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No.3

BNI Technology

a genetics-based solution to global challenges in the 21st century

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a genetics-based solution to global challenges in the 21st century

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Preface

Biological nitrification inhibition (BNI) is a special function of plants enabling them to suppress the nitrification process in the soil. It was discovered as a natural phenomenon in grasslands of Latin America and the wet savannah of West Africa. Thanks to great efforts by Japan International Research Center for Agricultural Sciences (JIRCAS) researchers led by Dr. Guntur V. Subbarao, this phenomenon had been intensively investigated and developed into an agricultural technology. BNI technology can contribute to the resolution of urgent global challenges, such as food production and climate change mitigation, through more efficient use of nitrogen in agriculture.

Ministry of Agriculture, Forestry and Fisheries, Japan (MAFF) has launched "Strategy for Sustainable Food Systems (MIDORI)," which has several numerical goals for 2050, one of which is to reduce chemical fertilizer use by 30%. BNI technology, as a Japan-made innovation, is placed in the "Sustainable Production" part, for the development of "super" plant varieties with high nitrogen-use efficiency and less environmental burden.

This report describes the background, concept, mechanisms, uniqueness, opportunities, and constraints of BNI technology. Because BNI is a new technology, the author describes specialized and professional areas for a better understanding of BNI. If there are any difficulties, it falls under the author's responsibility. It is the author's great pleasure to help readers understand BNI technology.

Abstract

The nitrogen cycle of the Earth has already been extended beyond its planetary boundary, as seen by the nitrogen pollution in terrestrial ecosystems and climate change at the global scale. A major reason for this is the high dependence of modern agriculture on chemical nitrogen fertilizers, a technology that supports the Green Revolution. Because of the rapid nitrification process in soil, more than half of the applied nitrogen fertilizer is not utilized by crops and is lost to the environment; thus, nitrogen-use efficiency (NUE) is low in agricultural production systems. Suppression of nitrification is key to improving NUE, allowing for more N to stay in the soil and to be used by crops. This will contribute to resolving the two major global challenges of increasing crop production and mitigating climate change, respectively.

Biological nitrification inhibition (BNI) is a function of plants that suppresses the nitrification process using special chemical compounds, that is, biological nitrification inhibitors (BNIs) from the roots to block the key enzymes of the nitrification process. BNIs are thought to be superior to synthetic nitrification inhibitors (SNIs), in terms of costs, effectiveness, safety for both humans and the environment, and accessibility to farmers. Plants and crops with high BNI capacity have been found and most of them belong to the Poaceae (Gramineae) family. Their nitrification-inhibiting compounds (BNIs) have been identified, brachialactone is from *Brachiaria humidicola*, sorgoleone is from sorghum, and zeanon is from maize. Regarding wheat, one of the most fertilized crops in the world, the wild relative *Leymus racemosus* was found to have a high BNI capacity, which was successfully transferred into international wheat varieties, such as Munal, by chromosome substitution. BNI-enabled Munal has been characterized as having the ability to suppress nitrification and utilize soil nitrogen more efficiently than original Munal. The BNI-Munal is currently used as a donor material for the development of BNI-enabled local elite wheat varieties in India.

The BNI-technology is a plant-based new technology; thus, recognizing its possibilities and limitations by ex-ante impact analysis is necessary. A map of the suitable regions was made for BNI-wheat with existing information on the performance of BNI-wheat; for example, preference for acidic soil pH. It is also important to show the impact of BNI-technology in a simulation model, based on the theoretical dynamics of nitrogen chemical species in the soil-plant-atmosphere continuum and empirical data from field observations. The impact of BNI-technology on enhancing agricultural production and mitigating nitrogen pollution and climate change is clearly demonstrated.

1: Nitrogen in the current environment

1.1: Rise of reactive nitrogen by the Haber-Bosch process

Nitrogen is one of the most essential elements for all biological systems, constituting proteins, nucleic acids, pigments, and neurotransmitters. Nitrogen is abundant and ubiquitous in the Earth's troposphere, and most of N is in the form of N_2 (N≡N), where two N atoms are strongly triple-bonded. Because N_2 gas is stable and unreactive, living organisms cannot directly utilize the N in the air. Biologically accessible N is categorized as reactive nitrogen (Nr), including all N forms other than N_2 gas, such as inorganic NH_3 and NOx and organic N. Nr is converted from unreactive N_2, under natural conditions, through biological nitrogen fixation (BNF) to produce ammonium-N by symbiotic microorganisms to leguminous plants, or through atmospheric deposition to produce oxides of N (NOx) by high-energy events such as lightning and wildfire. All organisms were dependent on the scarce Nr available on Earth, which was also the case in agricultural systems. The sources of nitrogen used to nourish the crops were only green manure, animal/human excreta, guanos, or saltpeters. Therefore, agricultural productivity had been stagnated until humans acquired the method of mass ammonia production in the early 20th century. This method is called the Haber-Bosch process (named after the two inventers, Fritz Haber and Carl Bosch) and is used to synthesize ammonia (NH_3) on an industrial scale using nitrogen from the air. The process proceeds under the condition of high temperature and pressure to break the triple bond; therefore, this process requires a large amount of energy (equivalent to 1% of worldwide energy consumption) (Capdevila-Cortada, 2019).

Since the Haber-Bosch process has been implemented, it has become possible to produce as much ammonia as desired. Ammonia is a versatile source of chemical fertilizers, explosives, and other N-containing chemical materials. Thus, the process dramatically increases the Nr inputs to anthropogenic activities and natural ecosystems. The annual global industrial production of ammonia was estimated to be 150 million metric tons of N (150 TgN) in 2021 (USGS, 2023), 80% of which is are used for making N fertilizer (mainly urea). Modern agriculture and food supply are highly dependent on this anthropogenic N. Erisman et al. (2008) estimated that approximately 50% of humans are alive because of the Haber-Bosch nitrogen production. They also mentioned the increasing influence of Haber-Bosch nitrogen on growing bioenergy and biofuel production.

1.2: Green Revolution by plentiful N chemical fertilizer

Historically, the agricultural benefits of Haber-Bosch nitrogen were realized as part of the Green Revolution, which occurred from the 1940s to the1960s. High-yielding varieties of wheat and rice bred by introducing of semi-dwarfing genes had a higher and more stable yields without lodging in response to higher N fertilizer application than traditional varieties. These varieties were disseminated along with sufficient chemical N fertilizer; thus, the production of wheat and rice was greatly increased to avoid food crises in Asia and support the growing world population. Mexico, which was once an importer of wheat in the 1940s, achieved self-sufficiency in 1950s and became an exporter of wheat in the 1960s. High-yielding wheat cultivars were introduced along with irrigation systems, to the Indo-Gangetic Plain, the northern part of the Indian Subcontinent, currently a food basket for India, Pakistan, and Nepal. The Philippines was the first country to benefit from the Green Revolution via the miracle rice cultivar, IR8, which requires N fertilizer and pesticides to double the yield as compared with traditional cultivars. The success of the Philippines has spread to other rice-producing countries in Southeast Asia in the 1970s and South Asia in the 1980s. Notably, the Green Revolution could not be achieved only by high-yielding varieties, but also by increased accessibility of chemical N fertilizer to local

