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Nitrogen transformations in modern agriculture and the role of biological nitrification inhibition

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The nitrogen (N)-use efficiency of agricultural plants is notoriously poor. Globally, about 50% of the N fertilizer applied to cropping systems is not absorbed by plants, but lost to the environment as ammonia (NH_3), nitrate (NO_3^-), and nitrous oxide (N_2O, a greenhouse gas with 300 times the heat-trapping capacity of carbon dioxide), raising agricultural production costs and contributing to pollution and climate change. These losses are driven by volatilization of NH_3 and by a matrix of nitrification and denitrification reactions catalysed by soil microorganisms (chiefly bacteria and archaea). Here, we discuss mitigation of the harmful and wasteful process of agricultural N loss via biological nitrification inhibitors (BNIs) exuded by plant roots. We examine key recent discoveries in the emerging field of BNI research, focusing on BNI compounds and their specificity and transport, and discuss prospects for their role in improving agriculture while reducing its environmental impact.

It has been estimated that by the year 2050, a 70–100% increase in global agricultural production will be needed to sustain a world population of nine billion people[1]. This will almost certainly require much greater reliance on global nitrogen (N) fertilizer synthesis via the industrial Haber-Bosch process, in which relatively non-reactive N_2 gas is hydrogenated to produce biologically available ammonia (NH_3). The amount of 'reactive N' (N_r) introduced into the biosphere by these means is already prodigious, amounting to 120 Tg yr^{-1} (Tg, teragram), or about twice as much as is fixed by all natural terrestrial processes combined (63 Tg yr^{-1}) (ref. 2). Of this, about 80% is used in fertilizer production[3], providing nourishment for half of the world's human population[4]. Unfortunately, modern agricultural systems are also highly inefficient in their N use, typically losing 50–70% of applied N_r to the environment[5,6] (Fig. 1). Moreover, the resulting increases in pools of excess N_r species, such as NH_3 and nitrous oxide (N_2O) in the Earth's atmosphere, and nitrate (NO_3^-) in terrestrial and aquatic ecosystems, have significant consequences for climate change and environmental toxicity[2,3,7,8]. These include changes to ecosystem productivity and biological diversity[9,10], eutrophication and nitrate contamination of freshwater resources[10,11], ozone and air quality degradation[11,12], and greenhouse gas (GHG)-driven climate change[3,7].

N_r losses from agricultural fields are driven by deprotonation of ammonium (NH_4^+) to NH_3, which is governed by fertilizer application rate, pH, moisture and temperature[13], and by an array of nitrification and denitrification reactions, which are catalysed by a diverse set of soil microorganisms (Fig. 2). Nitrification, the oxidation of NH_3 to NO_3^-, was long considered to be the domain of two groups of chemoautotrophic bacteria, the ammonia- and nitrite-oxidizing bacteria (AOB and NOB). The AOB subgroups β (for example, *Nitrosomonas* spp.) and γ (for example, *Nitrosococcus* spp.) initiate the nitrification process by oxidizing ammonia to hydroxylamine (NH_2OH), the rate-limiting step, which is catalysed by the enzyme ammonia monooxygenase (AMO), and then oxidize NH_2OH to nitrite (NO_2^-) via hydroxylamine oxidoreductase. NOB (for example, *Nitrobacter* spp.) complete the process by producing NO_3^- via the enzyme nitrite oxidoreductase[14–16]. More recently, however, a much wider cast of microbial players has become known, including archaeal and fungal nitrifiers[16,17], as well as unique 'commamox' bacteria (complete ammonia oxidizers) of the genus *Nitrospira*, that perform both oxidative steps[18,19]. The relative abundances and activities of nitrifiers vary with soil type, climate, vegetation and other environmental factors[20–22].

The discovery of ammonia-oxidizing archaea (AOA) is particularly noteworthy; no evidence had been found for such organisms until 2004, when J. C. Venter and colleagues found *amo* sequences in archaeal-associated genomic scaffold data from microbial populations in the Sargasso Sea[23]. Indeed, the existence of the entire domain of Archaea has only been known for 40 years[24]. Since the discovery of AOA, it has been recognized that they outnumber AOB in many habitats[25] and can play a dominant role in nitrification, particularly in acid soils[17,26] (see section 'Promises and drawbacks').

Ammonium-based fertilizers (urea, anhydrous NH_3, $(NH_4)_2SO_4$ and NH_4NO_3) comprise the most commonly used forms of N applied in agriculture[27]. While many soils can electrostatically retain an abundance of cations such as NH_4^+, due to the typically negative charges on soil particle surfaces, deprotonation of this ion produces ammonia gas that is volatized to the atmosphere in large amounts (globally, 18% of applied synthetic N_r (ref. 28)), while similar amounts (19% globally) are lost via the nitrification of NH_3 to NO_3^-, followed by leaching and runoff of this poorly soil-bound anion[29,30]. The reverse process, denitrification, that is the reduction of NO_3^- to NO_2^-, nitric oxide (NO), N_2O and N_2, is also catalysed by a diverse cast of bacterial, archaeal and fungal players[16,31] (Fig. 2). The partial reduction of NO_3^- to N_2O is environmentally adverse, because N_2O is not only a GHG with 300 times the heat-trapping capacity of carbon dioxide (CO_2), per molecule[32], it is also the most important destroyer of ozone in the atmosphere[12]. Currently, the majority (60–80%) of global anthropogenic N_2O emissions (as well as 10–12% of all anthropogenic GHG emissions) are attributable to agriculture[33,34]. Substantial quantities of N_2O can also be released during nitrification, via NH_2OH oxidation and via 'nitrifier denitrification', as in the reductive reaction sequence $NO_2^- \rightarrow NO \rightarrow N_2O$ (ref. 35). The latter process can account for as much as 97% of

