Trends in Plant Science

CellPress

Opinion

How Plant Root Exudates Shape the Nitrogen Cycle

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Although the global nitrogen (N) cycle is largely driven by soil microbes, plant root exudates can profoundly modify soil microbial communities and influence their N transformations. A detailed understanding is now beginning to emerge regarding the control that root exudates exert over two major soil N processes – nitrification and N_2 fixation. We discuss recent breakthroughs in this area, including the identification of root exudates as nitrification inhibitors and as signaling compounds facilitating N-acquisition symbioses. We indicate gaps in current knowledge, including questions of how root exudates affect newly discovered microbial players and N-cycle components. A better understanding of these processes is urgent given the widespread inefficiencies in agricultural N use and their links to N pollution and climate change.

Trends

Major advances in understanding the complexity of the N cycle have recently been made, with the discovery of previously unknown microbial players and N transformations.

The study of plant root exudates and their influence on the plant–soil microbiome in shaping nutrient cycles has greatly intensified in recent years.

Root exudates that specifically inhibit soil nitrification have been identified in important crop species, including rice, wheat, and sorghum, while others have been shown to stimulate root nodulation and N_2 fixation, even in neighboring plants.

By influencing soil N cycle dynamics, root exudates have been shown to improve N use efficiency and can help to mitigate environmental pollution and climate change.

The Nitrogen Cycle Today

To a great extent, the N cycle of the Earth can be described as a network of oxidation–reduction reactions catalyzed by plants, fungi, bacteria, and archaea. These organisms modulate the oxidation state (OS) of N between that of fully reduced amines (e.g., ammonium, NH_4^+; OS = −3) and fully oxidized nitrate (NO_3^-; OS = +5; Figure 1). The largest pool of N in the biosphere, atmospheric dinitrogen gas (N_2; OS = 0), is not directly available to most organisms, but enters the living world naturally via **biological N_2 fixation** (BNF, see Glossary) by diazotrophic prokaryotes (as well as geochemically, e.g., via lightning) [1]. These unicellular microorganisms can be bacterial or archaeal, free-living or in symbiotic associations (e.g., within plant root nodules), and reduce N_2 to NH_4^+, which can then be incorporated into amino acids and thence into a myriad of organic compounds [2,3]. NH_4^+ can also be readily oxidized by soil microbes, producing hydroxylamine (NH_2OH; OS = −1), nitrite (NO_2^-; OS = +3) and NO_3^- via the process of **nitrification**. This process is catalyzed by a host of microorganisms termed ammonia-oxidizing bacteria and archaea (AOB and AOA, respectively), nitrite-oxidizing bacteria (NOB), as well as the newly discovered **comammox** (complete ammonia oxidizers) that perform both oxidative steps in a single bacterium of the genus *Nitrospira* [4–7]. The reverse process, **denitrification**, involves the reduction of NO_3^- to NO_2^-, nitric oxide (NO; OS = +2), nitrous oxide (N_2O; OS = +1), and finally back to N_2, and is performed by bacteria, archaea, and fungi [7]. Two relatively under-reported reaction sequences that nevertheless contribute significantly to terrestrial N cycling include **dissimilatory nitrate reduction to ammonia** (DNRA), involving the use of NO_3^- as an electron acceptor by bacteria and fungi, which reduce it to NH_3 via NO_2^- under anaerobic or low-oxygen conditions [8], and **anammox** (anaerobic ammonium oxidation), the formation of N_2 from NO_2^- and NH_3 by bacteria via the intermediates NO and hydrazine (N_2H_4; OS = −2) [9,10].