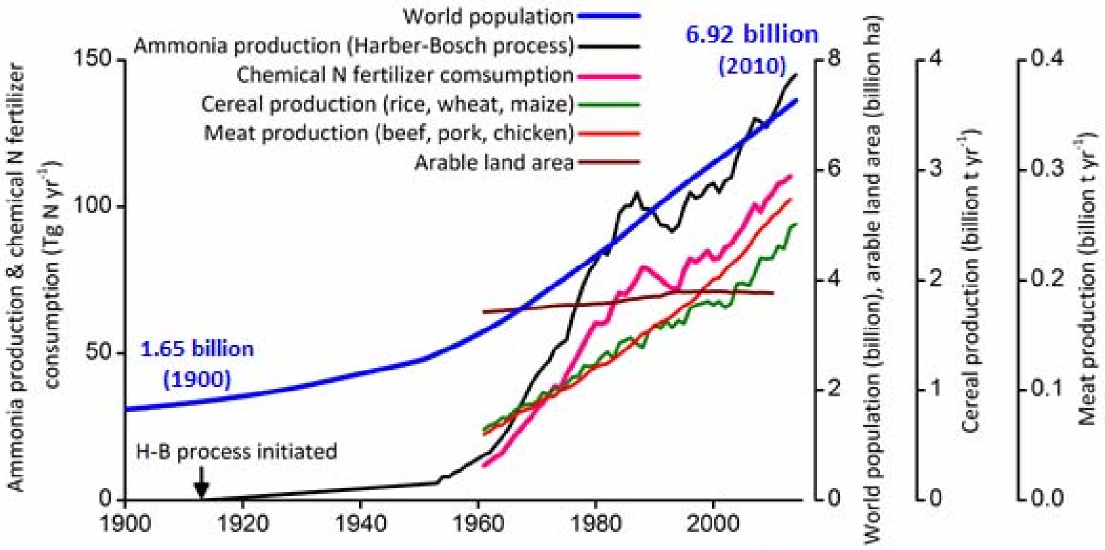

Figure 1. Relationships between ammonia production, N-fertilizer consumption, and agricultural production, and world population growth from 1900–2000 (Hayashi et al. 2021)

farmers, attributed to the Haber-Bosch process. Figure 1 shows the causal relationship; for example, world population growth is supported by an increase in food (crop and meat) production, or food production per unit area, as the arable land has not increased over the past century. Food production is proportional to the ammonia/N-fertilizer production via the Haber-Bosch process (Hayashi et al. 2021). With the Green Revolution, agricultural systems have changed in several ways. Diversified cropping systems with various crops, especially legumes, have declined, and crop cultivation has been separated from animal rearing. These are the reasons why crop production has accelerated to depend more on chemical N fertilizer.

1.3: Stagnating nitrogen-use efficiency

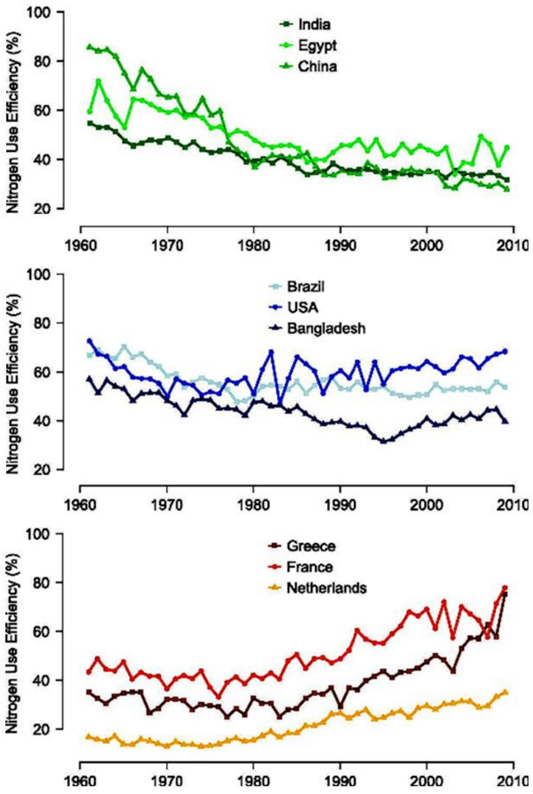

Figure 2. Changes in nitrogen-use efficiency over the past 50 years; compared by country (Lassaletta et al. 2014)

Nitrogen-use efficiency (NUE) is defined as the output/input ratio of N in an agricultural system. Lassaletta et al. (2014) calculated the NUE of 124 countries over the past 50 years using the Food and Agriculture Organization (FAO) database. They suggested that NUE was higher in countries with a higher ratio of symbiotic nitrogen (BNF) than in countries with a higher ratio of synthetic nitrogen (chemical fertilizer) among the total N inputs (Fig. 2). The regional scale N budget was estimated by Zhang et al. (2015), where Sub-Saharan Africa had the highest NUE of 72%, followed by the USA+Canada (68%) and the former Soviet Union (62%) in 2010. Comparing NUE among crops, rice and wheat that received a relatively high rate of N-fertilizer had a lower NUE of approximately 40%, whereas soybean had a high NUE of 80%. It has been shown that NUE was 65% in the early

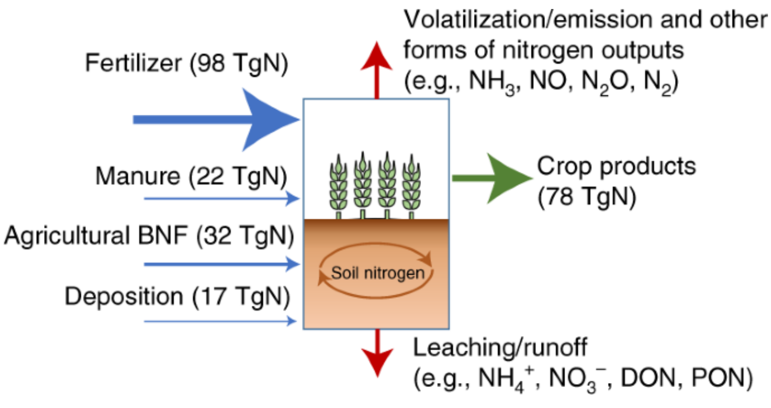

Figure 3. Quantitative estimation of global N budget in agricultural production systems (Zhang et al. 2021)

1960s and it decreased to approximately 45% in the 1980s, and became stable at approximately 47% in recent years (Lassaletta et al. 2014). Figure 3 shows a quantitative estimation of the nitrogen budget using datasets from 2011–2015 (Zhang et al. 2021), resulting in nitrogen assimilation by crops of only 78 TgN/year, whereas nitrogen inputs to the agricultural lands are 169 TgN/year in total, comprising of the synthetic N fertilizer (98 TgN) and manure (22 TgN) applications, and BNF (32 TgN) and atmospheric deposition (17 TgN). This means that more than half (54%) of the nitrogen provided to the agricultural lands is not used by crops and is not harvested. Although only a small portion of this unused nitrogen was incorporated as the soil organic nitrogen, most was lost to the surrounding environments. The loss of N from the root zone has significant economic consequences, accounting for \$17 billion US annually (Subbarao et al. 2006a). Nitrogen is released into the air as volatilized NH_3, and emitted as NO, N_2O, and N_2, and into the hydrosphere as inorganic N (NH_4^+ and NO_3^-) and organic forms of N (dissolved and particulate). Except for N_2 gas, the other chemical N species discharged into the environments are Nr.

