Review

Biological nitrification inhibition (BNI)—Is there potential for genetic interventions in the Triticeae?

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The natural ability of plants to release chemical substances from their roots that have a suppressing effect on nitrifier activity and soil nitrification, is termed 'biological nitrification inhibition' (BNI). Though nitrification is one of the critical processes in the nitrogen cycle, unrestricted and rapid nitrification in agricultural systems can result in major losses of nitrogen from the plant-soil system. This nitrogen loss is due to the leaching of nitrate out of the rooting zone and emission of gaseous oxides of nitrogen to the atmosphere, where it causes serious pollution problems. Using a newly developed assay system that quantifies the inhibitory activity of plant roots (*i.e.* BNI capacity), it has been shown that BNI capacity is widespread among crops and pastures. A tropical pasture grass, *Brachiaria humidicola* has been used as a model system to characterize BNI function, where it was shown that BNIs can provide sufficient inhibitory activity to suppress soil nitrification and nitrous oxide emissions. Given the wide-range of genetic diversity found among the Triticeae, and the current availability of genetic tools for moving traits/genes across members, there is great potential for introducing/improving the BNI capacity of economically important members of the Triticeae (*i.e.* wheat, barley and rye). This review outlines the current status of knowledge regarding the potential for genetic improvement in the BNI capacity of the Triticeae. Such approaches are critical to the development of the next-generation of crops and production systems where nitrification is biologically suppressed/regulated to reduce nitrogen leakage and protect the environment from nitrogen pollution

Key Words: Nitrification, *Nitrosomonas*, *Brachiaria humidicola*, *Leymus racemosus*.

Introduction

The biological oxidation of ammonia to nitrate is termed "nitrification", and is carried out by two groups of chemo-lithotrophic bacteria (*Nitrosomonas* spp. and *Nitrobacter* spp.), which are ubiquitous components of the soil microbial population (Norton *et al.* 2002). Nitrification and denitrification are critical processes in the removal of N from organic wastes where they facilitate N cycling in organic-based waste systems. However, in agricultural systems, rapid and unchecked nitrification results in inefficient N use by crops, leading to N leakage and environmental pollution (Clark 1962, Subbarao *et al.* 2006a, 2009a, Schlesinger 2009). Most plants have the ability to utilize either NH_4^+ or NO_3^- as their N source (Haynes and Goh 1978). Therefore, minimizing the role of nitrification in the N cycle in agricultural systems should not limit nitrogen availability to plants and is desirable as it reduces the amount of nitrogen leakage from these systems.

Need for nitrification suppression?

Nearly 90% of worldwide application of N-fertilizer is in the form of NH_4^+ that is rapidly converted to NO_3^- (within days or weeks) by nitrifier activity (Sahrawat 1980, Mason 1992, Strong and Cooper 1992). Being a cation, NH_4^+ in the soil is held by electrostatic forces to the negatively charged

clay surfaces and the functional groups of soil organic matter (SOM) (Amberger 1993). This binding is sufficiently strong to restrict N loss by leaching (Amberger 1993). In contrast, NO_3^- with its negative charge, does not bind strongly to the soil, and thus it is mobile and liable to being leached out of the root-zone (Amberger 1993). Several heterotrophic soil bacteria denitrify NO_3^- (*i.e.* convert NO_3^- into gaseous N forms: N_2O, NO, N_2) under anaerobic or partially anaerobic conditions (*i.e.* this often coincides with heavy rainfall or irrigation and/or improper drainage) (Bremner and Blackmer 1978, Mosier *et al.* 1996). Losses such as these reduce the effectiveness of N fertilization and presents serious pollution problems (Clark 1962, Jarvis 1996).

The rapid conversion of NH_4^+ to NO_3^- in soil results in the inefficient use of both soil N and applied N fertilizer. Soil organic N can also go through the nitrification process, making it subject to similar losses of N (Dinnes *et al.* 2002, Subbarao *et al.* 2006a, 2009a, 2009b). Nitrification is the single most important process in the N cycle that leads directly to N losses (Clark 1962, Barker and Mills 1980). Moreover, the assimilation of NO_3^- by plants requires more metabolic energy than the assimilation of NH_4^+ (20 moles of ATP per mole of NO_3^- *vs.* 5 moles of ATP per mole of NH_4^+) (Salsac *et al.* 1987) making the use of NH_4^+ by plants more efficient. In addition, the assimilation of NO_3^-, not NH_4^+, results in the direct emission of N_2O from crop canopies reducing further its N-use efficiency (Smart and Bloom 2001). Keeping N in the NH_4^+ form thus has a large number of advantages for improving N uptake in agricultural systems. Many of these advantages have been demonstrated using chemical nitrification inhibitors (Slangen and Kerkhoff 1984, Subbarao *et al.* 2006a).

The movement towards high-nitrifying systems

Nitrification plays only a relatively minor role in many natural climax communities as only a small portion of the total N may go through the nitrification process in the nitrogen cycle. In contrast, nitrification plays a dominant role in most agricultural systems (Vitousek *et al.* 1997, Nasholm *et al.* 1998, Smolander *et al.* 1998, Subbarao *et al.* 2006a). Modern agricultural systems rely heavily on large inputs of external N (through inorganic N fertilizer) to maintain high productivity as naturally fixed N is seldom adequate (Dinnes *et al.* 2002). In addition, several changes that took place during the 20th century as part of modernizing, led to rapid nitrification in agricultural systems (Rabalais *et al.* 1996, Poudel *et al.* 2002). These include a) Reduced use of diversified crop rotations, b) Separation of crop production systems from animal enterprises, c) Increased soil tillage, d) Irrigation and drainage of agricultural fields and e) Increased use of N fertilizers.

Current production systems that depend heavily on industrially produced inorganic N have replaced earlier production systems that relied primarily on legumes and/or animals for N inputs (Dinnes *et al.* 2002). In addition, the separation of crop and animal production enterprises has led to an even greater dependence on inorganic N fertilizers (*i.e.* bypassing agricultural systems for organic matter recycling), and has resulted in the reduction of SOM levels worldwide (Tiessen *et al.* 1994). This heavy dependence on mineral fertilizers has also contributed to the stimulation of nitrifier activity and the subsequent highly-nitrifying soil environments (McGill *et al.* 1981, Poudel *et al.* 2002, Lal 2003, Bellamy *et al.* 2005). This coupled with the installation of sub-surface drainage systems in many developed parts of the world has resulted in the acceleration of NO_3^- leaching, leading to further reductions in the efficiency of N-cycling in agricultural systems (Clark 1962, Pratt and Adriano 1973, Dinnes *et al.* 2002).

Consequences of high-nitrifying environments on global nitrogen-cycle

The green revolution which was largely founded on the application of industrially fixed nitrogen to semi-dwarf rice and wheat varieties, has doubled global food grain production and reduced food shortages, but at a high environmental cost (Tilman *et al.* 2001, Hungate *et al.* 2003). The rapid and unrestricted nitrification found in modern production systems, results in the loss of nearly 70% of N-fertilizer inputs to agricultural systems. This amounts to a direct economic loss of nearly US$ 17 billion worldwide annually from cereal production systems alone (Raun and Johnson 1999, Subbarao *et al.* 2006a). In addition, there are countless other costs related to environmental problems that are not yet addressed (Viets 1975, Ryden *et al.* 1984, Tilman *et al.* 2001, Schlesinger 2009).