In recent decades, our understanding of the N cycle has undergone two major modifications. First, the discovery of archaea has raised fundamental questions about the participation of this vast prokaryotic domain, distinct from bacteria, in the N cycle. While it is now known that

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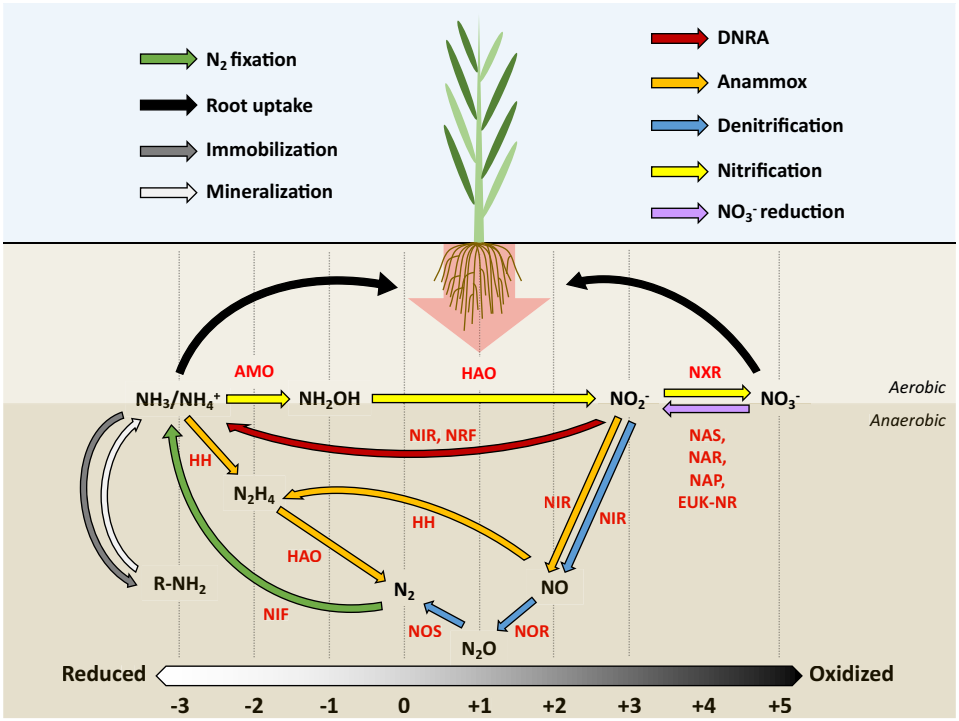

Figure 1. The Soil Nitrogen Cycle. A schematic overview of the major N transformations in the soil N cycle (adapted from [123]). Colored arrows correspond to specific N transformations (indicated at the top of the figure) that are catalyzed by specific enzymes, including various nitrate reductases (NAS, NAR, NAP, and EUK-NR), nitrite reductases (NIR, NRF), nitric oxide reductase (NOR), nitrous oxide reductase (NOS), nitrogenase (NIF), ammonia monooxygenase (AMO), hydroxylamine oxidoreductase (HAO), nitrite oxidoreductase (NXR), and hydrazine hydrolase (HH). Root exudates (denoted by the thick, pale-red arrow) influence some of these reactions (see text for details). Numeric scale at bottom indicates oxidation state (OS) of nitrogen-containing compounds.
For a Figure360 author presentation of Figure 1, see the figure online at http://dx.doi.org/10.1016/j.tplants.2017.05.004#mmc1

archaea are capable of N_2 fixation, this may be largely restricted to marine and freshwater sediments, and might not be of great significance to agricultural systems [11]. By contrast, nitrifying archaea (i.e., AOA) have recently been found to be widely distributed, particularly in acidic soils [4], although their activities relative to AOB might be inhibited at high-NH_4^+ concentrations [12].

The second major modification is due to a profound change in the nitrogen cycle itself, in other words its accelerating disruption by human activities. Collectively, the industrial production of reduced-N fertilizer using the Haber–Bosch process, the fixation of N_2 by cultivated legumes, and the combustion of fuels now result in more fixed nitrogen per year than all natural processes combined (210 vs 203 Tg N $year^{-1}$, respectively) [13]. While this has been immensely valuable to human commerce and nutrition [14,15], it has also come at the cost of a wide range of serious environmental problems, most notably the eutrophication of fresh and marine waters [16,17], and the production of N_2O, a potent greenhouse gas (300-fold more heat-trapping capacity than CO_2, per molecule) and the single most important ozone-destroying agent known [18,19]. These issues are directly linked to nitrification processes in fertilized soils, which generate the soil-mobile anion NO_3^- from relatively immobile NH_4^+ pools, causing massive losses of N from agricultural systems (Figure 2) and providing substrates for both nitrifiers and denitrifiers to produce N_2O [20,21].