1.4: Threatened planetary boundary of the Earth’s N cycle

As described previously, natural processes have produced 203 TgN of Nr annually; for example, 140 and 58 TgN is produced via BNF in marine and terrestrial ecosystems, respectively, and 5 TgN by lightning (Fowler et al. 2013). This amount of Nr has been managed by the Earth’s system to maintain the global N cycle by denitrification (return to stable N_2 gas) and by deposition into soil or the sea. However, in the modern world, anthropogenic processes produce a significant amount of additional Nr (210 TgN annually) through industrial N fixation (i.e., the Haber-Bosch process for fertilizers and raw materials), totaling 120 TgN, with BNF in the agricultural land (60 TgN) and combustion of fossil fuels (30 TgN) (Fowler et al. 2013). When the Nr of natural and anthropogenic origin are incorporated into the global nitrogen cycle, the Earth's system cannot properly maintain the appropriate quantity (or ratio) of global Nr. For this reason, Rockström et al. (2009) and Steffen et al. (2015) recognized that the global N cycle has already exceeded the planetary boundary (or zone of uncertainty) and is at high risk, along with the biodiversity loss, among the other Earth system processes (Fig. 4). Excess amounts of Nr species in the ecosystem have detrimental effects; for example, water quality may be degraded by ammonium-N and nitrate-N as well as dissolved or particulate organic N, whereas the air may be polluted by NO and N_2O gases, which accelerates climate change as strong greenhouse gases (GHGs). Therefore, we must pay attention to the increasing load of nitrogen on the Earth, and implement measures to reduce such threats caused by Nr. More importantly, agriculture contributes to 75% of the production of Nr, which is a major reason for the high risk to the nitrogen cycle in sensitive ecosystems. Low NUE is proof of this, therefore, improving NUE through agricultural technologies to mitigate the adverse effects of reactive nitrogen is a pressing issue. Inherently, NUE improvement leads to adequate N use to meet crop demands; thus, sustainable nitrogen use ensures food security for the increasing global population.

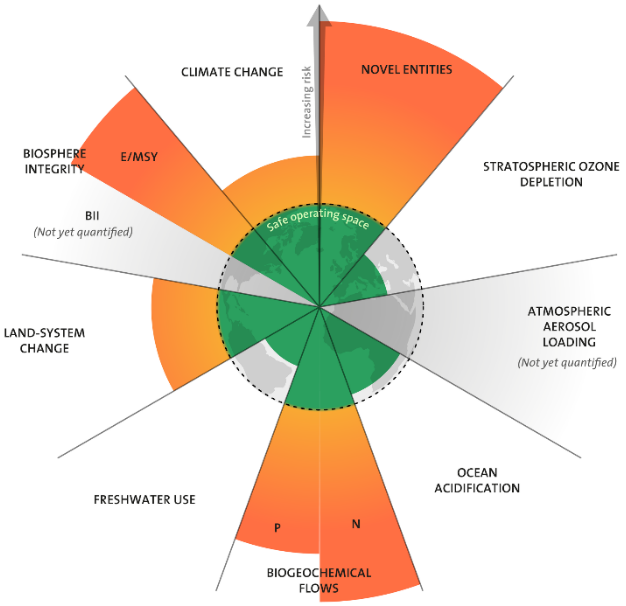

Figure 4. Earth system processes in planetary boundaries (Steffen et al. 2015).

Several research opportunities exist for technological development to improve NUE, as reviewed by Udvardi et al. (2021). These include: (i) improving the synchrony of soil N supply and crop demand both temporally and spatially (e.g., split N application); (ii) enhancing the efficiency of N fertilizers with novel formulations (e.g., polythene-coated and slow-release fertilizers) or containing effective inhibitors (urease and nitrification inhibitors); (iii) a mixture of inorganic-N (chemical) fertilizers and organic-N-based fertilizers (manure and crop residue); (iv) precision agriculture to use big data by monitoring crops and soil/environments (with drones or GreenSeeker®); (v) enhancing BNF and incorporation of legumes; and (vi) crop breeding for higher NUE based on plant-microbe interactions.

Hereafter, I emphasize biological nitrification inhibition (BNI) technology for the improvement of NUE, which may be categorized as (vi), as described in the previous paragraph. BNI technology is unique for utilizing and enhancing the inherent ability of plants, that is, the BNI ability, and is based on the understanding of N transformation mechanisms in the soil to explain why a significant amount of applied nitrogen is wasted and lost from the soil to the environment.

2: Biological nitrification inhibition (BNI)

2.1: Nitrogen transformation in the soil through nitrification and denitrification

For the improvement of NUE, it is important to comprehend the chemical transformations of N species in agricultural soils, because nitrogen lost to the environment emerges exclusively from the soil through the biochemical (enzymatic) activities of microorganisms underground, except for volatilized ammonia (NH_3), which accounts for 10–20% of the total N inputs (van Grinsven et al. 2015). Figure 5 shows the pathways of the chemical species of N in the soil, showing the fate of ammonium-N (derived from chemical N fertilizer and other forms of nitrogen inputs, such as BNF, manures, and crop residues) to nitrate-N (the nitrification pathway; left half), and then nitrate-N is reduced to form NO, N_2O, and N_2, (the denitrification pathway; right half). In modern agriculture, which depends heavily on synthetic N fertilizers, the rate of nitrification from NH_4^+ to NO_3^- is estimated to be less than 10 d (Subbarao and Searchinger, 2021). Since the anion NO_3^- does not generally bind to

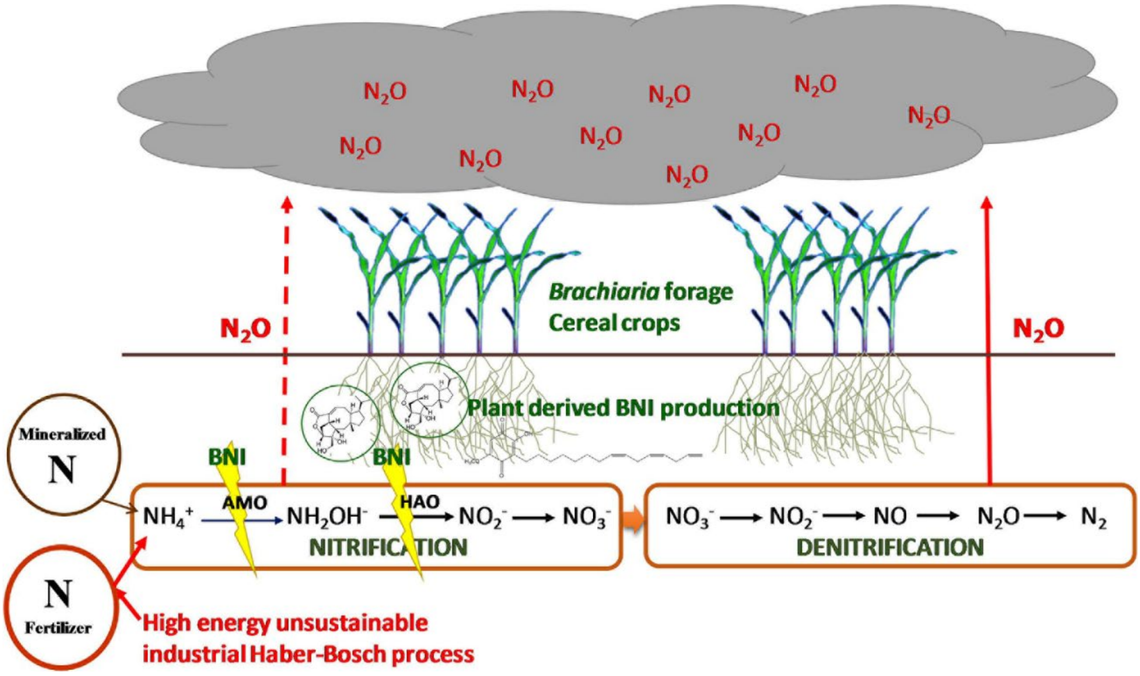

Figure 5. Transformation of N chemical species in the soil through biochemical processes. Blocking sites of BNIs are also shown (courtesy of JIRCAS).

the soil surface, unlike cation NH_4^+, it easily leaches along with the flow of soil water to the groundwater and enters the denitrification pathway, through which nitrogenous gases are generated. From the perspective of plant physiology, such a situation is not favorable for the growth of annual crops because most of the N minerals (both NH_4^+ and NO_3^-) would disappear around their roots at the highest demand, unless the timing and place of N fertilizer application are considered. This is a direct explanation for the low NUE, and an improvement in NUE can be achieved if we can keep NH_4^+ in rhizosphere for a longer period by decelerating the nitrification process.