Nitrogen fertilizer use is expected to triple by 2050 from the current 100 Tg N yr^{-1} in agricultural systems. This will further accelerate nitrogen leakage from production systems, placing a much heavier pollution loads on the environment (Vitousek *et al.* 1997, Tilman *et al.* 2001, IFA 2005, Schlesinger 2009). Leaching of NO_3^- from root zones with the subsequent NO_3^- contamination of ground water and surface waters is one of the major environmental concerns associated with nitrification (Scheperts *et al.* 1991, Tilman *et al.* 2001, Schlesinger 2009). Several studies have shown a close linkage between N-fertilizer usage, increased groundwater NO_3^- levels, human health and environmental problems (Broadbent and Rauschkolb 1977, Vitousek *et al.* 1997, Subbarao *et al.* 2006a, Schlesinger 2009). Nitrogen lost due to NO_3^- leaching from agricultural systems is currently estimated at 61.5 Tg N yr^{-1} (Schlesinger 2009).

In addition to the pollution of terrestrial and marine water bodies, agricultural systems contribute nearly 30% of the current nitric oxide (NO) and 70% of the nitrous oxide (N_2O) emissions to the atmosphere (Fig. 1) (Bremner and Blackmer 1978, Smith *et al.* 1997, Hofstra and Bouwman 2005). In the atmosphere, N_2O acts as a powerful greenhouse gas having a global warming potential 300 times that of CO_2 (Kroeze 1994, IPCC 2001). In addition, the NOs that reach the stratosphere can damage the protective ozone layer (Crutzen and Ehhalt 1977). During plant growth, assimilation of NO_3^- rather than NH_4^+ results in N_2O emissions
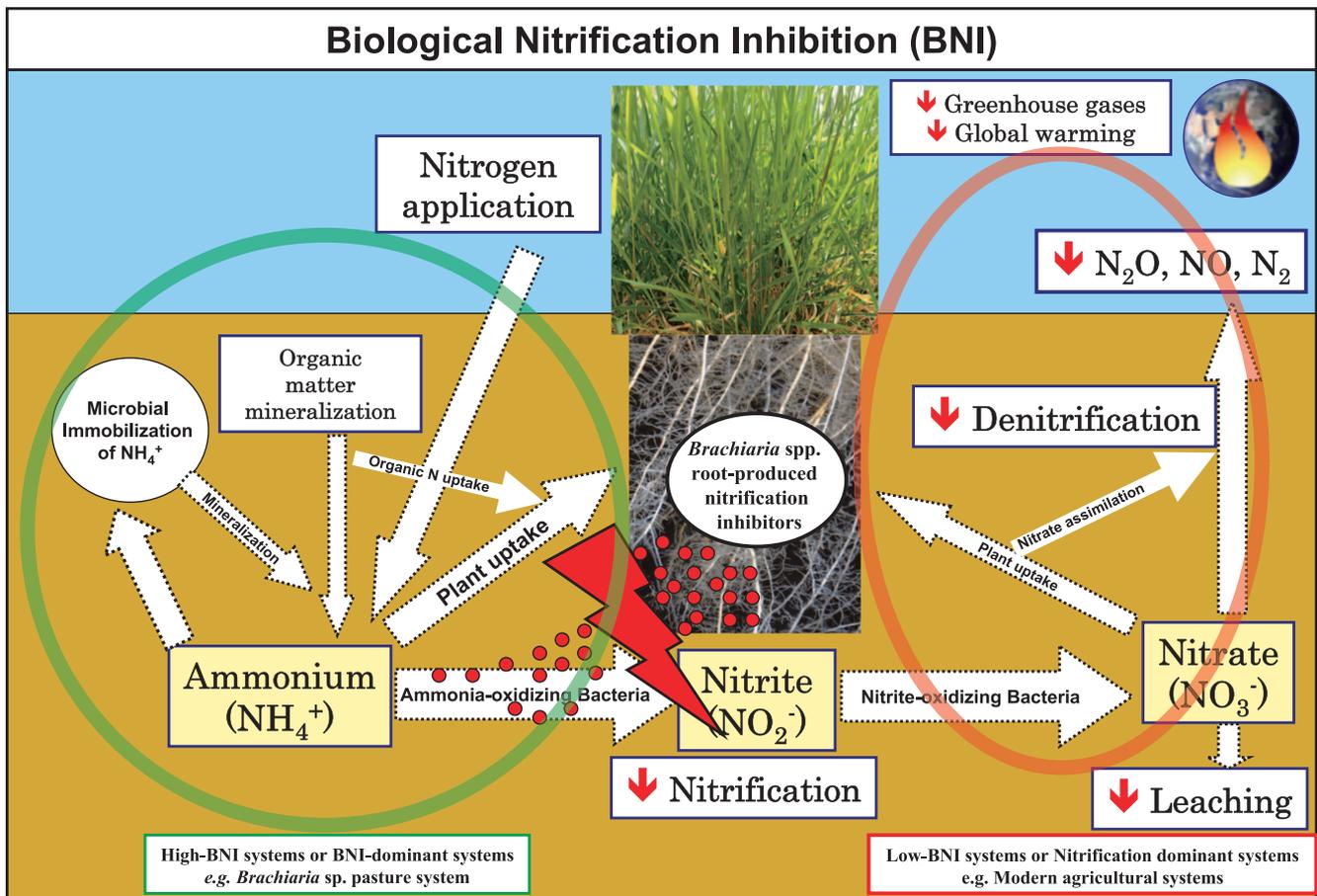
Fig. 1. Schematic representation where biological nitrification inhibition (BNI) interfaces with the nitrogen cycle. BNI's produced by the root inhibits the process that converts ammonium to nitrate. In ecosystems with large amounts of BNI, such as *Brachiaria* pastures, the flow of nitrogen from ammonium to nitrate is restricted and ammonium tends to remain or build up in the soil/root system. In systems with little or no BNI, such as modern agricultural systems, nitrification tends to occur at a rapid rate and ammonium is rapidly converted to nitrate that is very susceptible to be lost from the soil/root system. [adapted and redrawn from Subbarao *et al. in press*).

directly from crop canopies (Smart and Bloom 2001, Nishimura *et al.* 2005). Current estimations indicate that nearly 17 Tg N yr^{-1} is emitted to the atmosphere as N_2O (Schlesinger 2009). By 2100, the global N_2O emissions are projected to be four times greater than current emissions, due largely to increases in N-fertilizer use (Hofstra and Bouwman 2005).

Options for the control of nitrification in agricultural systems

A number of N-management strategies that utilize rate and/or timing of fertilizer applications such as "fall" *vs.* "spring", basal *vs.* split applications, banding of N fertilizers *vs.* broadcasting, deep placement of N fertilizer *vs.* surface application, point-injection placement of solutions, and foliar applications of urea have been used to enhance the use efficiency of applied N. Strategies have also been developed to synchronize fertilizer application with crop N requirements to facilitate rapid uptake, reducing N residence time in soil which help limit losses by denitrification and/or NO_3^--leaching (Newbould 1989, Dinnes *et al.* 2002). Many such

agronomic strategies have limitations, as they are associated with additional labor costs (for split applications) and other practical difficulties (Dinnes *et al.* 2002).

Synthetic Chemical Inhibitors: Nitrification inhibitors (NI) are compounds that delay bacterial oxidation of NH_4^+ by depressing activities of soil nitrifiers. In theory, reducing nitrification under conditions where there is a high risk of N loss from NO_3^- leaching or denitrification, should improve nitrogen use efficiency (NUE) (Hughes and Welch 1970, Hendrickson *et al.* 1978, Ranney 1978, Bremner *et al.* 1981, Rodgers 1986). Minimizing the rate of nitrification until the primary crop is in its log phase of growth will give the plants a better opportunity to absorb N while it still remains in the root zone. In addition, rapidly growing crops will absorb more water from precipitation/irrigation, thus lowering the risk of NO_3^- being leached out of the root zone (Dinnes *et al.* 2002, Liao *et al.* 2004).

