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REVIEW

The causes of the selection of biological nitrification inhibition (BNI) in relation to ecosystem functioning and a research agenda to explore them

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Abstract

Biological nitrification inhibition (BNI) has already led to several studies mainly focused on underlying molecular mechanisms and applications to agriculture. We argue that it is also important to study BNI more systematically from the ecological and evolutionary points of view to understand its implications for plants and soil nitrifiers as well as its consequences for ecosystems. Therefore, we propose here a dedicated research agenda identifying the most critical research questions: (1) How is BNI distributed across plant phylogeny and why has it been selected? (2) What are the costs-to-benefits balance of producing BNI compounds and the relative impacts on BNI evolution? (3) Can we understand the evolutionary pressures leading to BNI and identify the environmental conditions favorable to BNI plants? (4) How has BNI coevolved with plant preference for ammonium vs. nitrate? (5) Diverse BNI compounds and various inhibition mechanisms have been described, but implications of this diversity are not understood. Does it allow inhibition of various groups of nitrifiers? (6) Does this diversity of BNI compounds increase the efficiency, spatial extension, and duration of BNI effect? (7) What are the impacts of BNI compounds on other soil functions? (8) Can field experiments, coupled to scanning of the diversity of BNI capabilities within plant communities, evaluate whether BNI influences plant-plant competition and plant coexistence? (9) Can field quantification of various nitrogen (N) fluxes assess whether BNI lead to more efficient N cycling with lower losses and hence increased primary production? (10) Can the impact of BNI on N budgets and climate (through its impact on N_2O emissions and its indirect impact on carbon budget) be evaluated at the regional scale? We discuss why implementing this research program is crucial both for the sake of knowledge and to develop applications of BNI for agriculture.

Keywords Biological nitrification inhibition · Ecology · Evolution · Research agenda

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Introduction

Biological nitrification inhibition (BNI) is the capacity of plants to inhibit nitrification through the exudation and release of biological nitrification inhibitors (BNIs) (Subbarao et al. 2012, 2013, 2015, 2017). This capacity seems to be particularly developed in some perennial grasses (Poaceae) of tropical savannas (Srikanthasamy et al. 2018; Subbarao et al. 2007). The discovery, acceptance by the scientific community, and understanding of the mechanisms behind this capacity have taken several decades until the 2000s (Donaldson and Henderson 1990; Jordan et al. 1979; McCarty et al. 1991; Meiklejohn 1968; Munro 1966; Lata et al. 1999, 2000, 2004; Lodhi 1978; Purchase 1974; Rice and Pancholy 1972; Robertson 1984; Stienstra et al. 1994; Stiven 1952; Sylvester-Bradley et al. 1988). From the outset, it was hypothesized that BNI plants (mainly grasses but also trees, e.g., Laffite et al. 2020; Lodhi 1978) have evolved in situations with low availability of nitrogen (N). In N-limited ecosystems, such as tropical savannas extremely constrained by fire, rainfall seasonality, and nutrient-poor soils (Abbadie et al. 2006; Bond 2016), BNI could allow a better conservation of the N resource. More specifically, through BNI, ecosystems are thought to be more N-conservative and to maintain a higher primary productivity, as ammonium is less prone to leaching than nitrate and cannot be lost through denitrification (i.e., reduction of nitrate/nitrite to gaseous NOx/N_2) (Boudsocq et al. 2009).

Despite these strong possible implications for the productivity and N balance of ecosystems, BNI studies have from the outset struggled to establish themselves as a recognized (ecological) research field. Till the year 2000, it was not rare to see manuscript reviews or comments in congresses rejecting the possibility of BNI or objecting that this phenomenon was not ecologically significant because restricted to very specific ecosystems, e.g., particular savannas. The first reason is likely the variety of situations and types of ecosystems initially studied. The first studies (e.g., Munro 1966; Stiven 1952) indeed targeted mainly some natural tropical grasslands or savannas with specific questions about the consequences on pastures (Meiklejohn 1968). Then, forests (e.g., Jordan et al. 1979; Lodhi 1978; Rice and Pancholy 1972; Robertson 1984) were the focus of attention. After additional work on some tropical savannas with potential applications to fallows (Abbadie et al. 2000; Lata et al. 1999, 2000, 2004), efforts have shifted to grasslands and pastures (e.g., Abbadie and Lata 2005; Subbarao et al. 2006, 2009). Finally, the idea that BNI is an important phenomenon has led to expanded studies aiming at applying BNI to agriculture (e.g., Subbarao et al. 2012, 2017).

The second, and likely most important, reason for the difficulty to establish BNI as an important – or even valid – research topic is the initial difficulty to prove that plants are able to inhibit nitrification through a specific mechanism. Noncoherent results were obtained in particular on the dynamics of nitrification or nitrate content in soil along ecological successions. For instance, whereas nitrification was supposed to decrease along ecological successions (e.g., Rice and Pancholy 1972), others observed the contrary (e.g., Robertson 1984). This has been added to the criticism of root extraction techniques to measure the inhibitory potential of exudates (Purchase 1974). Moreover, it has been assumed that other mechanisms than the exudation of BNI compounds could explain BNI. In particular, it has been stressed that the competition between plants and microorganisms for ammonium could explain the decrease of nitrification under plant species rather than a real BNI capacity (Stienstra et al. 1994). Overall, during several decades, the major criticism against the recognition of the existence of BNI has been the lack of direct evidence for the biological inhibition per se and the involved molecular mechanisms.

The first step to respond to this issue was through fieldwork (savannas of Lamto, Ivory Coast), in particular through plant transplantations, demonstrating the long-term modification of soil nitrification by wild savanna grasses (Lata et al., 1999, 2000, 2004). These authors hypothesized that BNI could be, at least partially, responsible for the apparent contradiction observed in humid African savannas between very strong environmental constraints (in particular very low soil N and organic matter contents) and a primary productivity that can be as high as in tropical rainforests. These results paved the way to the use of BNI in fallows (Abbadie et al. 2000) and more generally in agriculture. The second step to prove the existence of BNI has involved, from the mid-2000s, the development of molecular biology techniques and the pioneering efforts made by Subbarao's team at JIRCAS (Japan) to identify BNI compounds. Studying the African grass *Brachiaria* sp. as well as *Sorghum* sp., this team has shown that BNI is indeed induced by several molecules exuded by roots (Subbarao et al. 2006, 2009, 2012, 2013, 2015). While BNI was initially thought to affect only bacterial ammonia oxidizers (AOB – above citations), recent results showed that BNI also inhibits ammonia-oxidizing archaea (AOA) (Kaur-Bhambra et al. 2022; Lu et al. 2019; Sarr et al. 2020; Srikanthasamy et al. 2018, 2021).

All these studies have led to the idea that the development of cereal varieties inhibiting nitrification through root exudates could promote more sustainable agroecosystems (O'Sullivan et al. 2016; Subbarao et al. 2017). Indeed, synthetic nitrification inhibitors are often inefficient (Gardner and Drinkwater 2009). This led several authors to suggest to use BNI crop varieties or to mix target crops with BNI plants within fields to better maintain N fertility under low N inputs (Cox et al. 2006; Subbarao et al. 2015). Coupled to the use of crop species with ammonium as the preferred N source, this could improve N plant nutrition and decrease N losses from agroecosystems (Subbarao and Searchinger 2021). This is particularly crucial in tropical areas where threats to soil fertility are also exacerbated because local human populations cannot afford fertilizers for subsistence farming. At the same time, the efficiency of chemical fertilizers is low due to rainfalls (Baligar and Bennett 1986) and to the ecological requirements of soil nitrifiers. Indeed, local nitrifiers being adapted to very low soil N are sometimes unable to benefit from fertilization and to transform efficiently ammonium into nitrate, though the latter N form is the one preferred by many local maize cultivars (Assémien et al. 2017). Together with the recent discovery of significant BNI activities in different wheat landraces (O'Sullivan et al. 2016), the growing number of species and varieties with BNI capacity (sorghum, wheat, rice, maize, etc.), and the new possibility of transferring BNI capacity from wild grass to elite wheats (Subbarao et al. 2021), all this knowledge is therefore pushing for a use of the BNI in agriculture (Coskun et al. 2017a; Subbarao et al. 2017; Subbarao and Searchinger 2021).

