI-activity to improve the N-economy of annual crops (a weak contributor of BNIs) that follow the pasture phase. For example, *Brachiaria* pastures that have high BNI-capacity, but receive little fertilizer inputs, can be rotated with annual crops (such as maize or upland rice that have low- or very low-BNI-capacity, but receive substantial N fertilization) in an agro-pastoral system to improve the recovery of applied fertilizer-N leading to overall N-economy. The stability of the residual BNI effects on soil nitrification potential (determined as soil-NH₄⁺ oxidation rate), where an annual crop such as maize is grown after a *Brachiaria* pasture, is depicted under various hypothetical scenarios (Fig. 17). However, it is assumed that the relative stability of the residual BNI effects may differ depending on the subsequent crop (e.g., maize vs. soybean) and also depending on

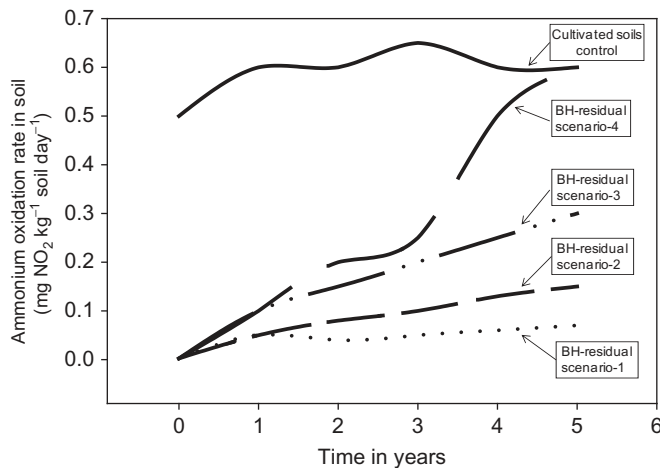


Figure 17 Various scenarios of stability of the residual BNI effects from *Brachiaria humidicola* (land under the *B. humidicola* pasture for several years) on soil nitrification potential (expressed as soil ammonium oxidation rate) on a cereal crop grown in a South American agro-pastoral system.

the cumulative N-fertilizer application amount in an agro-pastoral system, which needs to be characterized in a production system context.

In crop species where the production of NI is established in shoot tissues but is not released from roots (e.g., in some crucifers; [Bending and Lincoln, 2000](#)), the incorporation of plant residues into the soil may be considered part of a crop management strategy to control nitrification. Research on BNI-function requires a multi-disciplinary approach where crop improvement and agronomic management are combined to utilize this novel biological trait effectively and economically in practical agriculture. Moreover, the boundaries of the agro-ecosystems where the BNI-function can be effectively deployed will have to be defined with the help of crop ecologists and agronomists. This will help breeders and molecular biologists target BNI-traits in crops for genetic improvement from the perspective of an entire agro-ecosystem. Deployment of the BNI-function, thus, requires an understanding of both edaphic and climatic conditions of production systems in the target region.

3. CONCLUDING REMARKS

Modern agricultural systems are dependent on large inputs of mineral N as their primary N source ([De Wit *et al.*, 1987](#); [Subbarao *et al.*, 2006a](#)); this along with changes in crop management practices has resulted in the evolution of the present high-nitrifying soil environments ([Celik, 2005](#); [Elliott, 1986](#); [Poudel *et al.*, 2002](#)). Most high-yielding crop varieties bred for these environments were also inadvertently selected for their preference for NO_3^- over NH_4^+ . Moreover most of our staple crops seem to lack any functional BNI-capacity. These factors taken together seem to have presented a significant incentive for the development of the current nitrification-dominated N-cycle in agricultural systems ([Figs. 1, 2, and 18](#)). Of several approaches potentially available for reversing this trend, the introduction of BNI-capacity into field crops and pastures would provide a powerful new strategy for the regulation of nitrification in agricultural systems. Genetic exploitation of BNI-capacity and the preference for NH_4^+ , found in the wild relatives of some crops (such as wild wheat, *L. racemosus*) and forage grasses (e.g., *Brachiaria* spp.), could provide biological options for delivering the BNI-activity to agricultural systems. The next generation of cropping systems should exploit the BNI-function to improve the efficiency of N use in agriculture and to reduce the negative impact of N-fertilizers on the environment.

Recent findings indicate that a number of diverse chemical molecules with an inhibitory effect on *Nitrosomonas* sp. can be produced and released by plant roots. The AMO enzyme has a high affinity for a wide range of

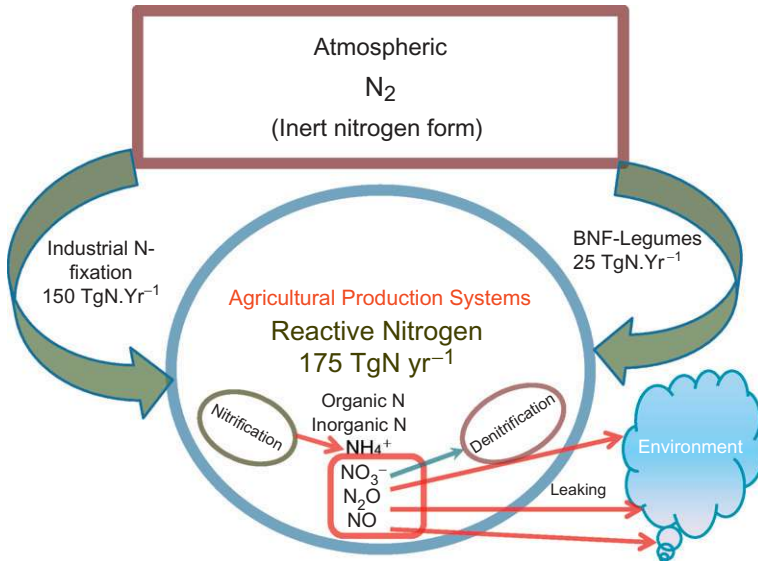


Figure 18 Current agricultural production systems largely driven by industrially fixed nitrogen with a very high risk for leaking reactive-N to the environment.