Numerous compounds have been proposed and patented as nitrification inhibitors (Malzer 1979, McCarty 1999, Subbarao *et al.* 2006a). Only a few nitrification inhibitors, nitrapyrin, DCD (dicyandiamide), and DMPP (3,4-dimethyl

pyrazole phosphate) have been thoroughly evaluated under field conditions (Goring 1962, Guthrie and Bomke 1980, Weiske *et al.* 2001, Zerulla *et al.* 2001, Di and Cameron 2002, Subbarao *et al.* 2006a). However, these synthetic chemical inhibitors have not been widely adopted by production agriculture as they are often not cost-effective. In addition, there are concerns over their lack of consistent performance across diverse agro-climatic and soil environments (McCall and Swann 1978, Gomes and Loynachan 1984, Subbarao *et al.* 2006a).

Slow and controlled-release nitrogen fertilizers: Slow- and controlled-release (SCR) fertilizers are forms of N fertilizer that extend the time of N availability for plant uptake (Shaviv and Mikkelsen 1993, AAPFCO 1997). SCR fertilizers release N into the soil solution at a reduced rate, which is achieved through special chemical and physical characteristics. SCR fertilizers are produced when conventional soluble fertilizer materials are encapsulated or given a protective coating (water-insoluble, semi-permeable or impermeable with pores), which controls water entry and rate of dissolution, thus nutrient release and its availability are more synchronized with the plant's requirements (Fujita *et al.* 1992). Because of the slow release of N to the soil, the availability of NH_4^+ to the nitrifiers is limited, thus N losses during and following nitrification can be reduced. Field evaluation of polymer-coated urea (POCU) indicates that N losses associated with nitrification can be substantially reduced, along with concurrent improvements in N recovery (Shoji and Kanno 1994). Because of the reduced N losses, the crop N application rates for POCU is about 40% less than the recommended level for normal N fertilizers (Balcom *et al.* 2003, Zvomuya *et al.* 2003). However, POCU is 4 to 8 times more expensive than normal urea, thus their adoption in production agriculture is limited (Detrick 1996).

Observations on interaction between plants and nitrification

Several studies have indicated that soil nitrification potential differed in different ecosystems. These differences in nitrification potential are not due to soil physical and chemical characteristics (Clark *et al.* 1960, Robertson 1982a, 1982b, Montagnini *et al.* 1989, Northup *et al.* 1995, Schimel *et al.* 1998, Hattenschwiler and Vitousek 2000, Lata *et al.* 2004, Lovett *et al.* 2004). Often ammonium levels exceeded nitrate concentrations by a factor of ten, indicating that ammonium was not limiting nitrification. Influence of vegetation in inhibiting nitrification was often suspected, but not proven (Lyon *et al.* 1923, Donaldson and Henderson 1990a, 1990b, Steltzer and Bowman 1998, Lewis and Likens 2000, Christ *et al.* 2002, Lovett *et al.* 2004). Certain forest trees (such as *Arbutus unedo*) are reported to suppress soil nitrification and nitrous oxide emissions, and it is hypothesized to be due to biological molecules (*i.e.* allelochemicals such as gallocatechin and catechin) added from the litter (leaves and roots) to the soil (Castaldi *et al.* 2009). Several earlier researchers observed a lack of or slow rate of nitrification in soils collected from certain tropical pasture grasses and for-

est soils; this led to the hypothesis that plant roots may influence nitrification (Jones *et al.* 1994, Laverman *et al.* 2000, Knops *et al.* 2002, Ishikawa *et al.* 2003, Subbarao *et al.* 2006a, Fillery 2007).

Mature grassland ecosystems are suspected of inhibiting soil nitrification (Boughey *et al.* 1964, Lata *et al.* 1999). Natural grasslands dominated by *Andropogon* spp., *Brachiaria humidicola* and *Hyparrhenia diplandra* have most of their inorganic soil N in the NH_4^+ form, a trait which is considered to be an indicator of the ecosystem's maturity (Meiklejohn 1968, Lodhi 1979, Sylvester-Bradley *et al.* 1988, Lata *et al.* 1999, Subbarao *et al.* 2006a, Castaldi *et al.* 2009). There have been a number of attempts to test this hypothesis, but they have achieved relatively little success due to the lack of suitable methodology (Robinson 1963, Munro 1966a, 1966b, Moore and Waid 1971, Purchase 1974, Rice and Pancholy 1974).

Unlike most agricultural systems, certain mature natural ecosystems are known to retain large amounts of added N despite little or no biomass increment, largely through incorporation of N into soil organic matter, but the underlying mechanisms are not well understood (Magill *et al.* 2000). The hypothesis that plants can suppress or stimulate nitrification has been debated for many years due to a lack of convincing evidence from *in situ* studies (Stienstra *et al.* 1994, Lata *et al.* 1999, 2004, Lovett *et al.* 2004, Fillery 2007). Recently, using two ecotypes of the tropical grass *Hyperrhenia diplandra* (high-nitrification ecotype and low-nitrification ecotype), it was demonstrated that nitrification can be stimulated or suppressed depending on the ecotype (Lata *et al.* 2004). Moreover, plant species that dominate some of the climax ecosystems with low nitrification were shown to produce organic compounds that inhibit nitrifier activity (Basaraba 1964, Likens *et al.* 1969, Jordan *et al.* 1979, Donaldson and Henderson 1990a, 1990b, Courtney *et al.* 1991). These inhibitory compounds when added to the soil from roots through exudation suppressed nitrification in the rhizosphere (Jordan *et al.* 1979). The degree of nitrification inhibition appears to increase with the ecosystem's maturity with little or no nitrification occurring in some mature ecosystems (Rice and Pancholy 1972, 1973, 1974, Lodhi 1982, Thibault *et al.* 1982, Baldwin *et al.* 1983, Cooper 1986, Howard and Howard 1991, White 1991, Northup *et al.* 1995, Schimel *et al.* 1996, Paavolainen *et al.* 1998, Ste Marie and Pare 1999, Erickson *et al.* 2000).

Since NH_4^+ assimilation by plants requires four times less metabolic energy than NO_3^-, it is hypothesized that inhibition of nitrification could be an ecological driving force for the development of low NO_3^- climax ecosystems (Rice and Pancholy 1972, Salsac *et al.* 1987, Lata *et al.* 2004). Among the inhibitory compounds proposed, phenolics, alkaloids, isothiocyanates, and terpenoids have received some attention (Lewis and Papavizas 1970, Zucker 1983, Putnam 1988, Choesin and Boerner 1991, Bending and Lincoln, 2000, Bertin *et al.* 2003, Gopalakrishnan *et al.* 2007, Subbarao *et al.* 2008, Zakir *et al.* 2008).

Biological Nitrification Inhibition (BNI)

BNI concept

The ability of certain plant species to release organic molecules/compounds from their roots that specifically inhibit the function of nitrifying bacteria in soil, is a phenomenon termed "biological nitrification inhibition" (BNI) (Subbarao et al. 2006a, 2006b, 2009a, 2009b). A schematic presentation of the BNI concept along with various processes of the soil N-cycle that are impacted is presented in Fig. 1. As nitrification can be the most important process that determines N-cycling efficiency (i.e. the proportion of N that stays in the ecosystem along a complete recycling loop) (Fig. 1), controlling nitrification through suppression will minimize various processes leading to N leakage (i.e. NO_3^- leaching and gaseous nitrous oxide emissions), and facilitate N flow through the NH_4^+ assimilation pathways (Fig. 1). Unlike NO_3^-, NH_4^+ is relatively immobile in the soil, so it can have a longer residence time in the root zone facilitating its uptake. The assimilation of NH_4^+ also requires much less metabolic energy than NO_3^-, leading to a higher NUE in agricultural systems.