In addition to the goal to develop BNI applications for agriculture, we argue that more in-depth comprehensive knowledge is required on the evolutionary and ecological determinants and implications of BNI in natural systems in particular, both from the microbial and plant point of view. While some of the mechanisms underlying BNI are indeed relatively well known, they have often been studied in controlled conditions, to identify molecular mechanisms, create BNI crops, or improve sown pastures. Information on the effects of BNI on ecosystem functioning remains scarce, and the evolutionary forces explaining its selection in some species/environments deserve better attention. This should help also identify interventions to encourage BNI in managed systems, in particular to determine its applicability to a wide range of environmental situations and of plasticity, feedback loops, and coevolution between plant populations and microorganisms. The corresponding spatial and temporal scales structure this article (Fig. 1) considering (1) the proximal effects on soil microbes and N dynamics, (2) the scale of plant communities and ecosystem structure and functioning, and the (3) biogeography and evolutionary perspectives. The objectives of this review article are therefore (1) to point to the main knowledge gaps regarding the implications of BNI for the functioning of plant and microbial communities and ecosystems, across spatial and temporal scales; (2) to discuss the selection forces that explain the evolution of BNI capacities in some plant species, in some types of ecosystems or regarding some microbial groups; (3) to propose a research agenda that could fill these gaps.

Knowledge gaps on BNI at the rhizosphere scale

The finest scale, at which the two types of organisms involved in BNI, plants and microorganisms, are directly connected, is the rhizosphere (Fig. 1, bottom left bubble), with proximal BNI effects on soil microbes and N dynamics. This is the scale that has been investigated by most of the approaches in a controlled environment (e.g., soil microcosms/mesocosms) so far. Despite these efforts, there are still a great number of gaps both plant-related and microbe-related.

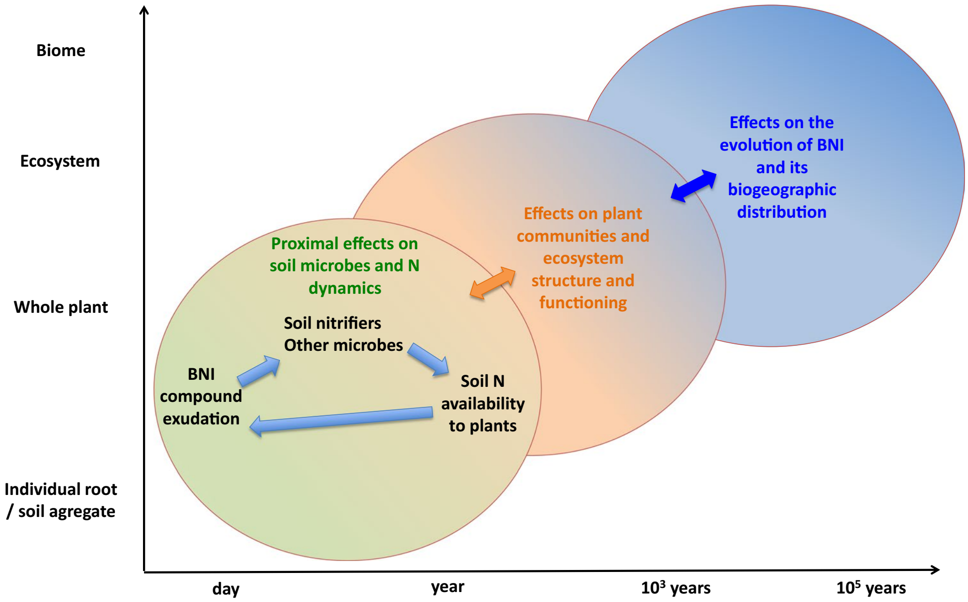

Fig. 1 Schematic representation of biological nitrification inhibition (BNI) effects across spatial scales (*y* axis) and temporal scales (*x* axis). Effects are distributed from proximal to large scales and can sometimes overlap, this double scale serving as a structuring for the arguments discussed in this article for reasons of clarity but also because scientific approaches are sometimes scale-dependent

Plant-related knowledge gaps

Many unsolved issues lie at the plant individual level, in particular regarding the action of BNI compounds released by the plant within the rhizosphere: (i) What is the diversity of the BNI compounds involved? (ii) What is the longevity of these compounds in the soil, and how is it related to soil characteristics? This longevity could influence the horizontal (potentially leading to inter-plant interactions) and vertical (potentially influencing nitrification along the soil profile and nitrate leaching to groundwater) mobility of the BNI compounds and the overall efficiency of BNI. (iii) What are the plant physiological characteristics and soil properties modulating BNI compound production?

Identified BNI plant compounds

So far, the relatively small number of exuded BNI compounds identified (about ten) is mostly polyphenolic compounds and fatty acids (Subbarao et al. 2015). Likely, the diversity of BNI molecules can be greater because the extraction and analysis methods and identifying new molecules are time-consuming and restricted to a small number of laboratories (notably the JIRCAS in Japan). However, each time a new BNI plant species has been discovered and checked for BNI exudates, new BNI compounds have been found. Further, several compounds are often found within a single species. The last most recent example concerns maize (Otaka et al. 2022) with two new BNI compounds (zeanone and HDMBOA). In addition to the already identified BNI root exudates, many BNI compounds have been extracted from plant tissues and leaf litter (Kaur-Bhambra et al. 2022; Subbarao et al. 2015). For example, Otaka et al. (2022) identified two other compounds in tissues, one of which being probably a precursor of the exuded zeanone. Likely, the action of exuded BNI compounds mainly occurs within the rhizosphere, where mineral N is taken up, while those coming from litter degradation could be active within the bulk soil (Kaur-Bhambra et al. 2022). The existence of different types of BNI compounds can thus be seen as an adaptation to different microbial communities (e.g., AOA vs. AOB) so as to induce different BNI effects.

Stability and mobility of BNI compounds

BNI compounds can be hydrophilic or hydrophobic (Di et al. 2018), and this potentially influences the mobility and thus the spatial distribution of the BNI compounds within soil – the hydrophilic ones being the most mobile, likely allowing them to inhibit nitrification outside the rhizoplane (Coskun et al. 2017a). The question arises whether compounds with different mobility might have been selected to optimize BNI. Indeed, the physicochemical conditions (e.g., pH, water) can lead to a selection of microbial communities (including those of the N cycle) very different between the rhizosphere and the bulk soil. The mobility of compounds should also have a significant impact on the vertical and horizontal spatial extension of BNI within the soil (Raynaud et al. 2006) but also on its stability and the persistence of the BNI effect. As stability can depend greatly on several physicochemical (organic matter content, pH, water, etc.) and biotic (microbial communities, exoenzymes) parameters that can spatially vary, it likely should depend on mobility. Conversely, the more stable the compounds the wider they could be dispersed. This stability is thus really one of the most important parameters to determine the ecological importance of BNI (Coskun et al. 2017a), but the duration periods of BNI in soils is poorly known. BNI effects likely disappear after 3 months but may persist for a very long time in some dried savanna soils (Subbarao et al. 2013), while residual in situ BNI effects in *Brachiaria* pastures have been observed for several years of maize cultivation (Subbarao et al. 2015).