substrates in addition to NH_4^+ (Hauck, 1980; McCarty, 1999). Moreover, by interfering with the functioning of AMO enzymatic pathway, biological molecules with diverse chemical structures can inhibit nitrifier activity. This unique feature of the AMO enzyme has been exploited during the development of synthetic chemical NIs (Subbarao *et al.*, 2006a). The unexplored chemical diversity of root exudates is an obvious place to search for novel NIs, which could be exploited to develop a range of biological and chemical strategies for controlling nitrification in agricultural systems. Beyond this, future research should also examine the second stage of nitrification occurring in the *Nitrobacter* bacteria.

There is sufficient evidence from recent studies to indicate potential differences in N_2O emissions among plant species, linked to their differing BNI-capacities (Fig. 16) (Subbarao *et al.*, 2009a). The comparison of inhibiting and non-inhibiting grass ecotypes in the savanna ecosystems showed that denitrification potential is nearly 10-fold lower in grasses from low-nitrifying sites than in those in high-nitrifying sites (Lata *et al.*, 2004). Presently, such differences are not considered by the Intergovernmental Panel on Climate Change (IPCC) in their estimation of projected N_2O emissions from agricultural systems (Stehfest and Bouwman, 2006). For example, there are >250 million ha of South American Savannas occupied by native grass or by pastures of introduced grasses such as *Brachiaria* spp.

(Fisher *et al.*, 1994), which have high BNI-capacity. These pastures are low-nitrifying and low-N₂O emitting systems, but if converted to crop production using species that lack BNI-capacity (such as soybean, wheat, barley, maize, rice), it could have major implications for N₂O emissions (Subbarao *et al.*, 2007a, 2009a). Such conversion is taking place. Hence, there is an increasing urgency to introduce adequate BNI-capacity into field crops and pastures to facilitate development of production systems that are low-nitrifying and low-N₂O emitting, but these systems must remain highly productive to meet the increasing food demands of the growing world population.

4. PERSPECTIVES

The availability of large amounts of industrially fixed-N (fertilizer-N) from the Haber-Bosch process has been a major driver of the Green Revolution that has doubled global food grain production during the last half-century. However, this high level of fertilization is responsible for the transfer of massive amounts of reactive-N (reduced forms of N, i.e., N-fertilizer) (Liu *et al.*, 2010) through agricultural ecosystems, even though these represent only 11% of the Earth's surface (Newbould, 1989) (Figs. 1 and 18). Currently, inputs from industrially fixed-N into agricultural systems (about 150 Tg year⁻¹) exceed the total biologically fixed-N in all natural systems of our planet (about 100 Tg year⁻¹) (Tilman *et al.*, 2001; Vitousek *et al.*, 1997). Further, fertilizer-N consumption is expected to double from the current levels by 2050 to reach 300 Tg year⁻¹ (Charles *et al.*, 2010; IFA, 2005; Schlesinger, 2009). N₂O emissions from agricultural systems are expected to reach 38.6 TgN year⁻¹ by 2050, contributing significantly to global warming (IPCC, 2007; Kroeze, 1994; Schlesinger, 2009; Smith *et al.*, 1997). There is also a growing concern about the environmental damage that would result, given the pervasive inefficiency of N-use by most crops (Hauck, 1990; Ju *et al.*, 2009; Schlesinger, 2009; Smil, 1999). The economic implications of this "wasted N" could be enormous and are expected to reach close to US\$ 81 billion at current fertilizer prices (estimated at US\$ 450 per Mg of urea-N) from the lost fertilizer even without considering the economic cost of potential environmental damage and the resulting ecological destruction. For example, for the European Union, which consumes only 11 TgN year⁻¹ (fertilizer-N) annually, the cost of damage from reactive nitrogen emissions on human health and ecosystems is estimated at US\$ 102–320 billion (Sutton *et al.*, 2011).

A major objective of this review is to increase awareness of inefficiency in nitrogen use, which is largely attributed to the dominance of nitrification

in the soil-N-cycle of current agricultural production systems. From a broader ecological perspective, the BNI-function can be exploited to tighten the N-cycle in agriculture. A fundamental shift toward an NH_4^+ -dominated crop N-nutrition could be achieved by using crops and pastures with high BNI-capacity. The next generation of production systems will require deployment of the BNI-trait as an integral part of a comprehensive strategy to defend the rhizosphere from nitrifying bacteria and brings a balance in soil-N forms (i.e., organic-N, NH_4^+ , and NO_3^-) absorbed and assimilated by crops and pastures to reduce N pollution and improve NUE.

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