Nitrogen-use efficiency ($NUE_{agronomic}$ = dry matter produced per unit of applied N) is a function of both intrinsic N-use efficiency ($NUE_{intrinsic}$) and total N uptake. Intrinsic N-use efficiency ($NUE_{intrinsic}$ i.e. dry matter produced per unit N uptake) of a plant is a physiologically conservative function (Glass 2003), thus difficult to manipulate genetically. Improvements in $NUE_{agronomic}$ mostly come through improvements in crop N uptake (Finzi et al. 2007). As described earlier, BNI function can improve N uptake due to its inhibitory effect on nitrification, which can positively influence $NUE_{agronomic}$ in production systems (Subbarao et al. 2006a). Recent modeling studies indicate that by inhibiting nitrification, nitrogen recovery, and hence nitrogen-use efficiency can be improved substantially. Primary productivity was positively impacted in tropical savannas dominated by native African grasses, Hyparrhenia diplandra that appears to have a great ability to suppress nitrification (Boudsocq et al. 2009).

Methodology to detect biological nitrification inhibitors (BNIs) in plant-soil systems

A bioluminescence assay using a recombinant strain of Nitrosomonas europaea was adopted to detect nitrification inhibitors released from plant roots (hereafter referred to as BNI activity) (Iizumi et al. 1998, Subbarao et al. 2006b). The recombinant strain of N. europaea carries an expression vector for the Vibrio harveyi luxAB genes, 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 demonstrated to be linear using a synthetic nitrification inhibitor, allylthiourea (AT) (Subbarao et al. 2006b). The inhibition caused by 0.22 mM AT in this assay, about 80% inhibition in bioluminescence and NO_2^- production, is defined as 1 ATU (allylthiourea unit) (Subbarao et al. 2006b). Using the response to a concentration gradient of AT (i.e. dose-response standard curve), the inhibitory effect of test samples (for e.g. root exudates or plant or soil extracts) can be expressed and compared in ATU units. With these methodological tools, it is now possible to determine and compare the BNI capacity of crops or pastures (Subbarao et al. 2006b).

Distribution of BNI function in plants

An evaluation of tropical pastures, cereal crops and legumes indicated that there is wide-spread BNI capacity among plant species (Subbarao et al. 2007b). The greatest BNI capacity was found in Brachiaria spp.. BNI capacity varied widely among plant spp.; there were substantial inter-specific differences among tropical pasture grasses. Pastures of B. humidicola and B. decumbens that are highly adapted to the low-N production environments of the South American savannas (Rao et al. 1996), showed the greatest BNI capacity (Subbarao et al. 2007b). In contrast, P. maximum, which is adapted to high N input showed the least BNI capacity (Rao et al. 1996, Subbarao et al. 2007b). Among the cereal crops evaluated, only sorghum showed significant BNI capacity (Subbarao et al. 2007b, Zakir et al. 2008); while other important cereal crops such as rice, maize and the Triticeae, including wheat and barley, lacked detectable BNI capacity (Subbarao et al. 2007b).

Inhibition of nitrification (i.e. BNI capacity in roots) is likely to be part of an adaptation mechanism to conserve and use N efficiently in natural systems having N-limiting environments (Lata et al. 2004, Subbarao et al. 2006a). Thus N stress could be one of the dominant forces driving the evolution of BNI function (Rice and Pancholy 1972, Lata et al. 2004). It is not surprising then that legumes did not show appreciable BNI capacity. For legumes, it is likely that the BNI attribute would have little or no adaptive value due to their ability to fix N symbiotically. Conserving N may not offer much of an advantage for legumes as it may attract non-legumes as competitors.

In fact, our preliminary studies indicate that soybean root exudates stimulate nitrification in laboratory soil incubation studies (Subbarao et al. 2007c). Several forest systems dominated by leguminous trees (Acacia mangium and A. auriculaeformis) have soils that either had no influence or stimulated nitrification. In contrast, forests dominated by non-legume trees such as Eucalyptus citriodora, Pinus elliotii, and Schima superba, have low nitrification rates in their soils (Li et al. 2001). Importantly, recent studies indicate that a wild relative of wheat, Leymus racemosus, has BNI capacity similar to that of Brachiaria spp., with BNI-activity release rates ranging from 20 to 30 ATU g^{-1} root dry wt d^{-1} (Subbarao et al. 2007e).

Regulatory requirements of the BNI function

Synthesis and release of BNIs is a regulated attribute in B. humidicola (Subbarao et al. 2007a). To some extent, the

release of BNIs is related to plant N status (Subbarao *et al.* 2006b). In particular the form of N applied (*i.e.* NH_4^+ *vs.* NO_3^-) 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, 2007e). Plants grown with NO_3^- as their N source did not release BNIs from roots (Subbarao *et al.* 2007a). The release of BNIs from roots was observed only from plants grown with NH_4^+ as their N source (Subbarao *et al.* 2007a, 2007e, 2009a, 2009b). Further, even for plants grown with NH_4^+, the presence of NH_4^+ in the rhizosphere is critical for the synthesis and release of BNIs from their roots (Subbarao *et al.* 2007a, 2007e). Though high levels of BNIs were detected in the root tissues of NH_4^+-grown plants, their release was observed only when their roots were exposed to NH_4^+ (Subbarao *et al.* 2007a, 2007e, 2009a, 2009b).

Recent reports characterize the plant's regulatory role in the expression of BNI in *B. humidicola*. The presence of NH_4^+ and the physiological consequences associated with its uptake (such as acidification of the rhizosphere) on the rhizosphere appears to play an important role in the synthesis and release of BNIs from roots (Subbarao *et al.* 2007a, 2009a, 2009b). The availability of NH_4^+ in the soil from either soil organic N mineralization or through the application of N-fertilizers such as urea or ammonium sulfate can enhance nitrifiers' activity and stimulate growth (Robinson 1963, Woldendorp and Laanbroek 1989). The regulatory role of NH_4^+ in the synthesis and release of BNIs suggests its possible adaptive role in protecting NH_4^+ from nitrifiers, a key factor for the successful evolution of BNI capacity as an adaptation mechanism (Subbarao *et al.* 2007a).

Stability of BNIs in soil systems

The BNI activity of roots was determined based on the inhibitory effect of root exudates (*i.e.* compounds released from roots) on *Nitrosomonas* biological activity, during a 30-min incubation period in the assay. However, to have a stable inhibitory effect on soil nitrification, the inhibitory compounds (*i.e.* BNIs) released from the roots must be stable in the soil environment for several weeks. This was confirmed by adding BNIs to the soil along with NH_4^+ as the N source to determine their inhibitory effects on soil nitrification over a 60 d period (Subbarao *et al.* 2006b, Gopalakrishnan *et al.* 2009).

These results indicated that BNIs released from *B. humidicola* roots are effective in inhibiting nitrification for at least 60 days (Subbarao *et al.* 2006b). Also, it is important to note that the inhibitory compounds in the soil need to reach a threshold level of about 5 ATU g^{-1} soil before the inhibitory effect becomes evident with a near total suppression of nitrification at about 20 ATU g^{-1} soil (Subbarao *et al.* 2006b). Subsequent studies indicated that BNIs partially lose their effectiveness in the soil after 80 days, and that the inhibitory effect is completely lost after 100 days (Subbarao *et al.* 2006b).

Molecules with BNI potential

Plants are known to release a wide range of substances that have biological activity (Bremner and McCarty 1988, Bending and Lincoln 2000, Subbarao *et al.* 2006a, Raaijmakers *et al.* 2009). These include molecules released from plant roots that belong to phenolic, fatty acid, isothiocyanate, and terpene groups (Choesin and Boerner 1991, Bennett and Wallsgrove 1994, Langenheim 1994, Bending and Lincoln 2000, Kraus *et al.* 2003, Subbarao *et al.* 2006a, 2009a, 2009b). The compounds responsible for BNI were not elucidated until recently despite the phenomenon having been speculated to occur since the early 1960s based on empirical field studies (Subbarao *et al.* 2006a). Recently, several nitrification inhibitors belonging to different chemical classes were successfully isolated and identified from plant tissues or root exudates using bioassay-guided purification with a recombinant *Nitrosomonas europaea* assay (Fig. 2) (Subbarao *et al.* 2006b, Gopalakrishnan *et al.* 2007, Subbarao *et al.* 2008, Zakir *et al.* 2008).