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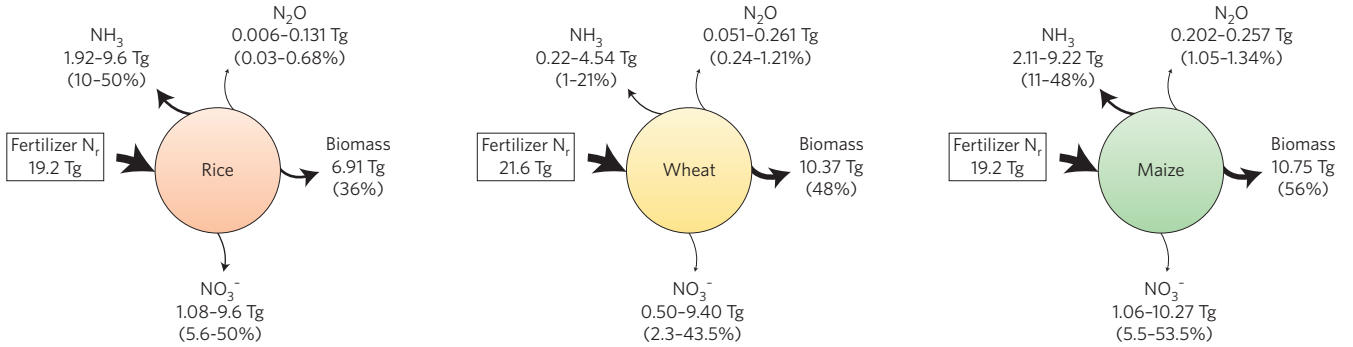

Figure 1 | Nitrogen budgets of the 'big three' crops. Of the *c.* 120 Tg N yr^{-1} fixed by the Haber-Bosch process[2], 50% is applied to the world's three major cereals, rice (16%), wheat (18%) and maize (16%) (ref. 119), which together provide more than 60% of human caloric intake[120] and cover approximately 546 million ha (36%) of global cropland[33]. Global averages of fertilizer N recovery (the proportion of fertilizer N retained as biomass) for the three cereals are shown[119]. The remaining N is lost to the environment through NH_3 volatilization, NO_3^- leaching and runoff, denitrification (producing NO, N_2O, and N_2 gases), and is also immobilized by other organisms or soils[30,33,121–130]. The proportion of N lost will vary depending on fertilizer type and environmental factors, including temperature, wind speed, rain, and soil properties such as cation exchange capacity and pH.

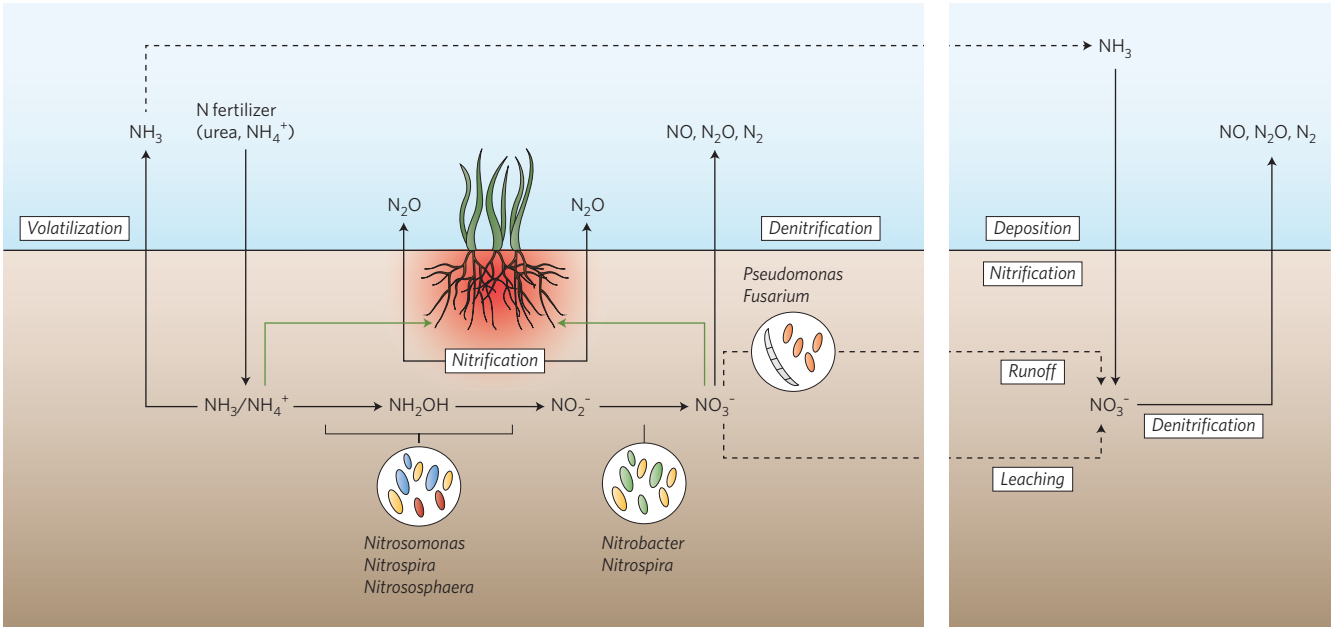

Figure 2 | Schematic overview of the fate of nitrogen fertilizers applied to agricultural systems. N fertilizers (applied mostly as urea or NH_4^+ salts, with the former hydrolysed to NH_3 by ureases) undergo nitrification, in which soil microorganisms (some genera of which are listed) oxidize NH_3 into NO_3^- via NH_2OH and NO_2^-. BNIs exuded from plant roots (shown in red) can inhibit these reactions (see main text for details). Plants can take up either inorganic N source (NH_3/NH_4^+ and NO_3^-; green arrows). N_2O, a potent greenhouse gas, is released predominately during nitrification in agricultural soils but can also stem from microbial denitrification. NO_3^- and NH_3 can travel long distances (dashed arrows) away from agricultural sites and into neighbouring ecosystems (via runoff/leaching and volatilization/deposition, respectively), causing indirect N_2O emissions and other problems locally (for example, soil nutrient losses, acidification, eutrophication and biodiversity loss).