The impact of various soil properties such as the redox potential/pH, organic matter/clay content, diversity and activity of bacteria, archaea and fungi, or soil fauna on BNI stability and efficiency remains to be analyzed. Even in controlled conditions, the studies done on known BNI compounds have also demonstrated highly contrasted BNI efficiencies (Coskun et al. 2017a). Overall, it is unclear whether the diversity of BNI compounds and BNI effects reflects the fact that BNI has evolved several times in different plant lineages, with different BNI compounds having similar effects (functional redundancy) and/or whether it reflects different adaptations to different environmental/soil conditions or different plant strategies to inhibit nitrification, representing a form of functional diversity. All these arguments support the need for a screening of BNI plant species and of their BNI compounds by first targeting key functional groups as perennials with a contrasted preference for ammonium versus nitrate. This will help to better document the biochemical and functional diversity of these compounds.

Plasticity and cost of synthesis and exudation of BNI compounds

The exudation of BNI compounds should at least depend on the physiological/energetical costs-to-benefits ratio of producing enough BNI compounds to significantly decrease nitrification within the rhizosphere. If the cost is high, plants should produce BNI compounds only when needed. Two arguments could however contribute to scale down the cost of BNI: (1) the cost of BNI molecules is, for a plant, likely low in comparison to the overall cost of all molecules released by this plant as rhizodeposits (Coskun et al.

2017a). Moreover, the fact that BNI molecules have often been found to have other ecological roles (e.g., membrane compound, action on root development, herbicidal effect on competitors, plant defense – Coskun et al. 2017a, 2017b) de facto reduces this cost. (2) When a plant efficiently inhibits nitrification, this increases (decreases) drastically the availability of ammonium (nitrate) (Lata et al. 1999), forcing the plant to switch its N metabolism towards ammonium. This allows plant to decrease the needs for nitrate reductase production and the relative cost of producing this enzyme (Lata et al. 1999). Nevertheless, (i) BNI compounds are complex molecules that could be costly to synthesize, (ii) the physiological mechanisms behind BNI exudation could also be costly, (iii) there are physiological constraints linked to the toxicity of ammonium within plant cells, and (iv) the absorption of ammonium by roots implies soil acidification within the rhizosphere, which likely decreases the bioavailability of plant nutrients and plant growth or increases the toxicity of certain ions (Britto et al. 2001). Assessing these pros and cons of BNI will be crucial, in particular to predict the consequences of BNI from the plant to the ecosystem scale. This also suggests that plant preference for ammonium versus nitrate and its plasticity is also a crucial plant trait because it influences the trade-off between the advantages and disadvantages of BNI (Boudsocq et al. 2012; Britto and Kronzucker 2013). This preference seems to be extremely variable between species and could also be plastic within species (Houlton et al. 2007). However, there is relatively little data available on the subject for BNI plants.

Impact of plant age and intraspecies variability

Plant age likely influences BNI activities through both a simple dose–effect due to the overall plant biomass (when BNI is linked to root biomass, e.g., Lata et al. 2000) and/or a possible plant stage effect if the different plant physiological stages are characterized by different inhibition activity per root mass. For instance, in sorghum, the BNI activity varies over time, with an inhibitory effect of root exudates increasing with age (2 first months after seed germination – Zakir et al. 2008). Further, hydrophobic- and hydrophilic-BNI activities are different between early and later growth stages (< 14 vs. > 30 days) (Subbarao et al 2015). The same pattern was observed for rice (Sun et al. 2016) with 6-week-old seedlings having stronger inhibition than 3-week-old seedlings. However, Tanaka et al. (2010) have shown on rice (21- to 70-day-old seedlings) that BNI tended to decrease with age likely due a change in the proportion of physiologically active root tissues. Temporal variation in BNI might be less important when plants are well established. For instance, field observations on a well-established, perennial cover of the savanna grass *Hyparrhenia diplandra* (Poaceae) able to live up to ca. 80 years (Koffi 2019) showed that its BNI capacity remains high and constant from several years to several decades (Lata et al., unpublished results) whatever the plant biomass. This capacity is quite stable even when plants are transplanted (Lata et al. 2004).

In addition to temporal variation, intraspecies variability is also a factor to consider. For instance, the same grass species *H. diplandra* has at least two ecotypes, one of which having BNI activity and the other one stimulating nitrification possibly through mixotrophy of nitrifying microbial communities (Lata et al. 2000). Similarly, Villegas et al. (2020) and O'Sullivan et al. (2016) demonstrated a variable inhibition within genotypes from the same species (*Megathyrsus maximus* and *Triticum aestivum*, respectively). However, this intraspecific variability and temporal trends in BNI capacity have been explored for only very few plant species.

Microbe-related knowledge gaps

There are many unsolved issues focusing on soil microorganisms under BNI influence: (i) What is the impact of BNI on nitrification activity (and how to measure this activity?) and its modalities of action at molecular level among ammonia oxidizers? (ii) What is the impact of BNI on other groups of nitrifiers? (iii) What is the impact of BNI on other groups of N-related microorganisms? (iv) What is the impact of BNI on the overall soil microorganism community?

BNI and ammonia oxidizer activity

Previous studies have shown that BNI compounds can suppress more than 90% of soil nitrification activity but that the inhibition level varies across plants and soils (Coskun et al. 2017a; Lu et al. 2019; Subbarao et al. 2013). However, a comprehensive analysis of the level of inhibition induced by known BNI compounds on the ammonia-oxidizing activity of different ammonia oxidizer strains is still missing. This prevents us from detecting any phylogenetic signal of the response to BNI across the tree of life of ammonia oxidizers and from assessing differences of sensitivity to BNI between nitrifier groups. The very first step in this direction was taken very recently by a study (Kaur-Bhambra et al. 2022) on the impact of three root-derived and three shoot-derived BNI compounds on 3 AOA and 4 AOB strains. In addition, some ammonia oxidizers are favored by low N availability while others perform better under high N conditions (Assémien et al. 2017; Prosser et al. 2019). As BNI intensity is expected to be higher in soils with low N availability and high NH_4^+/NO_3^- ratios (Subbarao et al. 2015), we could hypothesize that ammonia oxidizers have evolved resistance to BNI in these soils. This could lead to an inverse relation between resistance to BNI and adaptation to high N availability in ammonia oxidizers. Moreover, information on the level of in situ BNI-induced inhibition on soil nitrification is very scarce. Inhibition of nitrification has often been characterized by quantifying nitrification enzyme activity, i.e., potential nitrification, or net nitrification (Laffite et al. 2020; Lata et al. 1999, 2000, 2004; Moreta et al. 2014; Srikanthasamy et al. 2018, 2021; Subbarao et al. 2009), but it should also be assessed through a decrease in the in situ gross nitrification rate. Overall, BNI can be due to an effect on (i) the specific activity of nitrifiers, i.e., the average activity rate per nitrifying cell, (ii) the growth and abundance of nitrifiers since soil ammonia oxidation tends to be quantitatively linked to AO abundances (Hesselsøe et al. 2001; Le Roux et al. 2008), and/or (iii) the diversity of nitrifiers as different nitrifier taxa have different specific activities (Le Roux et al. 2016; Prosser 1989). However, most BNI studies have focused on total nitrification activity while studies on BNI effect on the abundances of nitrifier groups are still scarce (e.g., Sarr et al. 2020; Srikanthasamy et al. 2018). Moreover, BNI effect on the diversity of nitrifying groups remains largely unexplored.