NH_3-oxidizing reactions in the nitrification pathway are governed by three groups of soil microorganisms: ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA), and nitrite-oxidizing bacteria (NOB). The first two groups are responsible for the transformations of NH_3 to NH_2OH and from NH_2OH to NO_2^-, catalyzed by the corresponding enzymes ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO), respectively. NOB participates in the transformation of NO_2^- to NO_3^- with NXR (nitrite oxidoreductase [NOR]), as in Figure. 5. The inhibition of these microbial or enzymatic activities results in a reduction of nitrification rate.

2.2: Synthetic nitrification inhibitors

Synthetic chemicals such as nitrapyrin (NP), dicyandamine (DCD), and 3,4-dimethylpyrazole phosphate (DMPP) can inhibit nitrification. Although their usage and recommended doses differ, these inhibitors target the AMO, which is the first step in the nitrification process. As summarized by Ayiti and Babalola (2022), these synthetic nitrification inhibitors have several shortcomings, such as difficulties in application, high cost, environmental pollution, food safety, transient (easily washed out with water), and discriminatory effectiveness, which are major disadvantages compared with biological nitrification inhibitors (BNIs) as described in the following subsections.

2.3: Discovery of BNI phenomena

Reportedly in 1983, scientists of the International Center for Tropical Agriculture, Cali, Colombia (CIAT) found in Colombia and Brazil that fields of *Brachiaria* *humidicola*, a tropical grass species, also known as creeping signalgrass (Photo 1) generally had low levels of soil NO_3-N, as described in the first study on the BNI phenomenon (Ishikawa et al. 2003). They conducted comparative studies with other tropical forage grasses and indicated the possibility of nitrification inhibition in the soil by *B. humidicola*. In addition, they observed that the grass species was less responsive to the application of inorganic fertilizer-N, contrary to *B. decumbens* and *Melinis minutiflora*. The team from JIRCAS led by Dr. Guntur Venkata Subbarao, eventually succeeded in establishing BNI technology. They showed that the exudates of *B. humidicola* roots suppressed nitrification (delayed NO_3^- generation) in soil incubation experiments because of a decreased AOB population in the soil, as estimated using the MPN (most probable number) method (Ishikawa et al. 2003).

Photo 1. Creeping signalgrass (*Brachiaria humidicola*) grazing land in Colombia (©AgroActivo)

Consistent with the first report on the BNI phenomenon in Latin American tropical grasslands (Ishikawa et al. 2003, Subbarao et al. 2006b), a French group also reported the possible allelopathic inhibition of nitrification by root exudates from the grass *Hyparrhenia diplandra* (Hack.) Stapf in a wet tropical savanna in Côte d'Ivoire, West Africa (Lata et al. 2004). From an ecological perspective, they indicated that the nitrification process plays a key role in the functioning of savanna natural ecosystems for plant N acquisition and N loss, and nitrification remains low presumably owing to the nitrification inhibition ability of several savanna plant species, an adaptive trait to thrive in N-limiting environments (Lata et al. 2004).

The quantitative evaluation of the nitrification rate is critically important for BNI studies. Subbarao et al. (2006b) established a bioluminescence assay using recombinant *Nitrosomonas europaea* to detect and quantify the activity of nitrification inhibition in plant–soil systems, as expressed in AT Units or ATU. One ATU was defined as the inhibition of NO_2^- production caused by 0.22 μM of allylthiourea (AT), a synthetic nitrification inhibitor. Using this novel protocol, it was confirmed that 20 ATU of BNI activity released from *B. humidicola* roots completely inhibited NO_3^- formation during a 55-d incubation and remained functionally stable in the soil for 50 d. Both the AMO and HAO enzymatic pathways in *Nitrosomonas* were effectively blocked by BNI activity. Therefore, this assay has been used as an effective method to characterize and determine the BNI activity in exudates or extracts of plant roots.

2.4: Screening of genetic resources for higher BNI ability

Standardized the bioluminescent assay for the detection and quantification of BNI activity, the BNI research team at the JIRCAS has intensively promoted the screening of plant species or cultivars/varieties within a specific crop. In collaboration with CIAT, which commenced in the early 2000s, they tested several tropical food and forage crops (Subbarao et al. 2007b). The root exudates of the major leguminous crops (soybean, cowpea, and common bean) did not show any detectable inhibitory activity, except for that of groundnut. Among the cereal crops tested, sorghum exhibited the highest specific activity (5.2 ATU/g root dry weight), followed by pearl millet. None of the three major crops (wheat, maize, and rice), or barley showed nitrification inhibition. Regarding pasture grasses, all tested grass species had inhibitory activities. Among these, *Brachiaria humidicola* had the highest BNI activity, and *B. decumbens* had the highest specific activity (18.3 ATU/g). Other pasture grasses, such as *Brachiaria brizantha*, *Pennisetum maximum*, *Melinis minutiflora*, and *Lolium perenne*, had inhibited nitrification to a lesser extent. Thus, it was concluded that biological nitrogen inhibition is a widespread phenomenon observed in a range of plant species and is a trait that presumably evolved to increase plant BNI activity (i.e., BNI capacity) in N-limiting environments.

They also investigated BNI activity within *B. humidicola* accessions and showed a wide range of variation (46.3 to 6.5 in ATU/g) (Subbarao et al. 2007b). If this is the case for other food crops, such a variation or genetic diversity can be used to breed crops with higher BNI capacity, and secondly the difference would be beneficial for identifying the gene(s) responsible for BNI ability.

The screening of plant species or varieties/lines for BNI capacity has been conducted to evaluate BNI activity (expressed as ATU) of root exudates or homogenates. BNI influences the biochemical process of nitrification process performed by nitrifiers in the soil such as AOB and AOA. Therefore, the existence of BNI should prove how the plant suppresses the populations of AOB and AOA in the rhizosphere, as measured by the expression of *amoA* gene. Moreover, BNI is a phenomenon based on a mechanism where certain compounds from a plant such as, biological nitrification inhibitors, affect AMO or HAO enzymes (Fig. 5). Thus, studies on the isolation, concentration, characterization, structure determination, and ultimately identification of the compounds is an effective way to obtain physical evidence of BNI.

In the following chapter, we introduce plants with a higher BNI capacity (BNI plants), which have been extensively investigated. In addition, some implications for technology development of BNI capacity are discussed.

2.5: Plants with high BNI capacity

1) *Brachiaria humidicola* (creeping signalgrass)

The BNI phenomenon was the first reported in *Brachiaria humidicola* (Rendle) Schweick., and *Urochloa humidicola* (Rendle) Morrone and Zuloaga. Creeping signalgrass or Koronivia grass is a tropical pasture that is widely used in Latin America (approximately 118 million hectares) and originated in Africa. Its BNI activity (ATU) is the highest among all plants examined to date.