N_2O emissions in cropping systems[36], while indirect N_2O emissions, derived from N_r transported from agricultural soils via volatilization, deposition, leaching and runoff (Fig. 2), may account for 28–37% of global agricultural N_2O production[37].

For these reasons, attempts to understand and mitigate nitrification in agriculture have recently become the focus of intensive research initiatives. Several strategies have been put forward to curb agricultural N_r losses, including the more precise co-ordination of fertilizer application methods with plant growth patterns, improvement of cropland management practices, and use of synthetic nitrification inhibitors (SNIs)[30,38]. Some of these inhibitors, including nitrapyrin, dicyandiamide, 2-amino-4-chloro-6-methylpyrimidine and 3,4-dimethylpyrazole phosphate (Table 1), have been used to suppress nitrification and increase nitrogen-use efficiency (NUE)[39,40], but also have many drawbacks, including difficulties in application, cost, degradation, pollution and entry into the food system[41–43] (for comparison, ref. 44). Functionally similar compounds exuded from plant roots have long been known to influence biogeochemical processes, including the N cycle[45–48], although the precise chemical nature and mechanisms of action involved have been, until recently, essentially unknown. With the advent of novel technologies, in particular the development of a bioluminescent, recombinant strain of *Nitrosomonas europaea*, which has greatly facilitated the measurement of nitrification and its inhibition[49,50], promising new research into nitrification inhibitors from plant root exudates, termed biological nitrification inhibitors (BNIs)[49], has emerged. Here, we evaluate these advances, which have focused on the identification and synthesis of specific compounds, their release and

Table 1 | A survey of nitrification inhibitors and their efficacy.

Compound	ED_{80} (μM)	Refs
BNIs		
Sorgoleone	12	60, 117
Sakuranetin	0.6	60
MHPP	120–166.5	61, 64
Brachialactone	10.6	50
1,9-Decanediol	516	64
SNIs		
Allylthiourea	0.22	118
Nitrapyrin	17.3	64, 118
Dicyandiamide	2200–2973	64, 118
2-Amino-4-chloro-6-methylpyrimidine	522	64

Plant-derived nitrification inhibitors (found specifically in root exudates) and synthetic nitrification inhibitors are listed, along with their ED_{80} values (effective dose where 80% inhibition of nitrification was measured).

their mechanisms of action, and discuss how they may contribute to agricultural remediation.

A fresh look at an old issue

Evidence supporting the presence of BNIs in a wide variety of ecosystems and in the tissues of many types of plants, including grasses (in addition to trees, shrubs, herbs and mosses), can be found in many early studies, notably the work of Rice and Pancholy[47,51]. These authors postulated that nitrification inhibition by root-released substances was a key mechanism driving ecological succession, and used it to help explain how soils of climax ecosystems can have much higher NH_4^+ concentrations, and much lower NO_3^- concentrations and nitrifier populations, than disturbed, early successional sites, a paradigm that continues to be explored today[52]. More recently, two tropical grasses, *Hyparrhenia diplandra*[53,54] and *Brachiaria humidicola*[55,56], have received closer attention following field observations that strongly correlated their presence on land with low nitrification potentials and low soil nitrate content. The isolation of BNIs from *B. humidicola* root tissue extracts indicated that methyl and ethyl esters of ferulic acid powerfully inhibited nitrification (in the case of ethyl ferulate, the IC_{50} (the inhibitor concentration that produces 50% of a response) was 200 nM), whereas the free acid had no inhibitory effect[57]. Curiously, while this was attributed to the greater lipid solubility of the ester forms, it stands in stark contrast to the earlier findings of Rice and Pancholy[47], where free ferulic acid was the strongest nitrification inhibitor out of 17 tested, completely inhibiting nitrification by *Nitrosomonas* at a concentration of only 10 nM. This disparity might be explained methodologically; the older study used a soil suspension protocol followed by chemical analysis, while the more recent one used bioluminescent *N. europaea* in its BNI assay[49,58]. Although the high sensitivity of this assay has been instrumental in revealing the physiology and biochemistry of BNI exudation in several important plant species, concerns about the applicability of bioluminescence results to the soil environment have been an ongoing theme[59,60] (see section 'Promises and drawbacks').

Until less than a decade ago, identification of specific BNIs was generally limited to those extracted from plant tissues and soil litter, rather than from root exudates *per se*. In 2008, the first nitrification inhibitor isolated directly from root exudates was reported in sorghum (*Sorghum bicolor*)[61], a species considered to have high BNI capacity relative to other crops[62]. This compound was identified as methyl 3-(4-hydroxyphenyl) propionate (MHPP), a phenylpropanoid with moderate BNI activity (Table 1; Fig. 3). The study was additionally significant in that it illuminated a poorly understood process: the response of root exudate transport rates to external stimuli (N source and pH). Since these discoveries were made, four other BNI-active root exudates have been isolated and partially characterized. Two are from sorghum, identified as sorgoleone, a benzoquinone that is the dominant BNI compound in the hydrophobic fraction of root exudates, and sakuranetin, a flavanone, which, like MHPP, was isolated from the hydrophilic fraction[60]. A third has been named brachialactone (a cyclic diterpene), and appears to be the most important BNI in *B. humidicola*[50,63], while a fourth, the most recently discovered, is from rice: 1,9-decanediol (a fatty alcohol)[64] (Table 1; Fig. 3).

The presence of both hydrophobic and hydrophilic BNIs in sorghum root exudates suggests that their sites of inhibitory action are spatially separated, with less mobile, hydrophobic compounds predominating in the rhizoplane, and more mobile hydrophilic compounds able to diffuse greater distances from the root[60]. It has also been suggested that the co-presence of biochemically distinct BNIs in the soil could exert an additive or synergistic effect[59,65], a possibility that has been overlooked in laboratory assays. BNI diversity might also decrease the likelihood of selection for inhibitor-resistant strains of nitrifying microorganisms[50]. These possibilities warrant further investigation.