Molecular and physiological mechanisms of BNI in ammonia oxidizers

Thanks to different studies by Subbarao and colleagues and several other research groups, our knowledge of the molecular and physiological mechanisms explaining BNI effects on ammonia oxidizers is already well advanced (Coskun et al. 2017a; Nardi et al. 2020). In particular, it has been shown that known BNI compounds generally target the first enzymatic step of NH_3 oxidation that is catalyzed by the ammonia monooxygenase (AMO) (Sun et al. 2016). Actually, over 40 compounds have already been reported to inhibit AMO (McCarty 1999). Given the diversity of BNI compounds and of AMO enzymes, it is likely that there are diverse modalities of action of BNI at a molecular level. Consistently, Wright et al. (2020) compared the response of an archaeal and a bacterial AMO to inhibition by linear 1-alkynes and by phenylacetylene, an aromatic alkyne. Phenylacetylene inhibited the archaeal and bacterial AMOs at different concentrations and through different inhibition mechanisms, suggesting structural differences between the two forms of monooxygenase, which could induce different responses of AOA and AOB to nitrification inhibitors. However, these studies have been only performed on a few strains (Iizumi et al. 1998; Kaur-Bhambra et al. 2022).

BNI and other groups of nitrifiers than ammonia oxidizers

Beyond demonstrated BNI inhibitory effects on ammonia oxidizers, we increasingly suspect that BNI compounds can inhibit other groups of nitrifiers. While ammonia-oxidizing bacteria and archaea (AOB and AOA; Kowalchuk and Stephen 2001; Stahl and de la Torre 2012) oxidize NH_3 to NO_2^-, nitrite-oxidizing bacteria (NOB; Le Roux et al. 2016; Wagner et al. 2002) oxidize NO_2^- to NO_3^-. While long hypothesized (Munro 1966), a recent study reported that BNI effect by some temperate tree species was due to a knockout of the NOB populations belonging to the genus *Nitrobacter* (Laffite et al. 2020). Using soil core transplantations, these authors demonstrated that BNI-induced changes in net and potential nitrification were correlated to changes in *Nitrobacter* rather than AO abundances. A direct inhibition of the growth of a *Nitrobacter* strain by soil and litter extracts was also observed (Laffite et al. 2020). This shows that the exploration of the nitrifier groups that can be inhibited through BNI remains in its infancy. In addition, after a lapse of time since the 1980s, the importance of heterotrophic nitrification (Zhang et al. 2015) has generated a growing interest and should also be considered. Further, both steps of nitrification can be performed by a nitrifier group which was discovered very recently, i.e., complete ammonia-oxidizing bacteria (comammox; Daims et al. 2015). To what extent comammox are another target of BNI however remains unknown.

Cascading effects of BNI on other N processes and associated microbial populations

BNI effect in plant rhizosphere does not only directly knock down nitrifier populations and nitrification but also likely induces cascading effects on other N processes, e.g., the activity of free-living N fixers that depends on N mineral forms or denitrification through the BNI-induced decrease in NO_3^- availability to denitrifiers. Recently, comparing stands from tree species with or without BNI capacity, Florio et al. (2021) evaluated such a cascading effect of BNI on soil denitrifier groups (i.e., nitrite and N_2O reducers) and on denitrification and N_2O emissions in the field. They reported that BNI did not always translate into lower denitrification and reduced N_2O emissions in particular because soil denitrifiers could be influenced by other environmental conditions such as pH, moisture, and organic carbon (C) availability. Florio et al. (2022 – this special issue) showed that after soil transfers between Douglas fir and two BNI tree species, potential denitrification changes were mostly mediated by changes in nitrifier activity and abundance and ultimately nitrate availability, hence suggesting a cascading effect of BNI on denitrifier activity. Consistently, studies in African savannas with BNI grasses vs. non-BNI trees showed that BNI plant species were associated to reduced denitrification (but not reduced abundances of denitrifiers) (Srikanthasamy et al. 2018).

BNI and overall microbial communities

Besides, through a direct effect of BNI compounds on nitrifiers and indirect effects (cascading effects generated by changes in nitrifier abundances and reduced nitrification), BNI plants could impact the whole composition of soil microbial communities and other soil functions. By acting on the N cycle, BNI could influence the diversity of the total microbial community and the archaea/bacteria/fungi ratios (Srikanthasamy et al. 2018, 2021). By acting on the conservation of nutrients and therefore plant biomass and primary production, BNI could influence the rate of soil mineralization. Interactions between fungi and BNI should be tackled: a good part of heterotrophic nitrifiers are fungi, and mycorrhizae have an important role in the supply of nutrients to the plants. The question therefore arises of the links between BNI and mycorrhization in terms of benefits and costs and between BNI and the phosphorus (P) cycle. Indeed, Ma et al. (2019) reported that the majority of soil archaeal taxa, most of them being AOA, were limited by P or other resources than N. On all these promising aspects, the thorough evaluation of such cascading effects across a broad range of soils and functions – including on N_2O emissions at a larger scale (see below) – remains to be done.

Knowledge gaps on BNI in terms of plant community and ecosystem structure and functioning

The plant community scale (Fig. 1, middle bubble) is tackled by studies interested in the BNI influence on soil N dynamics (including the articulation with other plant attributes/traits like N uptake capacities), on soil-vegetation feedbacks, and for putting BNI into the perspective of plant response to disturbances. Here the main knowledge gaps are as follows: (i) What is the effect of BNI on the coexistence and competition between plant species, the invasion capacity of BNI plant species and the ecosystem structuration? (ii) What are the impacts of disturbances (herbivory, fire, water scarcity/seasonality, etc.) on BNI via soil microbial communities or via plant diversity and ecosystem functioning? (iii) How does BNI influence N dynamics throughout the soil profile and in the overall ecosystem?

Impact of BNI on plant coexistence and ecosystem structuration

The fact that BNI has been selected along the Darwinian evolution of plants (see next section) implies that BNI influences the competitive abilities of plants. Models confirm that BNI influences the outcome of competition between plant species for N in relation with their preferences for ammonium vs. nitrate (Boudsocq et al. 2012; Konaré et al. 2019). In a nutshell, other things being equal, two species can coexist if one absorbs more N in the form of nitrate and the other in the form of ammonium, and inhibiting nitrification favors the plant species that prefer ammonium. Such theoretical results should be tested using mesocosms and field experiments where both BNI and non-BNI plants, and with contrasted N form preference, are grown together for a period long enough to assess the outcome of competition in terms of both plant growth (biomass) and plant demography (survival, fecundity). They could be based on situations observed in the field as BNI is likely responsible for the high performance of imported African grasses in South American pastures (Lata et al. 1999) and their invasiveness in northern Australian savannas (Rossiter-Rachor et al. 2009, 2017).

Because plants with strong BNI capabilities tend to be savanna grasses, the question of the influence of BNI on the structure of savanna plant communities is important. In the West African humid savanna of Lamto (Ivory Coast), all perennial grass species strongly inhibit nitrification so that BNI is unlikely to structure their community (Srikanthasamy et al. 2018). However, in other types of savannas, e.g., drier savannas, savannas from Eastern and Southern Africa, or disturbed savannas, grasses (often with diverse growth forms, perennial tussock grasses vs. annual grasses vs. stoloniferous grasses) might have different BNI capabilities, e.g., some with strong BNI capabilities but other with no BNI capabilities. In such cases, the impact of BNI on the structuration of the grass community and the possible consecutive spatial structure of grass species and mineral N availability should be studied.