It was reported that within 3 years of establishment, *B. humidicola* pastures have suppressed AOB and AOA populations in the soil and emission of nitrous oxide (N_2O) gas from the soil was significantly lower, compared to those in soybean and other pastures (*Panicum maximum* and *Brachiaria* hybrid cv. Murato). Intraspecific variation has been observed and two varieties (BH-679 and BH-16888) that were reported to strongly suppress ammonia oxidation (Subbarao et al. 2009). The BNI activity is stable in low pH environments and is triggered and sustained by the availability of NH_4^+ (Subbarao et al. 2007a). Brachialactone (Fig. 6), a diterpenoid, was identified as a biological nitrification inhibitor in the root exudates of *B. humidicola*, and was shown to inhibit only AMO,

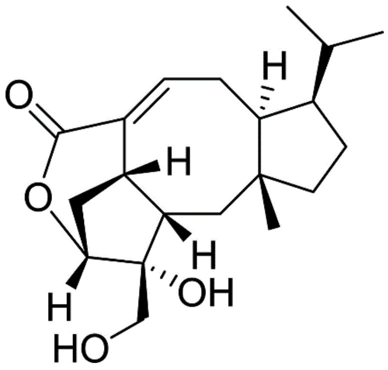

Figure 6. Brachialactone (©PNAS)

but not HAO (Subbarao et al. 2009).

In grazing systems, pasture grasses normally receive no nitrogen fertilizer, and the land receives a limited amount of nitrogen from the feces and urine of animals. Therefore, the benefit of the high BNI ability of *B. humidicola* has not been fully demonstrated in monocultured grazing systems. It could be effective to exploit the BNI ability of *B. humidicola* if the pasture was introduced to the agro-pastoral rotation systems as a pre-crop or a cover crop before planting cereals such as maize (as animal feed) and rice (for human consumption), which receive a considerable amount of inorganic N fertilizer, so that the NUE in the total agricultural production systems improve. In such cases, BNI compounds (inhibitors) must accumulate and remain active for prolonged periods. Maize grain yield was improved in a long-term plot (15 years) established *B. humidicola* pasture as compared with a plot of maize-soybean rotation as well as increased NUE, because of accumulated nitrification inhibitors (Moreta et al. 2013). The residual effects of BNI by *B. humidicola* have been investigated, where a significant increase in maize yield and N uptake was observed a year after the introduction of *B. humidicola* as compared with that of continuous maize monocropping, presumably because of lowered nitrification, whereas the residual effect was diminished (Karwat et al. 2017).

The existence of other BNI-active substances inside the plant tissues of *B. humidicola* has been reported, including polyphenolic compounds such as methyl ferulate and methyl-*p*-coumarate from root tissues (Gopalakrishnan et al. 2007), and fatty acids such as like linoleic acid and α-linolenic acid from shoot tissues (Gopalakrishnan et al. 2009). Therefore, the possible applications of the BNI ability of the tropical grass has expanded from the release of the inhibitors along with decomposition; thus, cropping systems should consider root mass and turnover (Photo 2), or the incorporation of shoots into the soil during land plowing (Nakamura, et al. 2020). Breeding efforts for *Brachiaria humidicola* have been intensively implemented by the CIAT team to combine good

Photo 2. *Brachiaria humidicola* plants in hydroponics (courtesy of JIRCAS).

agricultural performance and high BNI capacity (Villegas et al. 2022). As *Brachiaria humidicola* has the highest BNI capacity observed to date, it is worth identifying genetic regions responsive to the release of the nitrification inhibitor, brachialactone, to explore the possibility of expanding BNI capacity to other crops. By mapping populations derived from the crossing of *B. humidicola* accessions with contrasting BNI capacities, the genetic analysis was performed using QTL (quantitative trait loci) analysis.

2) Sorghum

Sorghum (*Sorghum bicolor* (L.) Moench) is the world's fifth most important cereal crop. It is grown in arid and semi-arid tropics, totaling more than 40 million hectares, and its harvest is approximately 60 million tons worldwide (FAOSTAT, 2023). Sorghum can be grown in a wide range of soil types, from heavy clayey soils to light sandy soils. It adapts to a wide soil pH range of 5.0–8.5.

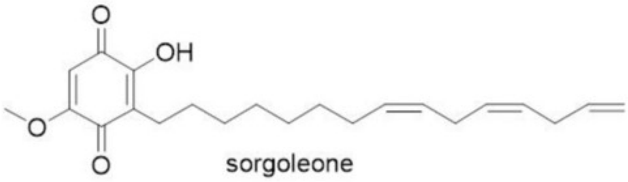

Figure 7. Sorgoleone (courtesy of JIRCAS).

Sorghum is recognized as the most promising cereal crop regarding its BNI capacity in a previous study (Subbarao et al. 2007b), although the plant is known to have a strong phytotoxic effect on weeds. Sorgoleone (Fig. 7) is an allelopathic compound found in the root exudates of sorghum and it has been the subject of many studies on potential bio-herbicides. Sorgoleone was isolated and identified as a biological nitrification inhibitor from the hydrophobic fraction of sorghum root exudates, and sakuranetin and methyl-3-(4-hydroxyphenyl) propionate were isolated from the hydrophilic fraction (Subbarao et al. 2013a). As sorgoleone explained most of the BNI activity in sorghum root exudates, the alterations in the soil microbial community as affected by sorgoleone release were investigated (Sarr et al. 2020). It was reported that the release of higher amounts of sorgoleone had great potential to inhibit the abundance of AOA (but not AOB) and soil nitrification.

Genotypic diversity in BNI ability has been observed among sorghum genotypes, and a very strong positive correlation has been demonstrated between BNI activity and sorgoleone release (Tesfamariam et al. 2014). Therefore, sorghum genotypes can be screened for high BNI by quantifying sorghum sorgoleone exudation, which is a relatively easier method than the evaluation of BNI activity (ATU). Breeding of sorghum lines with the ability to release higher amounts of sorgoleone could be a strategic way to improve the biological nitrification inhibition during cultivation (Sarr et al. 2020). In a collaboration between JIRCAS and the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), based in Patancheru, India, the mini-core collection of the global sorghum germplasm, including cultivars from African semi-arid regions, was evaluated for its high BNI capacity. Through genetic dissection of the BNI trait, the improvement in the BNI capacity is possible if alleles for superior sorgoleone exudation were identified (Subbarao et al. 2013b).

3) Wheat

The average value of NUE in cereals' production worldwide has remained low at 33% for two decades (Raun & Johnson, 1999). As the 2nd largest production crop, wheat (*Triticum aestivum* L.) has required more than 18% of the world's total nitrogen fertilizer in recent years, which was the largest percentage among crops until 2014 (IFA, 2022). Therefore, improving the NUE in wheat production systems is a principal global challenge for reducing N leakage into the environment. To address this issue, scientists from the JIRCAS and International Maize and Wheat Improvement Center (CIMMYT), based in El Batán, Mexico, have evaluated many wheat varieties with higher BNI ability. Unfortunately, to date, there is no significant BNI capacity among wheat genetic resources (Subbarao et al. 2007b). As part of the exploration of alien genes from wild related species for wheat improvement, a group of Japanese wheat breeders/geneticists found that a wild relative species of wheat, *Leymus racemosus* L. (Photo 3), had high BNI capacity, which effectively suppressed soil nitrification (Subbarao et al. 2007c). For the BNI capacity of *L. racemosus* to be transferred into cultivated wheat without any undesirable effects derived from alien genes, considerable efforts have been made

Photo 3. Natural population of *Leymus racemosus* in Bulgaria (©Bulgarian Flora Online).

to select interspecific hybrid lines with the minimum chromosomal segments of *Leymus* for a higher BNI capacity. Eventually, a genetic stock of wheat with *Leymus* chromosome (referred to as Lr#n-SA) showing high BNI capacity was obtained under the background of “Chinese Spring” an old wheat cultivar often used as a genetic and grain quality standard. Then the superior genetic stock was crossed with international wheat cultivars “Munal” and “Roelfs” to successfully produce BNI-enabled “BNI-Munal” and “BNI-Roelfs” (Subbarao et al. 2022). This indicated that the genetic barrier between the crop and its wild relatives was successfully removed. However, it is still worth exploring existing wheat landraces for their BNI capacity as a genetic source for improving NUE in elite wheat varieties, as reported by O’Sullivan et al. (2016).