Relatively few studies have investigated biological nitrification inhibition in the 'big three' agricultural crops, perhaps surprisingly, given the economic importance, and intensity of N use, of rice, wheat and maize (Fig. 1). This de-emphasis may partially be attributed to a preliminary, and in retrospect, overly constrained survey that asked how widespread BNIs might be among plant species[62]. In this study of 18 species, no BNIs were found in root exudates of the three major cereals. However, the discovery of substantial BNI activity in exudates from roots of *Leymus racemosus*, a wild relative of wheat, led to an ambitious project that aimed to transfer this characteristic to *Triticum aestivum* (bread wheat) by genomic addition of the BNI-associated Lr#n chromosome from *L. racemosus*[65]. While this approach was successful, the task of transferring genetic material among plant species, let alone genera, can be arduous[66,67]. Fortunately, the idea, based on a single cultivar, that wheat possesses little or no biological nitrification inhibition, has been overturned by a recent survey of 98 genotypes of *T. aestivum*[67]. This study showed that several landraces, and two commercial cultivars that are in use today, have significant BNI activity in their root exudates, although the chemical identities of these BNIs have yet to be determined[67] (Fig. 3). This discovery is likely to make the breeding of biological nitrification inhibition into modern wheat much more straightforward. Similarly, the idea that rice roots do not exude BNIs has also been overturned[64,68]. In a study of root exudates from 36 rice genotypes, Tanaka *et al.* showed that about half had significant biological nitrification inhibition,

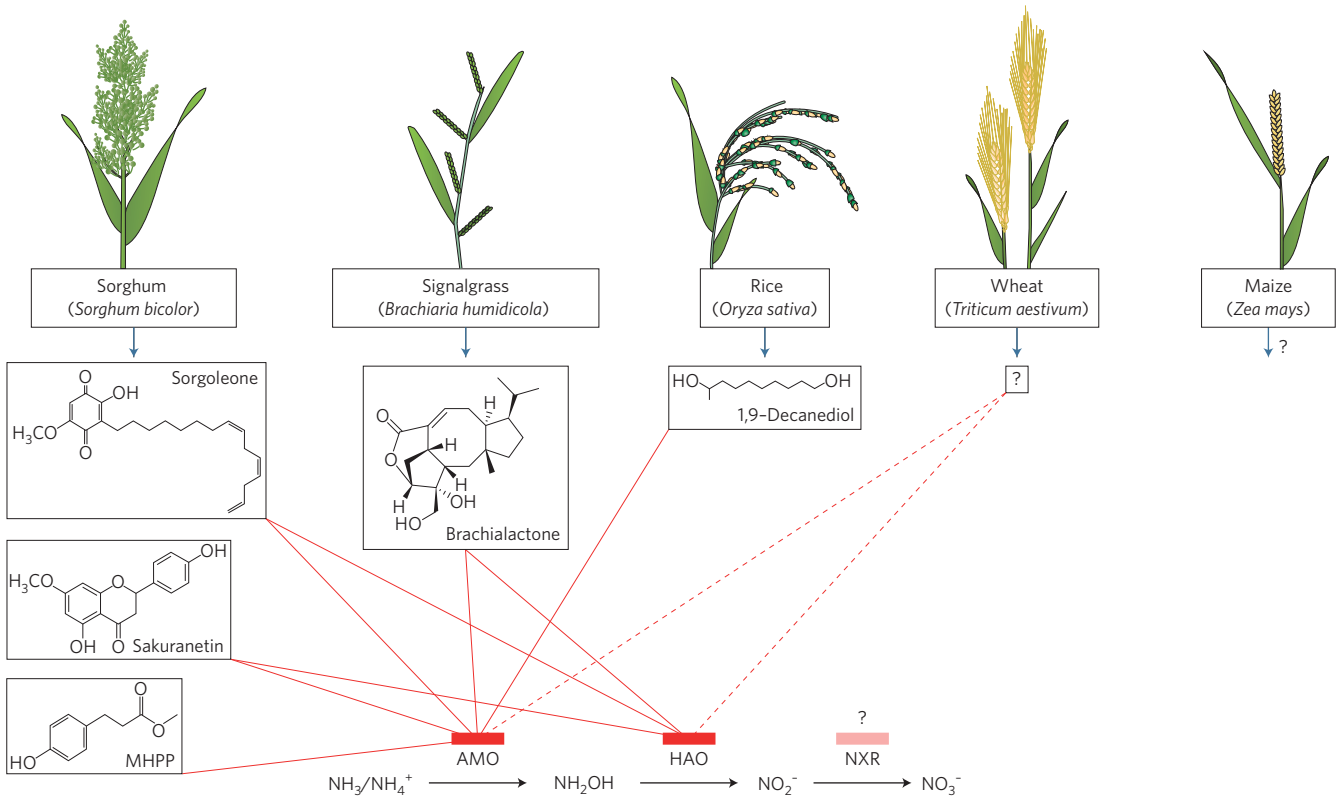

Figure 3 | BNIs from root exudates and their enzyme targets. BNIs from exudates that have been isolated to date are shown, along with the plants from which they are derived and their enzyme targets, which catalyse the nitrification reactions. Solid red lines denote known modes of action, dashed red lines denote unknown modes. BNIs have been demonstrated in the case of wheat, although specific compounds have not been identified. BNIs have not been demonstrated in maize, although it is one of the 'big three' global crops (see Fig. 1). Nitrite oxidoreductase inhibition by root exudates has also not been demonstrated. AMO, ammonia monooxygenase; HAO, hydroxylamine oxidoreductase; NXR, nitrite oxidoreductase.

particularly among upland rice[68]. More recently, a screening of 19 rice genotypes indicated that there was strong BNI potential in both indica and japonica subspecies[64]. Here, a new BNI was identified to be the fatty alcohol 1,9-decanediol, a compound with moderately strong potential to inhibit nitrification (Table 1). There has still not been a documented demonstration of BNI exudation by maize roots, but it is too early to rule out BNI potential in the vast genetic resources of this third member of the 'big three'.