In the same humid savanna of Lamto, while grasses inhibit nitrification, trees or some grass ecotypes are stimulating it. Modeling results (Konaré et al. 2019, 2021) suggest that BNI could facilitate the coexistence between trees and grasses which is the feature that defines savannas. In fact, trees that can have extended root systems would benefit from BNI by absorbing ammonium beyond their canopies, below BNI grasses in open areas. While other mechanisms of coexistence between savanna trees and grasses have been identified (Sankaran et al. 2004), it would be useful to test these modeling results, at least by measuring the involved nitrate and ammonium fluxes from the soil to trees and grasses. It must be noted that all these issues, i.e., the influence of BNI on competition between herbaceous plants or between herbaceous plants and trees, are relevant to any other type of ecosystem outside savannas and to the development of applications of BNI based on the addition of BNI plants in a cropping system (e.g., as cover crops).

BNI in the context of disturbance

All ecosystems are subject to perturbations such as drought, fires, and large herbivores. This has been particularly studied in savannas and grasslands where these perturbations play a key role in tree-grass coexistence and the ecosystem maintenance (House et al. 2003). A broad issue is thus to determine how perturbations influence plant capacity to inhibit nitrification both at the individual plant level and the consequences at the ecosystem scale in terms of N fluxes. Seasonality of water and temperature, and in particular drought-rewetting events, is known to impact soil microorganisms and plant functioning, by both stimulating (Birch effect) and decreasing mineralization and nitrification/denitrification activities (Kaisermann et al. 2013; Shi et al. 2021; Thion and Prosser 2014). AOA and AOB communities seem to respond differently to perturbations and to have different water and temperature niches (Gubry-Rangin et al. 2017; Thion and Prosser 2014). Seasonal fluctuation of nitrification in BNI-savanna ecosystems was also observed (Lata 1999; Srikanthasamy 2018) without deciphering whether it was due to seasonality of the exudation activities, switch of microbial communities and/or to the stability and mobility of BNI compounds linked to, e.g., soil water content.

Both fires and herbivores consume aboveground plant biomass and hence their photosynthetic surfaces, which could decrease on the short term the C resources they can allocate to BNI and therefore increase nitrification – in cases where fire intensity does not have a deleterious impact on microorganism communities. However, a study reported that nitrification decreases after the fire (Srikanthasamy et al. 2021), likely because savanna grasses resumed their growth quickly after the fire, at the end of the dry season, and even before the beginning of the rainy season, which would actually lead to an increased exudation of BNI molecules. The impact of herbivores on BNI has never been assessed, but the grazing impact on nitrification has already been shown in temperate and tropical grasslands (e.g., Le Roux et al. 2003). In the long term, impacts of herbivores and fire on soil nitrification may occur via many mechanisms and could interact with BNI via changes in the composition of the grass community: these perturbations would change the respective competitive abilities of BNI and non-BNI plants. For example, in savannas, BNI perennial grasses would be climatic species (i.e., present in a stable ecosystem in its final stage of ecological succession) adapted to a given regime of fire and herbivory and changes in these regimes could favor non-BNI grasses. Hence, it has been suggested for West African savannas that overgrazing by cattle could displace perennial BNI grasses by annual non-BNI grasses, which would increase nitrification and would contribute to the degradation of the ecosystem (Yé et al. 2017, 2021). These issues are very relevant to the management of pastures and the use of BNI grasses in these pastures.

Impact of BNI on overall N budget

BNI decreases N losses through leaching and denitrification, and this has been shown by both measurements (e.g., Ishikawa et al. 2003; Karwat et al. 2018; Lata 1999; Srikanthasamy et al. 2018) and models (Boudsocq et al. 2009), though these studies focused on mechanisms without quantifying accurate N fluxes at a relevant scale. Hence, the actual impact of BNI on N budgets and emissions of N_2O at the ecosystem and global scale has so far not been assessed. For example, bush encroachment is a worldwide phenomenon (Ward 2005) replacing BNI grasses by trees that have no BNI capabilities or that even stimulate nitrification, thus likely strongly modifying the N budget of large grassland, woodland, and savanna areas and increasing their N_2O emissions (Fig. 2). Finally, it also remains to measure how BNI influences N dynamics throughout the soil profile and, beyond the leaching of nitrate, and how it is linked to plant rooting pattern (e.g., between BNI and non-BNI plants – Konaré et al. 2021), to the pH and O_2 profiles and therefore the nitrification/denitrification ratio. For now, in situ measurements of N_2O at ecosystem or plot scale showed that BNI grass can strongly decrease N_2O emission in field plots (Subbarao et al. 2009). The only study currently available for temperate forests demonstrated that BNI decreases N_2O emissions only if soil denitrifiers are mostly limited by N rather than C or moisture (Florio et al. 2021). In a moist savanna from the Guinea zone, Assémien et al. (2019) showed that denitrifier abundances and activities as well as gross and net denitrification rates are mostly determined by nitrate availability rather than soil moisture or C. It is thus likely that BNI can contribute to decreasing N_2O production and emissions from this type of savannas. Given that the moist savanna zone covers $0.5\ 10^6$ km^2 for the sole West Africa (White 1986), this effect could be significant at large scale and evaluating the influence of BNI on the N_2O balance at a global scale is needed.

Knowledge gaps on BNI from a biogeography and evolutionary perspective

The biogeography and evolutionary scale (Fig. 1, right bubble), because of the long-term and large spatial consideration of BNI, is the scale that has been investigated the less so far. Here the main knowledge gaps are as follows: (i) To what extent is BNI present globally, and what environmental conditions might explain its evolution and expression? (ii) What coevolution has been possible between plant traits and BNI,

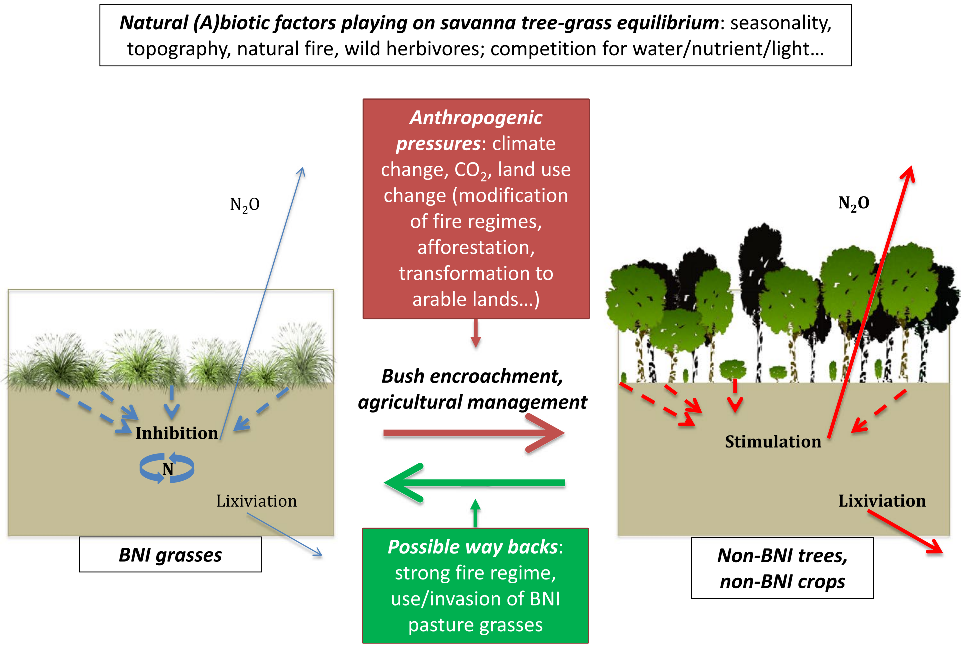

Fig. 2 Main shifts promoted by anthropic pressures impact between savanna/grassland ecosystems dominated by (left) BNI or (right) non BNI plant species, which could have huge implications for N cycling, including N_2O emissions and nitrate leaching, at local and large scales

especially for traits related to nutrient and N conservation in particular? (iii) What are the links between BNI evolution and BNI costs and benefices, and more generally what are the evolutionary dynamics of BNI?