BNI compounds in the aerial parts of *B. humidicola* were identified as the unsaturated free fatty acids, linoleic acid and α-linolenic acid (Subbarao *et al.* 2008). They are relatively weak inhibitors with IC$_{50}$ values of 3×10^{-5} M; while the IC$_{50}$ value of synthetic nitrification inhibitor 1-allyl-2-thiourea is 1×10^{-7} M. However, other free fatty acids having different chain lengths or numbers of double bonds, *e.g.* stearic, oleic, arachidonic and *cis*-vaccenic acid did not show inhibitory activity, indicating that there are specific chemical structural requirements to affect *Nitrosomonas* function (Subbarao *et al.* 2008). BNI compounds linoleic acid and α-linolenic acid may have the size and shape suitable to be an inhibitor. These two BNI compounds possibly inhibit both ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) enzymatic pathways, which catalyze essential reactions of the ammonia oxidation process in *N. europaea* (Subbarao *et al.* 2008). When linoleic acid and α-linolenic acid were mixed with soil, the mixture suppressed nitrification for several months (Subbarao *et al.* 2008). The BNIs released (determined as BNI activity) from roots of *B. humidicola* and *Leymus racemosus* (wild wheat) appears to block both AMO and HAO enzymatic pathways with equal effectiveness (Subbarao *et al.* 2007a, 2007e) (Fig. 3). In addition, BNIs could also disrupt the electron transfer pathway from HAO to ubiquinone and cytochrome (which needs to be maintained to generate reducing power, *i.e.* NADPH), that is critical to the metabolic functions of *Nitrosomonas* (Fig. 3), thus requires further research for clarity on this issue (Subbarao *et al.* 2009b). This is in contrast to synthetic nitrification inhibitors AT, nitrapyrin and DCD, which inhibit nitrification by suppressing only the AMO enzymatic pathway in *Nitrosomonas* (Subbarao *et al.* 2007a, 2007e) (Fig. 3).

In the root tissue of *B. humidicola*, two phenylpropanoids, methyl-*p*-coumarate and methyl ferulate (Gopalakrishnan *et al.* 2007) were found to be the major BNI compounds, in place of the two free fatty acids found
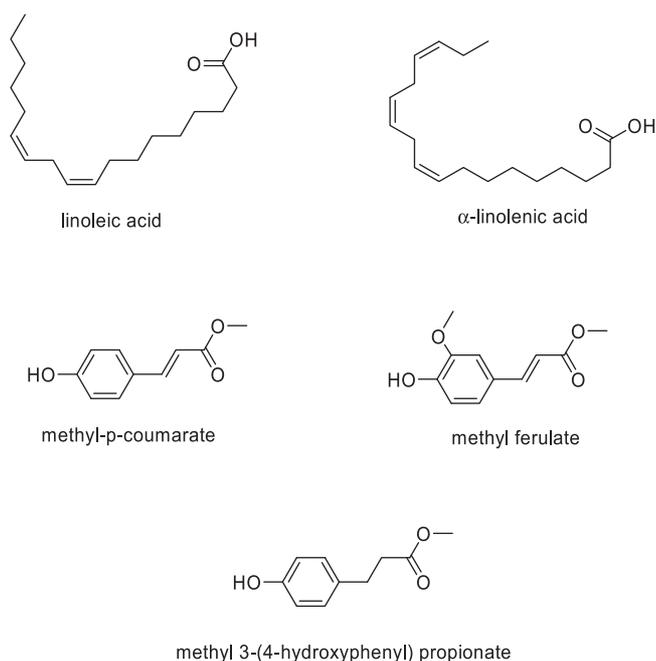
Fig. 2. Chemical structures of recently reported BNI compounds from plants

are involved in lignin biosynthesis, showed no inhibitory activity at concentrations less than 1×10^{-2} M (Gopalakrishnan *et al.* 2007). It is expected that *B. humidicola* releases methyl-*p*-coumarate and methyl ferulate, or simple metabolites derived from these BNI compounds, into the soil environment *via* degradation of root tissues in these pasture systems (Gopalakrishnan *et al.* 2007). However, these BNI compounds mentioned above (Fig. 2) were not detected in root exudates; thus BNIs released from roots are different from those isolated from the shoot and root tissues (Gopalakrishnan *et al.* 2007, Subbarao *et al.* 2008). It is expected that a major portion of the inhibitory effect (*i.e.* BNIs added to the soil) in *Brachiaria* systems come from root release (*i.e.* exudates); however, these BNIs need to be isolated and their chemical identities are yet to be determined (Subbarao *et al.* 2007a). From the root exudates of hydroponically-grown *Sorghum bicolor*, a BNI compound was isolated and identified as the phenylpropanoid, methyl 3-(4-hydroxyphenyl) propionate [MHPP]. The IC_{50} value for MHPP is approximately 9×10^{-6} M, which is similar to those of BNI compounds methyl-*p*-coumarate and methyl ferulate (Zakir *et al.* 2008).

Field studies showing BNI activity

To demonstrate BNI function under field conditions, field plots planted with *B. humidicola* (a high-BNI capacity species), *Panicum maximum* (a low-BNI capacity species) and soybean (a species that lacks BNI capacity) were fertilized with ammonium sulfate (200 kg N ha^{-1} y^{-1}) and monitored monthly for soil nitrification potential and N$_2$O

in the aerial part of the plant. 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 acids namely *p*-coumaric acid and ferulic acid, which
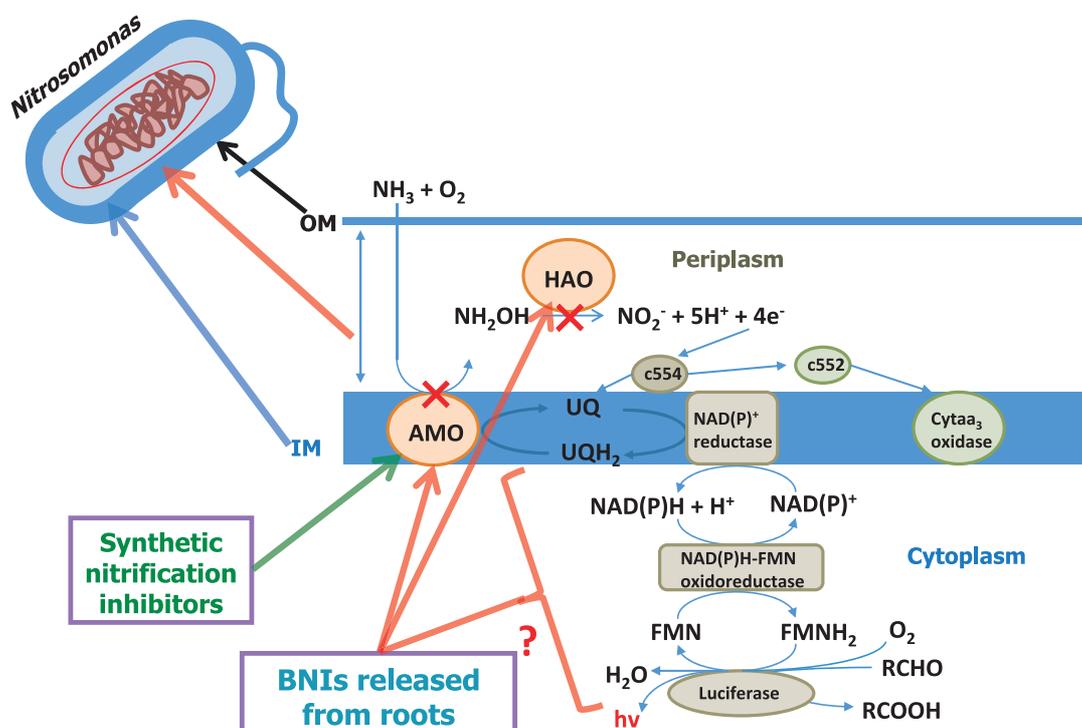

Fig. 3. Mode of inhibitory action by synthetic nitrification inhibitors and BNIs released from roots of *B. humidicola* (based on Iizumi *et al.* 1998, Subbarao *et al.* 2007a).

emissions for three years (Subbarao *et al.* 2007d). Nitrous oxide (N_2O) emissions were suppressed in the field planted with *B. humidicola* (Subbarao *et al.* 2007d, 2009b). In contrast, soybean that lacked BNI capacity had N_2O emissions nearly 10-fold higher than the *B. humidicola* plots (Subbarao *et al.* 2007d, 2009b).