The conundrum of BNI specificity

Have nitrification inhibitors that are produced by plants evolved as specific responses to nitrifying environments, or alternatively, are they produced by plants for very different functions, inhibiting nitrification only as a coincidental side effect? Conifer needles, for instance, can produce large quantities of terpenoids, which are capable of inhibiting nitrification, but the primary functions of these compounds are related to intracellular activities, including plant defence[69]. In fact, many of the BNIs so far isolated from both root exudates and plant tissues play roles that are unrelated to nitrogen metabolism. An obvious example is that of linolenic acid, which, although considered to be a 'major BNI compound' found in the leaves of *B. humidicola*[50], is also one of the most common polyunsaturated fatty acid species found in plant membrane lipids, comprising over 90% of the thylakoid lipid moiety in some species[70]. Moreover, all of the BNI compounds so far identified in sorghum (Fig. 3) appear to have other functions. Sorgoleone, in particular, has long been known to have a powerful herbicidal effect on competitors when exuded by sorghum roots, suppressing competitor photosynthesis via binding to the D1 protein and inhibiting photosystem II, as well as inhibiting mitochondrial electron transport, root H^+–ATPase activity, and water uptake[71]. Sakuranetin has been found to have phytoalexin activity in rice leaves, acting in plant defence[72], and MHPP was recently shown to have profound effects on the hormonal control of root development in *Arabidopsis thaliana*[73]. The ability of these compounds to also inhibit the nitrification of NH_3 to NO_2^- via NH_2OH (Fig. 3) may then, in some cases, be less indicative of a BNI-specific function, but instead could reflect the surprisingly diverse range of compounds, including CO_2, glucose and methane, that are able to inhibit AMO[74].

One way to resolve this conundrum was recently discussed by O'Sullivan *et al.*[67], who suggested that if a compound is 'actively' exuded by roots rather than released through more stochastic processes, such as litter decomposition and membrane leakiness, it is more likely to be specific to biological nitrification inhibition. In their own study in wheat cultivars, the authors noted a lack of correlation between the specific activities of BNIs in tissue and in root exudates, suggesting a functional decoupling of the BNI pools. In the case of rice, Tanaka *et al.* showed that release of BNIs from roots was due to membrane-regulated processes and not to non-specific leakiness of the tissue[68]. The presence of bioactive compounds in root exudates may reasonably indicate that they interact with the soil environment, but a further degree of confidence in their assignment as BNI-specific substances emerges from studies that indicate that root exudation is rapidly, and pronouncedly, increased only in regions externally exposed to NH_4^+. This has been demonstrated in sorghum[61], *B. humidicola*[75] and *L. racemosus*[65], as well as in wheat plants transformed with the Lr#n chromosome from *L. racemosus*[65]. Interestingly, in sorghum, this stimulation appears to be linked to the activity and expression of plasma membrane (PM) H^+–ATPases

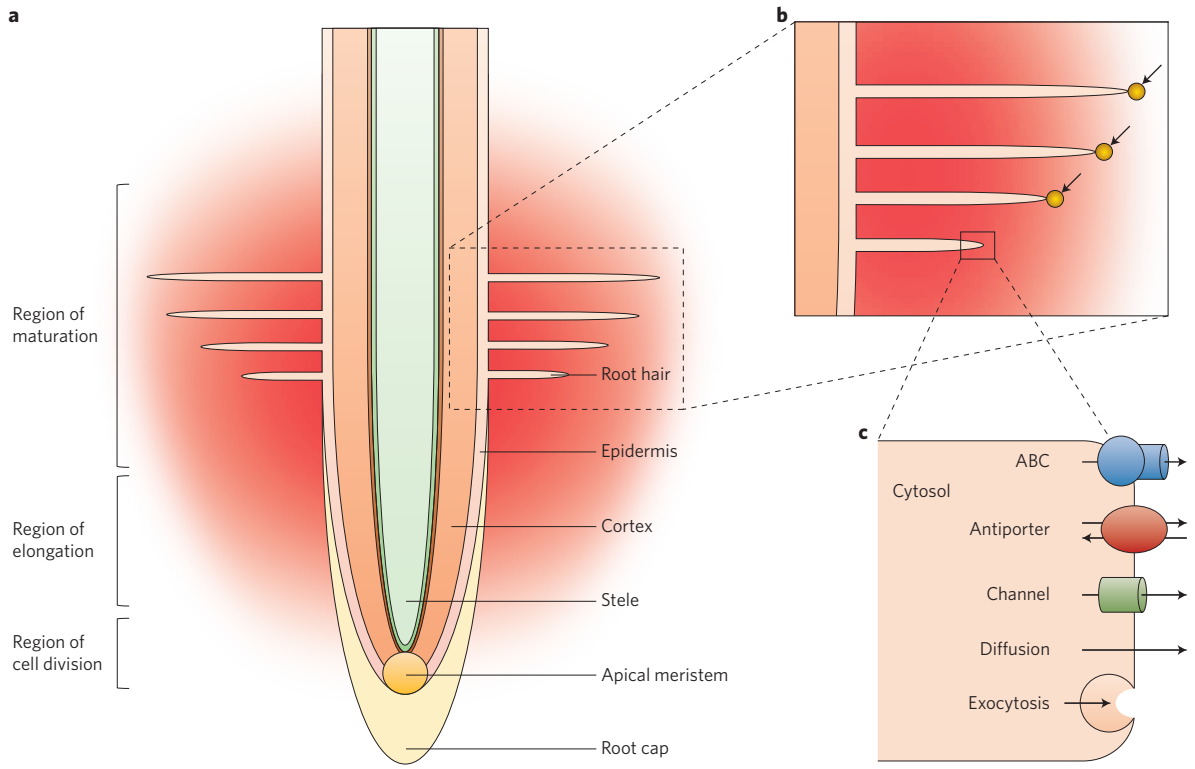

Figure 4 | Zonation and mechanisms of BNI root exudation. a, Longitudinal section of a root. The region of maturation is considered to be a major site of exudation of allelochemicals, including BNIs[79]. **b**, Root exudate droplets, such as sorgoleone, are excreted solely from tips of root hairs[80] (see arrows). **c**, Putative release mechanisms for root exudates include ABCs, antiporters (for example, MATE transporters), ion channels, simple diffusion, and vesicle release via exocytosis[76,79,80].