Biogeography of BNI and its emergence

A major knowledge gap concerns the extent of BNI capacity across the broad range of savanna grass species and the geographic extension of BNI in savannas worldwide. For instance, do most of the grass species in West African humid savanna soils, where nitrifiers are adapted to very low N availability (Assémien et al. 2017), inhibit nitrification? Is BNI also common in other types of African savannas, for example, in Sahelian savannas where water resource limits primary production or in savannas of Southern and Eastern Africa where both N and P can be limiting (Ludwig et al. 2004)? Similarly, it is not known whether savanna grasses from other continents possess BNI capacities. The success of some African grasses in the pastures of Australia (Rossiter-Rachor et al. 2009) and South America (Baruch et al. 1985) could be due to their capacity to inhibit nitrification, which suggests that native savanna and grassland grasses outside Africa would have not evolved BNI capabilities.

A second knowledge gap concerns the environmental conditions that have led to the evolution of BNI in some savanna grass species. By decreasing N losses, BNI increases the efficiency of N cycling (Boudsocq et al. 2009), and it can thus be hypothesized that ecosystems where N availability is particularly low should be the hot spots of the evolution of BNI. Overall, savannas are considered to have N-poor soils (Bate 1981), but, for example, savannas from Eastern and Southern Africa can be dominated by N_2-fixing trees such as acacias, which can lead to a limitation by P instead of N and could thus have hindered the evolution of BNI. Fire and herbivory could also be influential for the selection of BNI capacity. Both fires and large mammal herbivores are key factors for the maintenance of savannas because, as disturbances, they tend to impede the establishment of a complete tree cover (House et al. 2003; Sankaran et al. 2004). Fires are also known to lead to N losses through emission of N gases to the atmosphere during the burning (Cook 1994; Delmas et al. 1995), which reinforces ecosystem N limitation. Herbivores accelerate N cycling, which can lead either to higher or lower N losses depending on soil characteristics and herbivory intensity (Bardgett and Wardle 2003; de Mazancourt et al. 1998). In a recent study, fire decreased AOA transcriptional activities and nitrification activities, likely due to an increase in root exudation after fire when grasses resume their growth (Srikanthasamy et al. 2021). However, no systematic screening of BNI capacities of grasses has been made with regard to the prevailing environmental conditions and disturbance regimes.

Plant traits linked to BNI and N conservation

The evolution of BNI capabilities in plants likely depends on plant traits other than root capacity to produce BNI compounds, and BNI should have coevolved with these traits. This should lead to correlations between these traits and BNI capacity that would be observed in present grass communities. For instance, plants that efficiently inhibit nitrification should better absorb ammonium rather than nitrate as their preferred N source (Boudsocq et al. 2009, 2012; Lata et al.

1999). As plants with C3 vs. C4 photosynthetic pathways respond differently to climate (e.g., to aridity) and can have different water and N use efficiencies and nitrate vs. ammonium preference (Luo et al. 2018), linking BNI and plant photosynthetic pathways should be of interest. It could also be hypothesized that perennial grasses are more likely to evolve BNI capabilities since perenniality (1) would increase the capability to inhibit nitrification as perennial root systems can exudate BNI molecules continuously, favoring long-lasting and cumulative BNI effects, and (2) would enhance the positive feedbacks of BNI to plant growth because perennial plants can increase N availability through BNI on the long term, hence maximizing BNI-related benefits for plant over years. This hypothesis is linked to the general idea that perennial plants are more conservative for nutrients than annuals (Grime 2001). Some results comparing degraded grass communities dominated by annual grasses to communities dominated by perennial grasses in West African savannas suggest that this hypothesis holds in relation with BNI (Yé et al. 2015). Similarly, BNI could have coevolved with the capacity to inhibit denitrification (Bardon et al. 2017). For example, it could be hypothesized that the capacity to inhibit nitrification impedes the evolution of the capacity to inhibit denitrification. Consistently with such a trade-off, Bardon et al. (2018) reported that *Pteridium aquilinum* (bracken) growing in acidic soils stimulates nitrification while also inhibiting denitrification. However, the associations between BNI traits and other plant traits have never been thoroughly studied across species or genotypes.

Following Odum's theory (Odum 1969), it has been hypothesized that plants occurring at the end of ecological successions in climax vegetation tend to inhibit nitrification (Rice and Pancholy 1972), leading to ecosystems that better conserve N, but this has never been tested thoroughly. Nevertheless, it has been shown that some savanna tree species stimulate nitrification (Srikanthasamy et al. 2018) suggesting that diverging evolutionary selection pressures may select for contrasted strategies within the same ecosystem. Overall, another knowledge gap corresponds to the capacity to influence nitrification as a dimension of plant strategies (Craine 2009; Grime 2001) and to possible links with other dimensions of these strategies such as the leaf economic spectrum (Laughlin 2011; Wright et al. 2004).

Evolution of BNI and BNI costs and benefices

The evolution of the capability to inhibit – or stimulate – nitrification should depend on the balance between benefits and costs of the control of nitrification. The benefits of BNI have been well identified (Boudsocq et al. 2009, 2012). As already said above, stimulating nitrification would be particularly beneficial for nitrate-preferring plants in competition with ammonium-preferring species (Boudsocq et al. 2012; Konaré et al. 2019). However, the costs of nitrification stimulation/inhibition have hardly been assessed. BNI plants bear costs corresponding to the amount of specific BNI compounds produced and to the energetic and mineral nutrient costs of the production of these molecules. This cost has so far never been assessed but the fact that, apparently, only a few plant lineages have evolved BNI capabilities would be a hint that inhibiting nitrification has a cost. In the same vein, symbiotic N_2 fixation is not widespread in plants likely because of its cost (Menge et al. 2008). Plants inhibiting nitrification through the quality of their litter, e.g., tannin content, bear costs linked to the production of the involved particular compounds, but they also bear the ecological costs and benefits linked to these compounds, e.g., for tannin, defenses against herbivores, and a slower litter decomposition (Grime et al. 1996). Understanding the selection of plant BNI capacity thus requires the joint analysis of all costs and benefices, which remains challenging for scientists so far.

Evolutionary dynamics of BNI

More generally, the evolutionary dynamics of BNI capacity has never been studied. For example, it is not known whether BNI has evolved a single time in grasses or whether it has separately evolved several times in various grass lineages. The fact that different molecule types are involved would plead for multiple evolutionary events (Coskun et al. 2017a). In the same vein, the timing of the evolution of BNI in grasses (i.e., early or late apparition during grasses evolution) is not known. Because C_4 grasses evolved a few million years after the apparition of tropical savannas during the Early-Middle Miocene (Edwards et al. 2010), and because all known grasses with high BNI capabilities (i.e., suppressing nearly totally nitrification) are C_4 savanna grasses, it is tempting to hypothesize that there are links between the evolution of BNI capacity and the evolution of the C_4 photosynthetic pathway in highly productive African savanna grasses. For example, using a modeling approach, BNI has been shown to increase biomass production in West African humid savannas (Boudsocq et al. 2009). However, the relationship between the evolution of the C_4 photosynthetic pathway and of the BNI capacity remains to be tested.