Similarly, soil nitrification rates from field -plots of *B. humidicola* were substantially lower than those of soybean or *P. maximum* (Subbarao *et al.* 2007d, 2009b). Further, laboratory soil incubation studies provided direct evidence that adding soybean root exudate to the soil stimulated N_2O emissions. In contrast, root exudate from *B. humidicola* suppressed soil N_2O emissions (Subbarao *et al.* 2007c). Soil incubation studies showed that high-BNI capacity genotypes in 10-year old *B. humidicola* fields suppressed soil nitrification more effectively (based on laboratory soil incubation studies) and had nearly five times less soil NO_3^- (after the 60-d incubation period) than low-BNI capacity genotypes (Subbarao *et al.* 2007d). These results indicate that plants can suppress or stimulate nitrification, a biological attribute that could be exploited to control nitrification in agricultural systems (Subbarao *et al.* 2007d, 2009b).

Potential for genetic interventions to improve BNI capacity in the Triticeae

Members of the Triticeae are known to release a number of different biological molecules from their roots such as phenolic acids (*p*-hydroxybenzoic, syringic, vanillic, ferulic, *p*-coumaric, chlorogenic, caffeic, *p*-hydroxybenzaldehyde, gallic and protocatechuic), hydroxamic acids (BOA, DIBOA, DIMBOA, AZOB, DIMBOA-glc, MBOA and Cl-MBOA), alkaloids (hordenine and gramine) and quinones (sorgoleone, and *p*-benzoquinones). These biological molecules have a diverse range of chemical structures and have been identified from both cultivated and wild Triticeae (Bennett and Wallsgrove 1994). These compounds, when released from roots, can play a great variety of roles in the rhizosphere, such as nutrient acquisition, pest and pathogen defense, and most likely other unknown functions. Some of these compounds likely have BNI properties that should be evaluated and characterized. This information could be critical to the development of genetic strategies to introduce economically and environmentally important levels of BNI capacity to the roots of economically important Triticeae, such as wheat, barley and rye.

The existence of genotypic variability is a prerequisite for the genetic improvement of any trait by a breeding program. Significant genotypic variability exists for the BNI capacity in roots of *B. humidicola* (Table 1). Specific BNI activities (ATU g^{-1} root dry wt d^{-1}) ranged from 7.1 to 46.3 ATU indicating that there may be potential for genetic improvement of BNI capacity by selection and intermating. In cultivated wheat, preliminary evaluations suggest a lack of significant BNI capacity (Fig. 4) (Subbarao *et al.* 2007e). Recent results, however, indicate that the roots of a wild relative of

Table 1. Genotypic variation in BNI released from roots of *B. humidicola* accessions. Four plants per pot were grown for 180 d before collecting root exudate (Subbarao *et al.* 2007b)

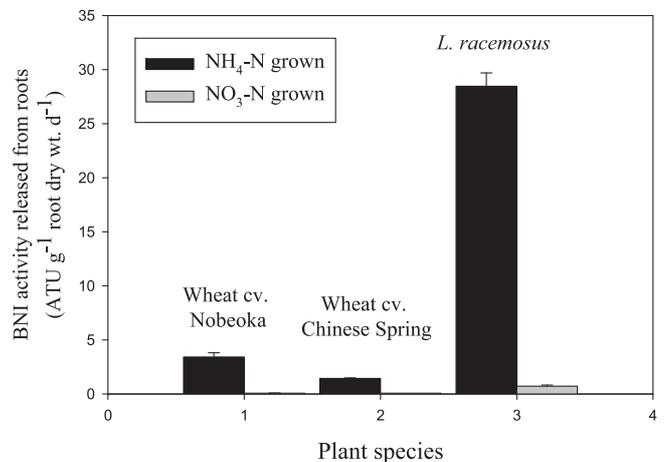| Serial No.	Accession No.	Total BNI released from four plants (ATU d^{-1})	Specific BNI (ATU g^{-1} root dry wt. d^{-1})
1	CIAT 26159	126.2	46.3
2	CIAT 26427	118.5	31.6
3	CIAT 26430	151.0	24.1
4	CIAT 679	68.8	17.5
5	CIAT 26438	93.5	6.5
6	CIAT 26149	22.3	7.1
7	CIAT 682	53.4	7.5
8	*P. maximum*	0.6	0.1
	LSD (0.05)	21.7	6.0

Fig. 4. BNIs (biological nitrification inhibitors) released from roots (*i.e.* root exudates) of two cultivars of wheat and their wild relative *L. racemosus*; plants were grown with either NH_4^+ or NO_3^- as the nitrogen source; root exudate was collected from intact roots in aerated distilled water over a 24 h period; vertical bars represent Fisher LSD ($P<0.001$) for the interaction term (N source × species) (based on Subbarao *et al.* 2007e)

wheat, *L. racemosus*, possess a high-BNI capacity (Fig. 4). Inhibitors released from roots of this wild wheat have been shown to effectively suppress soil nitrification for more than 60 days (Subbarao *et al.* 2007e). Using chromosome-addition lines derived from the hybridization of this wild relative (*L. racemosus*) with cultivated wheat, it was shown that genes conferring high-BNI capacity are located in chromosome Lr#n, and can be successfully introduced into and expressed in cultivated wheat (Fig. 5) (Subbarao *et al.* 2007e). These results indicate there is great potential for developing the next-generation of wheat cultivars with root BNI-capacity sufficient to suppress nitrification in wheat production systems. It needs to be emphasized however, that during this study only one accession of *L. racemosus* was evaluated and characterized for BNI capacity. Available acessions of *L. racemosus* are yet to be characterized for BNI-capacity, as some may well be superior for this trait compared to the accession evaluated. Further, several other wheat wild
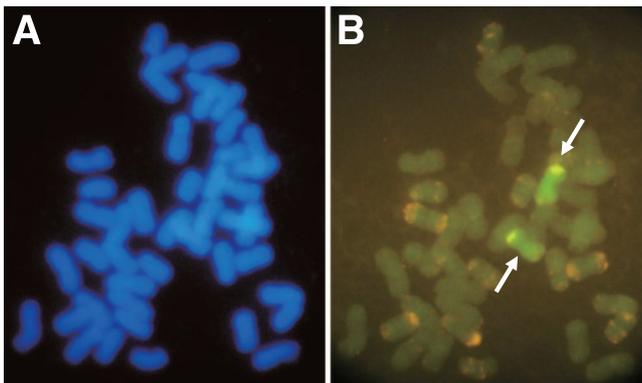
Fig. 5. Karyotype analysis of DALr#n, a chromosome-addition line derived from *L. racemosus* × *T. aestivum*; A. DAPI staining revealed 44 chromosomes; B. The probe of *L. racemosus* genomic DNA (green) and TaiI and Afa family repetitive sequences showed the presence of two Lr#n chromosomes; arrows indicate Lr#n chromosomes (based on Subbarao *et al.* 2007e).

relatives/progenitors in the Triticeae, need to be evaluated systematically and their BNI-capacity characterized to develop a more thorough understanding of the BNI trait/function and to identify multiple sources of high-BNI capacity for wheat improvement. In addition, evaluation of several mapping populations generated using *Leymus* sp. could provide valuable information that is expected to prove helpful in developing genetic strategies for improving BNI capacity of cultivated wheat. For example, the available mapping population from the cross *L. cinereus* × *L. triticoides* (Wu *et al.* 2003, Bushman *et al.* 2008), could provide a useful system for genetic research on the BNI trait.