in root cells, as well as to NH_3 assimilation[76,77], but the significance of these relationships is not yet clear (see section 'Zonation and mechanisms of BNI release). In the case of rice, the strength of nitrification inhibition in root exudates has been shown to be positively correlated with both ammonium-use efficiency and ammonium preference in 19 cultivars, which provides evidence for a BNI-specific function in this most important of crop species[64].

In terms of the functional assignment of putative BNIs, a particularly interesting case is that of MHPP, exuded by sorghum[59]. It was recently discovered that treatment of *A. thaliana* roots with synthetic MHPP resulted in a substantial remodelling of root system architecture (RSA)[73]. This took the form of both a substantial increase in lateral root development and a reduction in primary root elongation, and was attributable to greater recruitment of auxin to the root tip, mediated by nitric oxide and reactive oxygen species. The ecophysiological significance of this finding, however, is not clear, as endogenous production of MHPP has not been identified in *A. thaliana*. It is worth noting, however, that increased lateral root formation is generally associated with NH_4^+ nutrition[61,78], and that, in sorghum, both the production and exudation of MHPP are stimulated by external NH_4^+. This raises the intriguing possibility that NH_4^+ triggers both BNI production and root branching via the production of MHPP in sorghum, although the effects of MHPP on RSA have yet to be investigated in this species.

Zonation and mechanisms of BNI release

Recent investigations have begun to unveil the cell types and transport mechanisms involved in BNI exudation. As mentioned, BNI release in several species is confined only to parts of the root system exposed to NH_4^+, ensuring that BNIs are specifically exuded in the regions of the rhizosphere where nitrification is probably at its maximum. Root development may also play a significant role in determining the composition of root exudates, and the maturation zone (proximal to the root tip) has been found to be a major site of exudation for allelochemicals, including BNIs[79] (Fig. 4a). Sorgoleone, the most thoroughly studied of the BNIs so far identified (although predominately in the context of other functions[70]; see section 'The conundrum of BNI specificity') is produced solely by root hairs in sorghum and exuded as golden-brown droplets from root hair tips[71,80] (Fig. 4b). Droplets of this kind are much less frequently observed for root hairs of other graminaceous plants, such as wheat[80].

At a finer level of resolution, several molecular transport mechanisms governing BNI efflux across the plasma membrane of root cells have been proposed (Fig. 4c). As the presence of NH_4^+ stimulates BNI release[50,61,75–77], and also results in short- or long-term membrane depolarization (depending on species)[81,82], it has been hypothesized that efflux of anionic BNIs is mediated by voltage-dependent anion channels. However, a mechanistic demonstration of this model has not yet been published[76,77,83]. Nor is it clear how to reconcile it with a set of positive correlations that have been demonstrated among PM H^+–ATPase expression and activity, BNI efflux and NH_4^+ supply[76,77], given that greater ATPase activity will counteract depolarization due to NH_4^+. Moreover, it has also been demonstrated that fusicoccin, a stimulant of H^+–ATPase activity that results in membrane hyperpolarization[84], also stimulates BNI activity; and conversely, vanadate, an inhibitor of H^+–ATPase that causes membrane depolarization[85], has been shown to suppress BNI activity[76,77]. Alternatively, it is possible that the vanadate-induced suppression of BNI activity is indicative of ATP-binding cassette (ABC) transporter involvement, which has also been demonstrated to be vanadate sensitive[86]. In fact, ABC transporters have already been demonstrated to mediate the release of root exudates such as flavonoids[79,80,87], belonging to the same chemical class as sakuranetin. Whether such transporters are indeed involved in the release of

BNIs awaits further investigation, but we suggest that insights from the broader literature regarding root exudation of other compounds (for example, allelochemicals mediating plant–plant interactions, and organic acids such as malate and citrate)[79,80,88] will be instrumental in guiding future investigations into BNI-release mechanisms.

For example, some BNIs might be released via members of the multidrug and toxic compound extrusion (MATE) transporter family, which have also been implicated in the transport of various root exudates, including flavonoids[80]. MATE transporters are secondarily active exchangers that utilize ion gradients to drive substrate movement across the plasma membrane. They have been shown to confer aluminium resistance in sorghum (SbMATE1), barley (HvAACT1) and *A. thaliana* (AtMATE1) by facilitating an aluminium-activated efflux of citrate anions from root apices[89]. Another MATE transporter, PEZ2 (phenolics efflux transporter 2), which localizes to root tips in rice, has been shown to exude the phenolic compounds protocatechuic acid and caffeic acid, for the solubilization of apoplastic iron[90].

Two other proposed transport mechanisms underlying root exudation across plasma membranes are simple diffusion and vesicular trafficking[79,80] (that is, exocytosis; Fig. 4c). However, the low lipid solubility of many root exudates, due to their electric charge or polarity, would probably prohibit simple diffusion. In addition, many root exudates are potentially cytotoxic to the plants that produce them (for example, allelochemicals such as sorgoleone), so limitations on their ability to freely diffuse across membranes would be required to prevent metabolic interferences[91,92]. Vesicular transport models are often postulated[79,80], as in the case of sorgoleone, and ultrastructural analyses in sorghum root hairs have revealed cells enriched with vesicles[93]. However, the precise molecular mechanisms of sorgoleone release are still unknown[80,93], and it is unclear whether other BNIs are released by similar processes.