Soil nitrification activities, especially by AOA, was suppressed and N_2O emission was reduced in a laboratory incubation test with the soil collected from root zone of field-grown “BNI-Munal.” In an experimental field in Japan, grain yield of “BNI-Munal” was superior to “Munal” under different nitrogen fertilizer applications, including no N input, showing that NUE was improved by the ‘acquired’ BNI capacity (Fig. 8, Photo 4) and it might be possible to reduce N fertilizer application to gain the same grain yields. Grain quality was not negatively affected in terms of breadmaking properties (Subbarao et al. 2022). Therefore, “BNI-

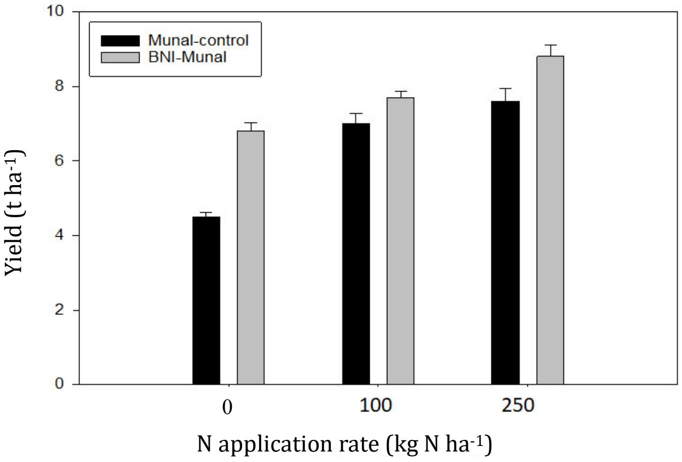

Figure 8. Yield response of wheat variety Munal and BNI-Munal to different amount of N applications (courtesy of JIRCAS).

Photo 4. Appearance of wheat variety Munal (right) and BNI-Munal (left) at heading stage (courtesy of JIRCAS).

Munal" itself can be disseminated to wheat fields as a nitrogen-efficient wheat cultivar upon adaptation, and can be used as a breeding material for nitrogen-efficient elite wheat cultivars in particular environments and economical requirements. A possible solution for the global N crisis.

4) Maize/corn

At the turn of the century, maize and corn (*Zea mays* L.) became the most cultivated crop worldwide in terms of yield (FAOSTAT, 2023). Accordingly, maize receives more than 20% of the chemically produced N fertilizer, followed by wheat (IFA, 2022). The impact would be beneficial if the NUE of the maize cultivation system was improved; thus, BNI-enabled maize has long been desired until Dr. Tadashi Yoshihashi, a chemist at JIRCAS found BNI activity in the root system of a commercial variety of sweetcorn (Honey Bantam) in 2017.

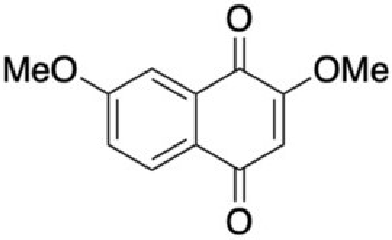

Figure 9. Chemical structure of zeanone (©Springer)

Since then, intensive studies have been conducted on the BNI activity of maize over a short period. Otaka et al. (2022) reported that two hydrophobic BNI compounds were isolated and identified from the root exudates, one of which was a new naphthoquinone and named "zeanone" (Fig. 9), showing strong nitrification inhibition (ED_{50} = 2 µM; indicating that 2 µM of zeanone suppresses half of the nitrification activity estimated by ATU). The other was a benzoxazinoid known as HDMBOA with average nitrification inhibition (ED_{50} = 13 µM). They also identified other BNI-active compounds in the root tissues, such as HMBOA, another benzoxazinoid, and a glycoside of HDMBOA. Based on these findings, the mechanism of BNI in maize has been discussed from the perspective of phytochemistry (Otaka et al. 2022).

5) Rice

Rice (*Oryza sativa* L.) is grown worldwide and is a staple food for people in East, Southeast, and South Asia. To support the increasing population in the region, rice is produced using a large amount of chemical fertilizer, which accounts for 15% of the world's use (IFA, 2022). Similar to other cereal crops, the NUE of rice can be as low as 30–50% (Patra et al. 2021). In addition to ammonia volatilization, nitrification could also be involved in the loss of N, even from rice field where an anaerobic environment is dominant, as the significance of the ammonia-oxidizing activity (*amoA* gene), mainly of AOA, was reported in the paddy rice rhizosphere (Chen et al. 2008).

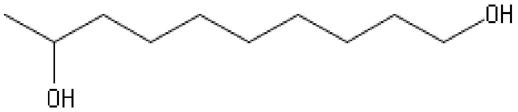

Figure 10. 1,9-Decanediol (© J-Global)

A group from the Chinese Academy of Science observed BNI activity in rice with a wide range of genotypic differences in their root exudates, and a compound, 1-9-decanediol (Fig. 10) was identified as a nitrification inhibitor that blocks AMO in the ammonia oxidation pathway (Fig. 5) (Sun et al. 2016). Further studies have shown that 1,9-decanediol is more effective than DCD, a synthetic nitrification inhibitor, which reduced N_2O emissions, especially from acidic red soils (Lu et al. 2019).

6) Other plant species

To date, most plant genetic materials with enhanced BNI capacity have been reported and investigated exclusively in Poaceae plants, mainly crops but also wild relatives or natural vegetation species. In recent years, an Australian research team published a report on the BNI capacity of weeds, including wild radish (*Raphanus raphanistrum*), a Brassicaceae plant, great brome grass (*Bromes diandrus*), wild oats (*Avena fatua*), and annual ryegrass (*Lolium rigidum*), among which, wild radish showed the highest BNI capacity, even higher than that of *B. humidicola* (BNI-positive control). Other weeds were similar in BNI capacity, as per of the evaluation protocol (O'Sullivan et al. 2017). Janke et al. (2018) also reported that the root exudates of *Hibiscus splendens* and *Solanum echinatum*, both native Australian plants, showed BNI activity similar to that of sorghum.

Another study was published by the CIAT group on Guinea grass (*Megathyrsus maximus* or *Panicum maximum*), the most promising fodder crop for beef and milk production in modern intensively managed pastures, which receive a relatively high rate of N fertilizer, in Latin America. They found wide variation in BNI capacity among *M. maximus* accessions, and accessions with low nitrification rates showed a lower abundance of AOB and a reduction in N_2O emissions compared with accessions with high nitrification rates (Villegas et al. 2020).

More evidence is expected to be published about the BNI capacity of the plants, because the BNI phenomenon has attracted attention in numerous research fields such as ecology, agronomy, microbiology, phytochemistry, weed science, and environmental science. the BNI International Consortium for Sustainable Development was established in 2015 by JIRCAS. Other than the Consortium, several international research activities have been formulated and implemented to explore this unique ability of plants, such as CATCH-BNI, a part of the EU-funded SusCrop-ERA-NET (Vanderschuren and Thonar, 2021).