Many other evolutionary issues are related to the evolution of nitrifying microorganisms in the rhizosphere of BNI plants. As nitrification is a key process that generates growth and maintenance energy for nitrifiers (Prosser 1989), the sensitivity of these organisms to evolutionary pressures linked to BNI molecules produced by grasses are expected to be particularly high. For example, soil nitrifiers could have evolved new physiological traits allowing them to resist or adapt to BNI compounds. This might have led to evolutionary arm races similar to the arm races between plants producing molecules against herbivores and herbivores producing new enzymes to bypass plant defenses (Pichersky and Raguso 2018). Such evolutionary dynamics have never been studied in the context of BNI.

Towards a research agenda for filling main knowledge gaps related to BNI

We built on the main knowledge gaps identified above to draw a comprehensive research agenda which could guide advances in our understanding of ecological and evolutionary aspects related to BNI and ultimately in the designing of new agroecosystems. The main knowledge gaps are summarized in Table 1 along with the types of studies which could address these gaps, distinguishing three levels.

Level #1: the rhizosphere scale

The proximal effects of BNI on soil microbes, plant functioning, and N dynamics are ironically intensively studied but so far not fully understood. Indeed, it requires, even more than for the other scales of study, a clever mix of scientific and technical skills from chemistry, biochemistry, genetics, molecular ecology, microbial ecology, plant physiology, theoretical ecology, etc. In this perspective, the creation of a BNI consortium (Subbarao and Searchinger 2021), led by the Japan International Research Center for Agricultural Sciences (JIRCAS), is a key aspect to share experiences and practices across disciplines. The experimental needs will first of all require molecular and chemical analysis of microbial strains and of plant individuals grown in hydroponic medium or plant clones or lines (with a view to understanding BNI genetics of BNI). They will also require to go through greenhouse and common garden experiments and should mobilize functional ecology methodologies such as isotopic labeling and different, complementary measures of nitrification rates (gross or net; potential or not). For example, measurements of potential nitrification lead to robust results regarding temporal variability but can miss important aspects in the response of nitrifying communities (e.g., between AOA and AOB) due to ammonium input in non-limiting quantity. Finally, all the targeted knowledge gaps at this scale (Table 1) should be analyzed across a large range of plant species or ecotypes, soils, and environmental conditions as it is backbone knowledge to understand BNI effect on ecosystem functioning. The most critical points which should be given priority in a short time here are the measurement of the plasticity of BNI compounds production according to environmental conditions, the question of their persistence, and their impact on groups of nitrifiers other than AOBs. Testing these conditions for achieving BNI will be necessary in particular in an application framework in agroecosystems.

Level #2: plant communities and ecosystem scales

Predicting the influence of BNI on the N budget at the level of plant communities/ecosystems, and on losses of N through nitrate leaching and emissions of N_2O, would require the combination of heavy field measurements and modeling (Table 1). It is indeed critical to measure real fluxes (including the estimation of gross N transformation rates) in the field at relevant temporal and spatial scales. For instance, N_2O emissions can be assessed using static or dynamic chambers placed at the soil surface (Clough et al. 2020) or at larger scales (ecosystem) using flux towers (Oertel et al. 2016). Assessing the leaching of nitrate should ideally be achieved using various types of lysimeters or nitrate-absorbing devices inserted within the soil at various depths (Webster et al. 1993). Because inputs of N to ecosystems and N losses tightly interact, fully understanding the impact of BNI on the N budget of ecosystems (and their primary production) and comparing these effects between ecosystems require assessing many N fluxes, using ecosystem/biogeochemical models. This is also required to make predictions at large scales (from the local plant community to the continent). Simple differential equation systems could be used (Boudsocq et al. 2009; Konaré et al. 2019), while more complex and realistic models would allow taking the diversity of underlying factors into account and upscaling results (Bouwman et al. 2013; Del Grosso et al. 2000). An important issue will be to implement results of a worldwide sampling of grass BNI capabilities in such models and to use them to make prediction on N budgets and N_2O emissions according to realistic scenarios of vegetation change. The most critical points which should be given priority in a short time here are the links between BNI and seasonal/regular measurements of in situ N fluxes (including vertically through leaching) to match (or not) what is already estimated under controlled conditions and perform a benchmark of the methodologies used for N stock and flow measurements.

Level #3: biogeography and evolutionary processes

Understanding the evolution of BNI in grasses and the environmental causalities of this evolution requires an ambitious, e.g., worldwide, intensive sampling of grasses, and the assessment of their BNI capabilities (Table 1). This sampling should prioritize savanna ecosystems, but temperate grasslands should also be included. This would allow assessing the frequency and variability of BNI capabilities in grasses and the geographic and phylogenetic extension of these capabilities. Combining this data with data on ecosystem properties (such as N availability, fire frequency, and

Table 1 Summary of the main knowledge gaps concerning the ecological and evolutionary aspects related to biological nitrification inhibition, BNI, and proposed research agenda detailing the approaches that could help filling these gaps

Knowledge gap	Research approaches to tackle this gap
# Rhizosphere scale	
Knowledge on BNI compound production	
Level of diversity of BNI compounds	Systematic screening (identification and quantification) of BNI compounds using metabolomic approaches on root exudates and plant litter, across a broad range of plant species or ecotypes known or suspected to have BNI capacity versus non BNI plants. Build open access metabolomics libraries of BNI compounds
Contribution of BNI compound production in the rhizosphere to the ecological strategies of different plant species	Comparison of the levels of BNI compound production by different plant species encompassing a broad range of ecological strategies (e.g., Grime strategies)
Plasticity of BNI compound production in the rhizosphere according to soil environmental conditions	Quantification of BNI compounds produced by different plant species across environmental conditions (gradients of NH_4^+ or NH_4^+/NO_3^- balance; pH; water regimes; etc.)
Knowledge on the fate of BNI compounds in soil	
Fate of BNI compounds in soil, and persistence of their effects on nitrifiers	Survey of the fate of different BNI compounds in plant-soil systems, and analysis of their degradation products through biochemical analyses and ^{13}C labeling. Study of the possible cascading effects of degradation products derived from BNI compounds on nitrifiers
Knowledge on the effects of BNI compounds on soil microorganisms	
BNI effects across soil ammonia oxidizers	Quantification of BNI effects on ammonia oxidation rate for pure cultures of AOB and AOA isolated from soil, comparing strains adapted to high or low N availability
Molecular and physiological mechanisms explaining BNI effects on ammonia oxidizers	Physiological, genetic, and biochemical/enzymatic analyses of the mechanisms underlying BNI effects on a wide diversity of pure cultures of AOB and AOA and across BNI compounds
BNI effects on nitrifier groups other than ammonia oxidizers, and underlying molecular and physiological mechanisms	Quantification of BNI effects on pure cultures of *Nitrobacter*, *Nitrospira*, heterotrophic nitrifiers, and Comammox isolated from soil. When relevant, physiological, genetic, and biochemical analyses of the mechanisms underlying BNI effects on *Nitrobacter*, *Nitrospira*, heterotrophic nitrifiers, and Comammox
Level of inhibition induced by BNI on nitrification	Quantification of BNI effects on soil nitrification enzyme activity (by potential nitrification assays) and on gross nitrification rate (by the ^{15} N-dilution technique) across a range of plants, soils and environmental conditions
BNI effects on the activity, abundance and diversity of nitrifiers	Simultaneous analyses of the activity, abundance, and diversity of soil nitrifier groups inhibited by BNI through biochemical assays, quantitative PCR, and high-throughput sequencing, respectively, to relate BNI effects on nitrification activity to effects on nitrifier group abundance/diversity
Cascading effects of BNI effects on other microbial groups than nitrifiers and other functions than nitrification	Quantification in particular of (i) ammonification and ammonifiers and (ii) denitrification (i.e., gross and net N_2O production) and denitrifiers (mainly nitrite and N_2O reducers), in the rhizosphere of plants with and without BNI capacity. In situ comparison and in common garden of the links between above- and belowground diversities (including free-living N fixers and mycorrhizal fungi) and soil functions (mineralization, P cycle, etc.)
# Plant community and ecosystem scales	
Spatial distribution of BNI effects in soil in relation with root spatial distribution and soil characteristics	In situ measurements of BNI compound concentrations, root density, and nitrification, along vertical and horizontal gradients. For multispecies plant covers, use of, e.g., NIRS technology, to assess the local biomass of roots from different species. Use isotopic measurements of the ^{18}O/deuterium ratio to measure water exchanges between soil depth and plant to understand the depth of root activity. Measure redox potential and nitrification/denitrification activities along soil profile