Improvement of BNI capacity in wheat and barley

Demonstrating that the high-BNI capacity of *L. racemosus* can be expressed in chromosome addition line of cultivated wheat provides the opportunity to further explore the introduction of the BNI trait into elite wheat cultivars. As wheat utilizes nearly a third of the total global nitrogen fertilizer output (Raun and Johnson 1999), introducing high-BNI capacity into cultivated wheat could have a large impact on reducing nitrogen leakage from wheat production systems globally. However, the alien chromosome of this chromosome addition line also carries many undesirable traits that reduce grain yield potential. This is an example of negative linkage drag, which is commonly observed in products of crosses, including early-generation backcrosses, of elite cultivars with wild relatives or other exotic germplasm. It will be necessary to transfer to wheat only small segment/s of this *L. racemosus* chromosome containing favorable alleles of genes controlling the BNI trait in order to minimize negative linkage drag that is associated with this introgression.

There are various chromosomal manipulation methods to induce a translocation between wheat and alien chromosomes, including irradiation (Sears 1993) and the use of a

gametocidal chromosome system (Endo 2007). Reciprocal exchange of alien chromosome segments with the corresponding wheat chromosomes (maintaining homoeology) without disrupting the genetic balance would be preferred. Centromeric or robertosonian 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 the BNI trait belongs to 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 can be done by crossing the Lr#n chromosome addition line with group 3 and group 7 monosomic lines of wheat, in which one of the group 3 or group 7 chromosomes is missing, to produce an F1 hybrid. Translocations can be obtained in the F2 generation at a certain frequency (probably low) because of spontaneous breakage and fusion of Lr#n and group 3 or 7 chromosomes of wheat that occurs during meiosis in the F1 generation. Development of a stable homozygous translocation stock with BNI capacity will likely require several additional generations of selfing and selection (for both BNI capacity and good seed set). The resulting centromeric translocation homozygote with high-BNI capacity could be readily utilized as a new cultivar if the genetic background of the parental chromosome addition line and monosomic line are sufficiently elite and the translocation line had lost the undesirable traits expressed in the original Lr#n chromosome addition line. However, it is likely that the stable translocation will then need to be transferred to more elite backgrounds by a series of backcrosses. Historically, one good centromeric translocation can have a huge impact on wheat breeding (Lukaszewski 2000, Singh *et al.* 2006).

During the 1980's it was shown that more than 50% of wheat varieties bred by CIMMYT had the 1RS.1BL translocation involving the short arm of the 1R chromosome from rye (*Secale cereale* L.), which has provided multiple disease resistance (Singh *et al.* 2006). 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. This can be achieved by suppressing the effect of the *Ph1* gene in wheat (which prevents homoeologous recombination between wheat and alien chromosomes), using the *ph1* gene mutant of common wheat (Sears 1977) as has been demonstrated in wheat-rye crosses (Lukaszewski 2000).

Introduction of the BNI trait from *L. racemosus* to barley would be difficult following this strategy, because diploid barley is very sensitive to chromosome manipulation (compared to tetraploid durum wheat 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 the *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 would 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.

Deployable genetic tools for introduction of high-BNI capacity into wheat and barley

With the plethora of forward and reverse genetic techniques at our disposal to characterize genotypic variation and generate isogenic lines (transgenics, mutants, RNAi, smiRNA) and near-isogenic lines (NILs) differing in their capacity to modify the rhizosphere environment (Hash *et al.* 2002, Neumann *et al.* 2009), it is possible to understand the genetic control of BNI function, and deploy it as a trait into elite wheat/barley germplasm. This will require a) availability of adequate genetic variation in BNI capacity of roots within crossable crop germplasm, b) optional identification of candidate genes controlling the trait and heterologous expression to verify their role(s) in BNI function, and c) introgression of genes controlling the trait into elite germplasm.

The availability of the entire genome sequences of *Arabidopsis* and a range of crop and agricultural plants (rice, sorghum, potato, barley, tomato, *Populus*, *Medicago*, *Lotus*, papaya and maize) could facilitate the use of tools such as 'genome wide expression profiling' to identify candidate genes controlling mechanisms associated with the rhizosphere (Vij and Tyagi 2007). These emerging tools can be applied for the identification of candidate genes associated with the BNI trait, followed by gene function analysis using mutant populations (if available) (Caldwell *et al.* 2004). Once verified, expression of 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 large numbers of transcriptomic studies on interactions between plants and soils, to date only a handful have identified genes with function that have been successfully deployed in elite germplasm (Oh *et al.* 2007). Indeed, in most cases successful deployment of the trait has preceded identification of the genes controlling it as plant breeding can be very effective with a "black box" approach provided that there is adequate genetic variation and an efficient protocol for screening large numbers of individuals (or progenies) for the trait of interest (*e.g.* squeezing mature spikes of triticale plants standing in the field to rapidly assess seed set). With the rapid advances in sequencing and data analysis capability (Varshney *et al.* 2009), however, a transcriptomic approach could have great potential for identification of candidate genes associated with BNI

function. In addition, integrated map-based approaches can be adopted for traits for which high throughput phenotyping systems are available (Sasaki *et al.* 2004, Raman *et al.* 2006, Magalhaes *et al.* 2004, 2007).

Use of plant populations to understand the genetic control of BNI in the Triticeae

Significant genotypic variation for traits associated with the rhizosphere has been reported for various crops. Traditional varieties/landraces often have traits that do not exist in elite germplasm (Manske *et al.* 2000). Wild progenitors and wild relatives have been extensively used in wheat as sources of traits such as disease resistance and tolerance to salinity and aluminum tolerance (Friebe *et al.* 1996, Munns *et al.* 2000). The discrepancy between wild relatives and elite germplasm with regard to rhizosphere traits is often attributed to the impact of decades of breeding and selection under agronomically favorable growing environments (*i.e.* water and nutrient sufficiency) (Buso and Bliss 1988, Rengel and Marschner 2005).

For wheat, the availability of the high-BNI *L. racemosus* chromosome addition line can provide a starting point for expression profiling studies to identify candidate genes for the BNI trait in the Triticeae. However, there is likely to be substantial reduction in genetic recombination near the introgressed chromosome segment even in derivatives of the chromosome addition line carrying the gene(s) controlling BNI on a much smaller block of *L. racemosus* chromatin. Thus conventional forward genetic approaches are not likely to help much in identifying the genes involved. Nevertheless, marker-based approaches for the deployment of the *L. racemosus* chromosome segments containing favorable alleles for genes controlling BNI in diverse cultivated wheat backgrounds can be an option once linkages with deleterious traits from *L. racemosus* have been overcome. As the genomic regions associated with BNI variation are likely to differ from cultivated wheat for DArT (diversity array technology), SNP (single nucleotide polymorphism), 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.

Another possibility for genotype screening is to use association mapping populations, which will allow screening of genetically diverse elite cultivars for specific BNI traits and association of variation in these traits with chromosome maps of the cultivars annotated with several thousand SNP and/or DArT markers. This will facilitate the rapid identification of QTLs and specific markers for genes. However, this is contingent upon the availability of a) a trait-phenotyping protocol that can handle large numbers 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 high enough to detect linkage genetic disequilibrium between cultivars having high and low values

for the target trait. If the trait of interest has been 'bred out' of the elite cultivars, or if there is relatively limited marker variation between the cultivars (even when target trait variation is present), in the genomic regions in which the genes controlling BNI traits are located, then the amount of relevant variation available may be inadequate for association mapping unless the study is based on evaluation of a panel of landrace germplasm accessions of diverse origin. Use of association mapping populations has previously elucidated potential QTLs for unknown N- and P-use efficiency mechanisms in wheat (Liao *et al.* 2008) and for resistance or tolerance to barley yellow dwarf virus in barley (Kraakman *et al.* 2006).