3: Toward the deployment of BNI technology

3.1: BNI technology as “Genetic mitigation strategies”

The goal of BNI technology is to develop low-nitrifying, low-N_2O emitting, N-efficient agricultural production systems, through the application of plants with high BNI capacity at a large scale.

As seen in the previous chapter, the BNI capacity is the ability of plants to suppress the nitrification process in the soil by nitrification inhibitors (BNIs), such as exudates from their roots or compounds released from decomposed roots and/or shoots. Plants with high BNI capacity and their BNIs are listed in Table 1.

Table 1. Plants with BNI capacity and biological nitrification inhibitors (BNIs) investigated to date.

Plant species	Origin	BNIs	Chemical properties	Mode of action (blocking enzymes)
Brachiaria humidicola	root exudates & root tissues	**brachialactone**	hydrophobic	AMO & HAO
	root tissues	**methyl ferulate**	hydrophobic	AMO
		methyl-*p*-coumarate	hydrophobic	AMO
	shoot tissues	**linoleic acid**	hydrophobic	AMO & HAO
		α-linolenic acid	hydrophobic	AMO & HAO
Sorghum	root exudates	**sorgoleone**	hydrophobic	AMO & HAO
		sakuranetin	hydrophilic	AMO & HAO
		methyl-3-(4-hydroxyphenyl) propionate	hydrophilic	AMO
Maize	root exudates	**zeanone**	hydrophobic	(under survey)
		HDMBOA	hydrophobic	(under survey)
	root extracts	**HMBOA**	hydrophobic	(under survey)
		HDMBOA-β-glucoside	hydrophobic	(under survey)
Rice	root exudates	**1,9-decanediol**	hydrophobic	AMO

Several BNIs have been identified, most of which have been scientifically proven to block key enzymes (AMO and HAO) of the nitrification process. This results in an improvement in NUE; more N stays longer in the soil and less N is released into the environment.

It is important to note that BNI technology employs “plants” themselves and does not use the nitrification-inhibiting compounds. This concept of BNI technology is primarily practical in terms of dissemination, because farmers may perform the same practice of crop cultivation and only replace the crop variant with a novel enhanced BNI-enabled crop without additional expenses, as surplus is anticipated a from the reduction in chemical N fertilizer application. Furthermore, the plant-level application of BNI technology is superior because the BNI-enabled plants produce a cocktail of BNIs and different compounds with different chemical attributes, such as hydrophobic and hydrophilic compounds.

Therefore, the key component of the BNI technology is crop varieties with high BNI capacity. That is why BNI technology is referred to as a “Genetic Mitigation Strategy” to reduce GHG emissions from agriculture (Subbarao et al. 2017).

As mentioned previously, there are currently some promising crop varieties; however, breeding efforts should continue to increase the possibility and applicability of BNI technology to broader areas, with more crop species (food, pasture, and energy) which have low NUE. Specifically, breeding for higher BNI capacity can be achieved by 1) expanding genetic stocks of BNI-enabled varieties/accessions of the target crops (e.g., in sorghum research) and exploiting of wild relative species as a novel source of BNI-traits (e.g., in wheat research), 2) utilizing genetic markers linked to BNI-traits for marker assisted selection (e.g., in *Brachiaria* and sorghum research), and 3) genome/gene editing in the future for the accelerated production of BNIs.

3.2: Requirement for more evidence of BNI technology

BNI technology has been deployed in several ways. BNI-enabled crops with can be incorporated into diversified agricultural production systems. Wheat for human consumption, and maize and sorghum for food, fodder, and energy, are mostly cultivated annually in monocultures or rotations with other crops or fallow. Pastures are traditionally managed in grazing lands for beef and milk and sometimes in more sustainably intensified silvopastoral and agrosilvopastoral systems. When BNI technology is implemented, these agricultural production systems are expected to improve their NUE. Therefore, it is necessary to show the benefits of BNI. The productivity and quantity of N uptake would remain unchanged under the reduced nitrogen input to the system and the environmental benefits are shown by decreased N_2O emissions from the soil as well as decreased N leaching deeper beneath the rhizosphere.

It is also important to demonstrate the positive effect of BNI in a quantitative simulation model to determine the nitrogen dynamics. To establish the model, efforts should be made to acquire data by monitoring N chemical species (NH_4^+ and NO_3^-) and microbial activities in the soil, as well as soil properties and aboveground environments, to explain the model theoretically and empirically. Therefore, we can simulate the amount of nitrogen used by crops, leached into groundwater, and emitted as N_2O, and then estimate the benefits of BNI technology at a certain site and crop management.

The benefits or impacts of BNI technology should be evaluated ex-ante with the life cycle assessment (LCA) methodology especially from a global environmental perspective, because the technology receives a high level of interest.

3.3: Ex-ante impact assessment of BNI technology

It is generally understood that the BNI technology is still developing. Such emerging technologies are often shown to function only at laboratory or pilot field levels, and the available data are limited at the farmer fields, households, markets, or government levels (Cuccurachi et al. 2018). It is worth upscaling the emerging technology using possible scenarios of future performance, defining the applicable places for the technology, and finding any ideas that would increase the practicality and effectiveness of the technology, i.e., ex-ante life cycle assessment (LCA).

Among several options of the BNI technology, BNI-wheat and BNI-pastures are thought to be most advanced in terms of readiness for practical application. Dr. Ai Leon, an economist of JIRCAS in collaboration with CIMMYT, conducted an ex-ante LCA of the BNI-wheat technology (Leon et al. 2022). They used scenarios based on the evidence of nitrification inhibition and N_2O emission reduction by BNI-enabled crops. They found that BNI-enabled wheat plants could effectively inhibit nitrification by 40% by 2050, and that the release of BNIs by roots occurs in soil pH of 5.5–7.0 at a depth of 30 cm. It was shown that when nitrification was suppressed by 40%, N fertilization and LC-GHG emission (including GHG emission associated with fertilizer production) would be reduced by 15.0 and 15.9%, respectively, and NUE would be improved by 16.7% at the field level (Leon et al. 2022). Regionally, the reduction rate of the fertilizer-induced GHGs was estimated to be the highest in Sub-Saharan Africa, followed by Europe and Central Asia, as these regions were thought to be more suitable for BNI-enabled crops. However, South Asia, which has the highest GHG emissions, has a relatively small area suitable for BNI-wheat because of the high pH of soils in this region (Fig. 11). Therefore, it is a challenge for BNI research to develop a BNI-enabled wheat variety adapted to alkaline soil. The ex-ante LCA methodology should be applied to other options of BNI technology, not only to assess the environmental benefits in the future, but also to identify possible limitations of this technology, so that we can uncover research opportunities to overcome such limitations by upgrading the technology. Ex-ante LCA studies have commenced for BNI-sorghum in collaboration with ICRISAT.