Promises and drawbacks

As understanding of biological nitrification inhibition improves, it is worthwhile to consider its potential utility in agriculture, as well as its drawbacks and limitations in this context. Clearly, BNI production and exudation holds the promise of increased agronomic NUE in crop plants, by reducing the amount of N required per unit of production. It seems probable that the positive correlation between BNI exudation and NUE recently demonstrated among rice cultivars[64] may be a general phenomenon, as it is also reflected in the high rates of BNI production among species such as *B. humidicola* and *H. diplandra*, which are adapted to low-N conditions[53–56]. Moreover, promising early findings indicate that BNIs can significantly suppress nitrification and N_2O emissions in the field[50], as well as increase crop yields in plants with low or unknown BNI production when planted in rotation with strong BNI producers. Recently, a fourfold yield increase was reported in a maize crop in rotation with *B. humidicola* (compared to maize alone) when N was a limiting factor[94]. From the perspective of the farmer, BNIs offer significant potential benefits by increasing agronomic NUE and crop yield, reducing N fertilizer overuse, and avoiding the shortcomings and potential risks with SNIs. The development of BNI-enabled future crop/pasture varieties can facilitate scaling-up of these new technologies for effective adoption by farmers (see section 'Perspectives').

The discovery of biological nitrification inhibition in modern wheat and rice lines is encouraging in terms of breeding this trait into other (for example, elite) cultivars and implementing it successfully into agriculture. However, more fundamental work is needed to understand BNIs before these goals can be fully realized. This includes greater understanding of BNI specificity, biosynthetic pathways, locations and mechanisms of release, and interactions among BNIs and the biotic and abiotic complexities of the soil matrix and greater environment. There is also a need for more research into biological nitrification inhibition in other species, particularly major food crops (for example, maize, barley and potato), and also into older germplasm, which may possess beneficial ancestral traits bred out of modern cultivars[67,95]. Traditional breeding methods for new 'Green-Revolution' crop varieties have typically occurred under high-N conditions, rendering the likelihood of selecting high-BNI/high-NUE germplasms low. A redirection toward selection procedures that take into account low-N conditions, such as those found in smallholder farmer environments in many parts of Africa[96–98], is thus urgently needed. Despite the small likelihood of breeding high rates of BNI production from low-NUE germplasm, a careful re-examination of existing cultivars *vis-à-vis* the BNI trait can nevertheless produce successes[64,67].

It has been proposed that agriculture could benefit from a "fundamental shift towards NH_4^+-dominated agricultural systems", were nitrification rates kept low enough to limit N losses via runoff, leaching and nitrifier denitrification[99]. This viewpoint is given some urgency by evidence suggesting that elevated atmospheric CO_2 suppresses NO_3^- assimilation and subsequent growth of C_3 plants[100]. It is important, however, to consider the complexity and variability of N source utilization among species[52], especially the preference for NO_3^- found among many crop plants, and the risks of soil NH_4^+ concentrations reaching toxic levels[101]. It is also important to consider N source effects in relation to major interacting factors[52], such as soil potassium (K^+) supply. In both rice and barley, for example, two important cereal grasses generally considered tolerant and sensitive to NH_4^+, respectively, optimal biomass, yield and agronomic water-use efficiency can only be achieved under relatively high external NH_4^+ concentrations if K^+ levels are appropriately elevated[101,102]. Moreover, while nitrification suppression is often desirable, it is also important to consider that some level of nitrification can be advantageous, as in rice cultivation, where the co-presence of NH_4^+ and NO_3^- can act synergistically to promote growth[103,104].

In addition, the potential side effects of nitrification inhibition need to be explored. In a recent meta-analysis of agricultural systems, it was found that, although SNIs could effectively decrease direct N_2O emissions and NO_3^- leaching (by 39–48% and 38–56%, respectively), they could also lead to sizeable increases in NH_3 emissions (33–67%)[105]. In another study, it was found that SNIs can increase NH_3 emissions from agricultural fields by 0.2–18.7 kg NH_3–N ha^{-1} (3–65%); downstream deposition and subsequent oxidation of the emitted NH_3 can contribute significantly to indirect N_2O emissions, potentially outweighing the mitigation of direct N_2O emissions[106]. Such side effects clearly warrant further investigation. However, given the possibility that BNI exudates are released from regions near root tips (see section 'Zonation and mechanisms of BNI release'; Fig. 4), it is possible that inhibition would occur mostly in deeper soil layers (for example, within 50 cm depth) and thus preclude NH_3 volatilization, which occurs predominately in surface layers (for example, up to a depth of 15 cm), and where topdressing with SNIs would typically occur. This also warrants further investigation.

It is also worth considering what the photosynthetic carbon costs of increased root exudation might be, and importantly, whether they could reduce agricultural yield. One comprehensive study estimated that 2–4% of net fixed C can be lost as root exudates (with the caveat that soil assays tend to overestimate rhizosphere C flow, while hydroponic methods underestimate it)[107]. This is distinct from the broad category of 'rhizodeposition', in which roots can release as much as 40% of net photosynthetic carbon[108], and includes compounds of low molecular weight such as amino acids, organic acids, sugars, phenolics and other secondary metabolites[92], high-molecular-weight compounds such as mucilage and proteins, and even entire cells (for example, border cells)[91]. While it is not yet clear how much additional C loss will be associated with increased BNI production and exudation in crop plants, it will probably be small compared to the sum of all rhizodeposits.