Other populations that can be used for assessing the impact of plant traits on rhizosphere processes include populations saturated with mutations. Such mutant populations exist not only in *Arabidopsis* but also in wheat, barley and sorghum. These populations have their genomes saturated with mutations (produced by chemical, radioactive or molecular techniques) either "knocking-out" genes or up- or down-regulating genes downstream of the mutation. Such an approach produces thousands of individual mutants and subsequent phenotypic screens to identify a specific trait of interest can allow rapid elucidation of the genes controlling expression of that trait. However, with the current phenotyping tools for the BNI trait, characterizing thousands of individual mutants for the BNI function could be a daunting task. Nevertheless, it is possible to use such an approach (with the assumption that refinement of phenotyping tools will be possible for BNI in the future) to identify the gene(s) controlling BNI traits. Several such mutant populations have been used to establish reverse genetic tools [TILLING (targeted induced local lesions in genomes) populations] that can be used to identify small numbers of mutants having DNA sequence variation in a particular candidate gene for the trait of interest (McCallum *et al.* 2000). This small subset of the mutant population can then be screened phenotypically to determine whether it includes individuals with an interesting trait variation (Till *et al.* 2007, Xin *et al.* 2009). The BNI screening protocol currently available should be adequate for screening the modest number of mutants in a particular candidate gene that might be identified from such a TILLING population, so this approach could be used to assess mutants in a small number of genes previously identified by genome-wide expression profiling studies.

Such an approach would be particularly relevant in barley as the extremes of an initial screen for BNI (Subbarao and George, unpublished) were in genotypes *cv* Optic (high-BNI) and *cv* Bowman (low-BNI) that have pre-existing mutant populations (Caldwell *et al.* 2004, White *et al.* 2009). Screening of these populations for loss (Optic) of function (which is more likely) or gain (Bowman) of function (which is less likely) will help in elucidating genetic control of the trait. Examples of successful uses of this approach include the use of mutants to estimate the contribution of ammonium or urea transporters to nitrogen uptake (Yuan *et al.* 2007,

Kojima *et al.* 2006). Similarly, the contribution of root hairs to nutrient acquisition was investigated by use of barley mutants without root hairs (Gahoonia *et al.* 2004).

A conventional bi-parental mapping population of about 400 double haploid lines derived from the cross of barley cultivars Optic (high-BNI) and Bowman (low-BNI), genotyped across the genome with DArT, SNP, AFLP, and/or STMS markers could be a complementary tool for determining the genetic basis of BNI variation in this species, assuming that the BNI phenotyping protocol can be modified to permit phenotyping of the large number of samples that is required. Such conventional bi-parental mapping populations have contributed to the determination of the genetic basis of Al tolerance in both wheat (Sasaki *et al.* 2004) and sorghum (Magalhaes *et al.* 2007), which is conferred by root exudation of organic acids.

Genes identified using these population screening approaches can then be validated by over-expression using transgenic approaches coupling the gene with specific promoters, or by monitoring their loss of function after down-regulating the gene of interest by RNAi (RNA-interference) or with the use of smiRNA (synthetic micro RNA) technologies (Miki and Shimamoto 2004, Alvarez *et al.* 2006). Some traits that have a role in influencing the rhizosphere are often thought to be highly heritable and simply controlled by one or two genes of large effect [(*e.g.* tolerance to Al toxicity in wheat) (Delhaize *et al.* 1993, Sasaki *et al.* 2004, Raman *et al.* 2005, 2006) and sorghum (Magalhaes *et al.* 2004, 2007) or heavy metal tolerance in *Thlaspi* (Eapen and D'Souza 2005)]. If genetic control of BNI proves to be simple, then such transgenic, RNAi or smiRNA approaches would be appropriate for candidate gene validation.

However, it is important to consider that traits such as BNI may be multi-genic in nature, and thus more difficult to manipulate by altering single genes alone. For example, tolerance to Al toxicity in both wheat and sorghum is simply inherited, with a large portion of the available genetic variation controlled by a small number of genes having large allelic differences. However, this same trait is inherited in a more quantitative manner in maize (Magalhaes *et al.* 2004). In such cases, individual candidate genes that might be targeted and/or identified from a genome-wide expression profiling study, association mapping study, or TILLING study, are not likely to account for large portions of the observed phenotypic variation for the target trait. Further complexity due to gene by environment (including genetic background) interactions can markedly increase the difficulty of trait deployment into elite genetic stocks. This was highlighted recently with attempts to improve nutrient efficiency traits using a single QTL (Liao *et al.* 2008, George *et al.* "*in press*"). In species where BNI capacity is found to be largely under quantitative genetic control or shown to have strong genotype by environment interactions, it will likely to be difficult and/or expensive to deploy this trait in a wide range of elite cultivars. There are thus, many challenges ahead for successful deployment of BNI function as a target trait in

routine breeding programs to control nitrification in wheat and barley production systems.

Concluding remarks and future outlook

Modern agricultural systems are dependent on large mineral N inputs as the primary N source for crop production (De Wit *et al.* 1987). In addition, the changes in cropping systems (*i.e.* moving away from diverse crop rotations to monocultures) and crop management practices have resulted in the development of high-nitrifying environments in modern production agriculture (Poudel *et al.* 2002). Many modern high-yielding crop varieties bred for these production environments have inadvertently been selected for their preference for NO_3^- over NH_4^+. It appears that most staple food crops completely lack BNI capacity or lack sufficient BNI capacity to control nitrification. These factors have possibly contributed towards evolution of the current nitrification-dominated N-cycle in agricultural systems, which is an inefficient and extremely leaky system that is already causing serious environmental problems (Tilman *et al.* 2001, Hungate *et al.* 2003, Schlesinger 2009). Genetic exploitation of the BNI capacity in relatives of major crops and pastures could provide a range of biological options to deliver BNIs to the nitrifier sites as a means to reduce soil nitrification. Developing the next-generation of crops with built-in genetic BNI capacity should be an integral part of strategies to improve N-cycling efficiency in production agriculture and reduce the negative impact of human activities on our environment.

Recent findings indicate that a number of diverse chemical molecules have an inhibitory effect on nitrification, that these can be produced by plants, and that they could be released into the soil to control nitrification. The AMO (the critical enzyme involved in *Nitrosomonas* for ammonia oxidation) enzyme has affinity to a wide range of substrates apart from ammonia as its primary substrate, thus it is a very unique enzyme indeed (Hauck 1980, McCarty 1999). This fundamental weakness in the functioning of AMO enzyme opens the way for nitrifiers to be influenced by a wide range of molecules with diverse chemical structures that could act as inhibitors of nitrification (Hauck 1990, Subbarao *et al.* 2006a). This has been exploited during the development of chemical nitrification inhibitors (Subbarao *et al.* 2006a). Recent findings indicate that biological molecules with diverse chemical structures inhibit nitrifiers' activity by possibly interfering with the functioning of the AMO enzymatic pathway. Thus there is enormous potential for the identification of new biological molecules with novel chemical structures that are yet to be discovered and identified as powerful nitrification inhibitors. These BNIs can be exploited by both plant scientists and natural product chemists to develop a range of biological and chemical strategies for controlling nitrification in agricultural systems. Moving away from the current NO_3^- dominated production systems will contribute towards development and adoption of more environmentally responsible and sustainable production systems. Such production systems would serve to reduce the undesirable impact of N-fertilizers on the global environment.

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