Table 1 (continued)

Knowledge gap	Research approaches to tackle this gap
Effects of BNI on plant community	In situ and mesocosm experiments, with measurements and modeling of N cycling in mixed BNI vs non BNI (possibly nitrification stimulating) plants. In situ and mesocosm experiments, with measurements and modeling of N cycling in mixed BNI perennial plants vs non BNI annual/biannual/weak perennial plants. In situ and mesocosm experiments, with measurements and modeling of N cycling in BNI plants of mixed growth forms (e.g., tuft vs mat-forming). In situ and mesocosm experiments, with measurements and modeling of N cycling in mixed BNI plants with different NH_4^+/NO_3^- preferences (gradient). In situ and mesocosm experiments, with measurements and modeling of N cycling in mixed BNI vs N-fixing plants. In situ and mesocosm experiments, with measurements and modeling of N cycling in mixed BNI vs mycorrhizal plants. These experiments should also analyze the role of BNI on plant-plant competition and on the plant community dynamics
Impact of BNI on N budget and N_2O emission at ecosystem scale	Comparison of different ecosystems/plant communities with contrasting BNI capabilities and field assessments of N fluxes (especially N_2O emissions and leaching of nitrate). Modeling approaches to complement information derived from field data
Interactions between the effects of BNI and of disturbances (whole plant or community scale)	In situ and mesocosm measurements to understand the interaction between BNI and drought/rewetting events. In situ and mesocosm measurements to understand the interaction between BNI and seasonality. In situ and mesocosm measurements to understand the interaction between BNI and different fire regimes. In situ and mesocosm measurements to understand the interaction between BNI and herbivory
# Biogeography and evolutionary processes	
Knowledge on the distribution of BNI in relation to biogeography, phylogeny, and functional traits	
Types of ecosystems where can BNI be encountered	Worldwide sampling of plant (mostly grass) species, assessment of their BNI capabilities
Ecosystem properties favorable to the existence/dominance of BNI plants	Building on worldwide screening of plant species for their BNI capabilities (above), quantify BNI plant species biomasses and test correlations with native ecosystem characteristics
Modalities of BNI evolution in the plant tree of life	Building on worldwide screening of plant species for their BNI capabilities (above), analyze the relationships between BNI capacity and the grass phylogeny
Knowledge on the link between BNI and plant/nitrifier traits	
Co-evolution of BNI with other traits such as perenniality and the preference for nitrate vs. ammonium	Building on worldwide screening of plant species for their BNI capabilities (above), quantify traits like perenniality, preference for nitrate/ammonium, etc. for each species, and test correlations between BNI capabilities and other plant traits in the perspective of the plant phylogeny
Co-evolution of BNI and BDI	Screening of a large number of plant species (in particular those known for BNI or BDI capacity to date, plus non BNI/non BDI species) to assess the relationships between their BNI and BDI capabilities
Co-evolution between BNI plants and nitrifying organisms	High-throughput sequencing of the rhizosphere microbiome, targeting nitrifier groups, across a broad range of BNI and non BNI plant species, analyzed in the perspective of plant phylogeny. Analysis of the de novo recruitment of soil nitrifiers by the roots of BNI versus non BNI plant species

mammal herbivore biomass) could suggest environmental properties favoring the selection of BNI plants. Further, combining these data with data on other functional traits of grasses could help in generating hypotheses on the coevolution of BNI with some plant traits. In addition, combining all these data with data on the phylogeny of the grasses would allow testing hypotheses on the modalities of the evolution of BNI, its coevolution with other grass characteristics (perenniality, capacity to inhibit denitrification, etc.), and the influence of ecosystem characteristics (Charles-Dominique et al. 2016; O'Meara 2012). This general approach implies to reliably compare BNI capabilities of species living in different native environments. A solution would be to grow all grass species in the same environment, i.e., in a common garden experiment, which might be challenging due to logistic problems. The results of such an experiment would also likely depend on the characteristics of the common environment (e.g., soil type(s) used for the common garden experiment). Another solution would be to use soils sampled in situ in the rhizosphere of the different grass species from their native ecosystems compared to non-rhizospheric soils. From these samples, BNI abilities should be assessed with a method integrating environmental and temporal variability arising, e.g., due to variations in soil humidity and nitrate availability.

Studying the evolution of BNI and testing simple hypotheses related to this evolution is also possible through modeling approaches such as the adaptive dynamics framework (Geritz and Gyllenberg 2005). This approach could allow predicting whether BNI can evolve and whether it leads to a stable fixed strategy (e.g., a unique intensity of BNI) or a diversification of strategies (i.e., plant species with diverse BNI intensities or non-BNI species and BNI species). However, such predictions require data (that are still missing) or assumptions on the ecological costs of producing BNI molecules. The approach would also allow predicting the impact, on the evolution of a plant trait such as BNI, of simple environmental features such as N availability (Barot et al. 2016), herbivores (Loeuille and Leibold 2008), and fire (Srikanthasamy et al. 2021). An interest of this approach is to predict the outcome of competition (including coexistence) between different species with different BNI strategies (Boudsocq et al. 2012; Konaré et al. 2019). This would allow predicting the possible influence of BNI on the functional diversity of grass communities. The most critical point which should be given priority in a short time here is an estimate of the energy and resource cost of BNI for plants, and of this cost is plastic, which we totally lack to establish the adaptive tradeoff between BNI and non-BNI.

Conclusions

This review article is a call to more systematically study the ecological and evolutionary aspects of BNI. Although the corresponding research agenda raises fundamental questions to be addressed, we advocate that answering them is also required to develop applications of BNI. For example, we need a thorough understanding of the physiological implications of BNI for plants, and of its consequences for soil functioning and biodiversity, to safely implement BNI in agricultural systems. We also need a comprehensive assessment of BNI capabilities in plants and some understanding of the underlying evolutionary dynamics to find the suitable species and genotypes to develop BNI crops or tree stands and agroecological applications of BNI such as mixtures of BNI plants with non-BNI crops or mixtures of varieties (Barot et al. 2017). BNI studies were initiated as addressing a purely fundamental issue of ecosystem functioning, and then have become a hot topic bearing potential paradigm shift in agronomy. Now is the time for these two aspects to walk together hand in hand and use the fundamental understanding of BNI in relation to ecosystem functioning to benefit N cycling and N use efficiency in agricultural systems.

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Declarations

Conflict of interest The authors declare no competing interests.

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