Shortcomings in current methods for measuring biological nitrification inhibition must also be taken into consideration, in order

to facilitate the translation of *in vitro* analyses to field conditions. As S. Winogradsky, a pioneer of nitrification ecology, warned long ago, laboratory cultures of microorganisms, particularly those grown on artificial (and often nutrient replete) media for generations, "cannot tell us much about microbial activity in nature"[109]. This holds true for microorganisms responding to root exudates. Thus far, most, if not all, BNI assays have been limited to bacterial cultures of *N. europaea* and *Nitrosospira multiformis*[49,59,60,64,67], undoubtedly overlooking the complexity of agricultural soils and the involvement of bacterial nitrite oxidizers, as well as archaeal players.

More specifically, the 'trapping solutions' used to collect root exudates are highly artificial (typically double-distilled water, or simple NH_4Cl solutions, which maximize BNI release and activity[49,60,64,68]), and will probably not reflect the quality and quantity of root exudates in soil-based systems. In one study, exudation rates of MHPP from sorghum roots were reported to be 10.8 mg g^{-1} root dry weight day^{-1} (ref. 61), but this has yet to be related to MHPP release rates in the soil. Using the *in vitro* rates as a guide, Nardi *et al.* applied synthetic MHPP to soils and demonstrated a concentration-dependent inhibition of soil nitrification, but questioned how realistic the applied concentrations were[59]. It is unknown, for example, how MHPP diffuses in the soil, making concentration determinations based on soil mass difficult. Moreover, exudation rates are known to vary with plant age[61] (see also ref. 64). Problematically, a related study showed that sakuranetin had a 500-fold-lower effective dose (ED_{50}) than MHPP in a bioluminescence assay but was found to be non-functional in a soil assay, whereas MHPP was effective in both assays[59,60]. Regardless of whether this reflects differences in BNI stability in soils, or differences in microbial activity between assays, it highlights the hazards of applying *in vitro* studies to the field. Nevertheless, this assay has accelerated progress in the field and when supplemented with soil-based assays, offers considerable potential for future discovery.

Experiments with root exudates from *B. humidicola* using a *N. europaea* assay have suggested that BNIs can be classified into three groups on the basis of their pH sensitivities (pH insensitive, reversibly inhibited by high pH, irreversibly inhibited by high pH)[75]. While the rationale of this work was to investigate biological nitrification inhibition in relation to the naturally acidic soils in which *B. humidicola* is found, the well-known inhibition of ammonia oxidation by *N. europaea* at pH 5 and below[110,111] was not taken into account. Therefore, it is possible that the pH profile of nitrification inhibition in *B. humidicola* exudates, which indicated strongly maximal BNI activity between pH 3 and 5, might have been largely a result of a measurement artefact and the lack of proper controls. In this context, however, it is interesting to note that, for over 100 years, scientists were perplexed by the contrast between the strong nitrification potential of acid soils, and the weak potential of bacterial cultures under acidic conditions in the laboratory[26]. This 'century-long paradox' may at last be close to being resolved, with the strong possibility that archaea, not bacteria, dominate nitrification in acid soils, but this type of organism had been elusive until recently, as well as resistant to cultivation[17,26]. This could be of very high practical significance, given that agricultural soils are predominantly fertilized by NH_4^+, which results in soil acidification when taken up by plants[101]. The advent of high-throughput molecular genotyping techniques will further clarify this important question[26], and help lead the development of BNI assays that accurately reflect soil conditions and the diversity of nitrifying communities.

Perspectives

While research into BNI exudation is still in its infancy, considerable progress has been made in this area in the past decade, from the identification of BNI compounds in root exudates of *B. humidicola*, sorghum and rice, to their discovery in modern wheat and their close relationship with NUE. As we have discussed, however, much more fundamental work will be required to fully bring such discoveries to bear upon today's pressing problems of agriculture and environmental degradation. This will include the continued search for BNIs in important crop species (for example, maize) and the need for large-scale field trials to produce a clear data framework. The means to stimulate BNI synthesis and release via breeding and growth conditions will also need to be further explored, as will the precise mechanisms of release and the optimization of BNIs in the context of trade-offs, such as BNI-induced NH_3 losses. In addition, it may prove fruitful to examine root exudates in the context of inhibiting or stimulating other key soil N transformations, such as urea hydrolysis and denitrification[112].

BNI release and activity in agricultural soils will not be a panacea, but must rather be considered as one element in a 'toolbox' of approaches to reducing N loss and improving NUE in pastures and croplands. Currently available elements of such a toolbox include improved fertilizer quality and application practices (for example, broader use of urea coated with urease and nitrification inhibitors, controlled-release fertilizers, improved timing and placement of N), and improvements in other farming practices, such as irrigation management, soil amendment and residue retention. Ideally, future agriculture would involve precise control over all facets of soil nitrogen dynamics, as well as drawing upon a detailed knowledge of the microbial community within a given volume of soil. Emerging technologies, such as soil micro-profiling and rapid genotyping of the soil microbiome, are already proving useful in this context[59,113,114]. Moreover, selective breeding and genetic modifications to introduce or optimize biological nitrification inhibition in crops should be carefully approached to target specific rhizosphere processes and possibly even specific soil layers. Indeed, breeding may be the more attractive option to bypass the politically controversial engagement of GMO (genetically modified organism) technology. Ultimately, the precise matching of N supply with plant demand over the lifetime of a crop will provide the highest NUE, while minimizing N wastage and pollution.

Despite longstanding warnings by the academic community[2–4,7–11], the consequences of overloading agricultural and natural ecosystems with N_r for the health of humans and the environment are still largely unrecognized by the general public. This is in stark contrast to broad public awareness of the global carbon footprint. With increased cognizance of the 'nitrogen footprint', future governments may find it prudent to implement nitrogen-offset/trading systems and agricultural subsidy programs that encourage the use of crop varieties with high NUE and low N wastage[115], akin to the widespread subsidies and global trading systems that limit carbon emissions and encourage low-carbon agriculture. We anticipate that such progressive steps will occur as crop scientists and plant physiologists intensify efforts to increase NUE in crops[116], including the understanding and practical deployment of biological nitrification inhibition.

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Competing interests

The authors declare no competing financial interests.