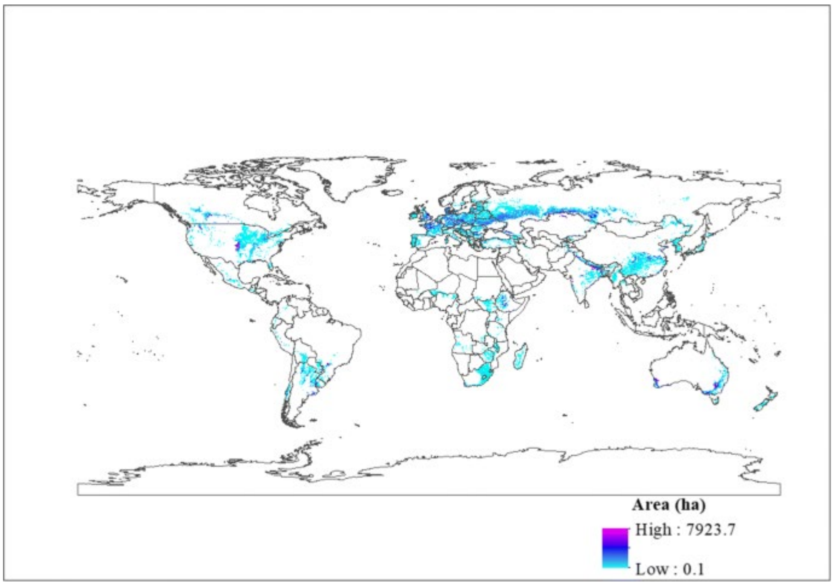

Figure 11. Harvested area suitable for BNI-wheat (Leon et al. 2021)

Recently, scientists in India observed better growth and yield of the BNI-enabled wheat lines under N-deficient conditions as compared with the corresponding non-BNI varieties even under soils of higher pH (approximately 8.0), in a preliminary pot experiment (Dr. Ajay Bhardwaj, in personal communication). If this can also be reproduced with exact evidence under field conditions, the applicability of BNI technology would be expanded to more wheat-growing regions, such as those shown in Fig. 11. Meanwhile, the molecular and biochemical mechanisms of BNI action in the soil, especially the interaction between the inhibitors and soil nitrifying enzymes, AMO and HAO, occur in a wide range of soil pH.

3.4: The International BNI Consortium

The International BNI Workshop held at JIRCAS, Tsukuba, Japan, from 2–3 March, 2015, was attended by 40 researchers representing four CGIAR Centers (CIAT, CIMMYT, ICRISAT, and ILRI) that have four CGIAR Research Programs, [CRPs: Climate Change (CCAFS), Wheat (WHEAT), Dryland-Cereals, Livestock-Fish] and national agricultural research institutes and universities in Japan. JIRCAS, together with CGIAR partners, formed the Consortium on BNI Research for Sustainable Development, with JIRCAS playing a convening and coordinating role. Subsequently, researchers of the Consortium, that is, CGIAR Centers and advanced research institutions from Europe, Japan, Asia, and the US, have hosted biannual meetings, with specialists from science-policy cooperation, such as the World Resources Institute (WRI) and International Nitrogen Initiative (INI). They interact together to exchange information and research findings, and discuss research strategies in several directions, primarily extend BNI capacity into new crops, deepen the knowledge on BNI mechanisms, realize the BNI technology on local sites by garnering political attention, and accumulate evidence of the benefits of BNI to more food production and a better environment, which are two of the greatest challenges for global agriculture (Searchinger et al. 2018).

Photo 5. Group photo of attendees of the 3rd International BNI Meeting, on 25 and 26 October 2018, Tsukuba, Japan.

3.5: BNI technology for sustainable development

Nitrogen is a double-edged sword for humans and all other living organisms on Earth. Life benefits from N nutrients (Nr), whereas N surplus (also Nr) has adverse environmental effects. Therefore, for a sustainable future, nitrogen must be properly managed. In agricultural systems, increasing food production and minimizing environmental impacts are pressing challenges. Modern agriculture heavily depends on N chemical fertilizer, and substantial amounts of N are not used and released into to the environment; therefore, NUE improvement is a holistic and justified solution to these challenges. Improving NUE will directly contribute to the Sustainable Development Goals (SDGs) of the UN 2030 Agenda, specifically, the 2nd "Zero Hunger" for food production, and 13th "Climate Action" and 14th "Life below Water" goals for the environment.

As BNI technology is an effective and practical option for improving NUE in the agricultural systems, it contributes to SDGs 2, 13, and 14, accordingly. In addition, BNI technology also contributes to the 12th goal "Responsible Consumption and Production" because it promotes the sustainable use of chemical fertilizer, and 15th goal "Life on Land" because the technology is based on the function of richly diversified terrestrial plants.

Regarding sustainable development in agriculture, the Ministry of Agriculture, Forestry and Fisheries, Government of Japan, has launched the "Strategy for Sustainable Food Systems, MIDORI –Innovation will enhance potentials and ensure sustainability in a compatible manner"– in 2021 (MAFF, 2021). This strategy has several goals for 2050, one of which is to reduce of the use of chemical fertilizers by 30%. All goals including this one may contribute to the MIDORI's overall goal of accomplishing zero CO_2 (GHG) emissions from the agriculture, forestry, and fishery sectors. BNI technology, as a Japan-made innovation, is placed in the "Sustainable Production" part, for the development of "super" plant varieties with high NUE and less burden to the environment. It is assumed that the impact of BNI technology would be of significant value in Japan, but an ex-ante assessment has not yet been conducted. To realize BNI technology in Japan, a research network should be organized with national and prefectural agricultural research centers, as well as universities.

Photo 6. Multiplication of the genetic materials of BNI-Munal and local elite varieties for hybridization in Samastipur, Bihar, India (courtesy of BISA).

Finally, it would be good to introduce a bilateral project between Japan JIRCAS and the Borlaug Institute for South Asia (BISA), and the Indian Council of Agricultural Research (ICAR), A SATREPS project titled “Establishment of Nitrogen-efficient Wheat Production Systems in Indo-Gangetic Plains by the Deployment of BNI-technology” (JST, 2022) officially commenced in 2022. India is the country that benefits the most from the Green Revolution, especially in Indo-Gangetic Plains, currently known as a food basket (wheat and rice) for 1.4 billion population (in the latest statistics). For wheat, farmers apply extremely high rates of N fertilizer beyond the crop demand in this area (Sapkota et al. 2020); therefore, there is sufficient rationale to introduce BNI-enabled wheat in this area to improve its low NUE (<30%). Ultimately, the project aims to demonstrate no yield reduction under 30% in N application in wheat fields. The first step of the project is to develop BNI-enabled elite wheat varieties adapted to local environments and market requirements by crossing ‘BNI-Munal’ or ‘BNI-Roelf’ with local elite varieties in a couple of project sites. Moreover, in India the central government has a tangible policy to reduce the consumption and import of N fertilizers because farmers in India use too much urea (NITI Aayog, 2015). This supports the project politically, so it is expected that the research outputs (BNI-enabled local elite wheat lines) could be smoothly implemented in the society; the lines are registered and distributed as recommended varieties and farmers accept them.

4: Conclusions

- Suppression of the nitrification process in the soil is key to improving nitrogen use efficiency (NUE), for more N to stay in the rhizosphere and be used by crops, and less N to be lost, which leads to increased crop production with the same amount or less N fertilization, and to minimize nitrogen pollution and climate change, both of which are global challenges in the 21st century.

- Biological nitrification inhibition (BNI) is a function of plants that suppresses the nitrification process by special chemical compounds (biological nitrification inhibitors [BNIs]) from the roots or shoots, has been observed in several Gramineae species including major cereal crops such as wheat, maize, and rice.

- The implementation of BNI-technology is expected to contribute to the resolution of pressing global challenges, food security (SDG 2) and climate change (SDG 13), through the improvement of NUE in agricultural production systems.

- BNI functions are mainly manifested under neutral to acidic soil pH and more ammonia-prone conditions; therefore, further studies are required to expand BNI technology to broader areas.

- To clarify the impact of BNI technology, more evidence and data from the field are required to construct a simulation model, based on the theoretical dynamics of nitrogen chemical species in the soil-plant-atmosphere continuum.

- BNI-enabled crop varieties, such as wheat, which is the most advanced, may be disseminated to develop low-nitrifying, low N_2O emitting, N-efficient agricultural production systems.

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