Glossary

Anammox: anaerobic ammonium oxidation, the formation of N_2 from nitrite (NO_2^-) and NH_3/NH_4^+ via the intermediates nitric oxide (NO) and hydrazine (N_2H_4).
Arbuscular mycorrhizal fungi (AMF): endosymbiotic fungi that form arbuscules and vesicles in roots of vascular plants. Plants provide photosynthates to AMF in exchange for nutrients (e.g., P and N).
Biological N_2 fixation (BNF): the conversion of N_2 into NH_3 by diazotrophic (N_2-fixing) microbes (bacteria and archaea), in contrast to geochemical processes (e.g., lightning) or the industrial Haber–Bosch process.
Biological denitrification inhibitors (BDIs): compounds found in plants that inhibit denitrification reactions. Thus far, only procyanidins from root extracts of *Fallopia* spp. have been shown to have BDI activity.
Biological nitrification inhibitors (BNIs): compounds found in plants that inhibit nitrification reactions. In root exudates, five compounds with BNI activity have so far been identified, in sorghum, *Brachiaria humidicola*, and rice.
Comammox: complete ammonia oxidizers, bacteria from the genus *Nitrospira* that perform complete nitrification of NH_3 to NO_3^-.
Denitrification: the reduction of NO_3^- to N_2 via the intermediates NO_2^-, NO, and N_2O. Denitrification is performed by bacteria, archaea, and fungi that use NO_3^- as an electron acceptor during anaerobic respiration.
Dissimilatory nitrate reduction to ammonia (DNRA): the bacterial and fungal reduction of NO_3^- to NH_3 via NO_2^-.
Nitrification: the bacterial and archaeal oxidation of NH_3 to NO_3^- via the intermediates NH_2OH and NO_2^-.
Plant growth-promoting rhizobacteria (PGPR): free-living bacteria that reside on root surfaces and in extracellular spaces. Some PGPR are diazotrophic and provide fixed nitrogen to plants.
Rhizosphere: the narrow interface between plant roots and soil in which complex interactions occur between root exudates and soil microorganisms.

Although plants cannot themselves fix N_2, or directly engage in nitrification, they do take up and assimilate both NO_3^- and NH_4^+, displaying substantial variations in preference for one inorganic N form over the other among different genotypes and environments [3,22]. Moreover, it is becoming increasingly clear that plants can exert control over N transformations catalyzed by the fungal and prokaryotic populations in and near the **rhizosphere** by releasing **root exudates** [23,24]. These are diverse chemicals that appear to be part of a belowground, inter-species language between plants and other plants, or other types of organisms [25,26]. In this article we discuss recent developments in the study of root exudates and their roles in altering the microbial pathways of N_2 fixation and nitrification, with special emphasis on improving agronomic N-use efficiency (ANUE, a ratio of yield to N-fertilizer input) and ameliorating the environmental problems brought about by an excessively N-rich world.

Root exudates: chemical compounds secreted by plant roots, some of which act as signals in the communication between plants and neighboring species (e.g., prokaryotes, fungi, herbivores, and other plants).

Biological Nitrification Inhibition: A Means to Curb N Losses?

In modern agricultural systems, ANUE tends to be very low. Of the total amount of fertilizer N applied to crop systems (~115 Tg N $year^{-1}$, globally [27]), 50–70% is lost to the surrounding environment [28–30]. These environmentally deleterious losses take the form of NH_3 volatilization (up to 64%, and, as a global average, 18% of N-fertilizer application [31,32]), NO_3^- leaching and runoff (globally, an average of 19% of application [33,34]), and denitrification (e.g., direct N_2O emissions account for 1% of fertilizer application, globally [30,35] (cf. [36]) (Figure 2). To

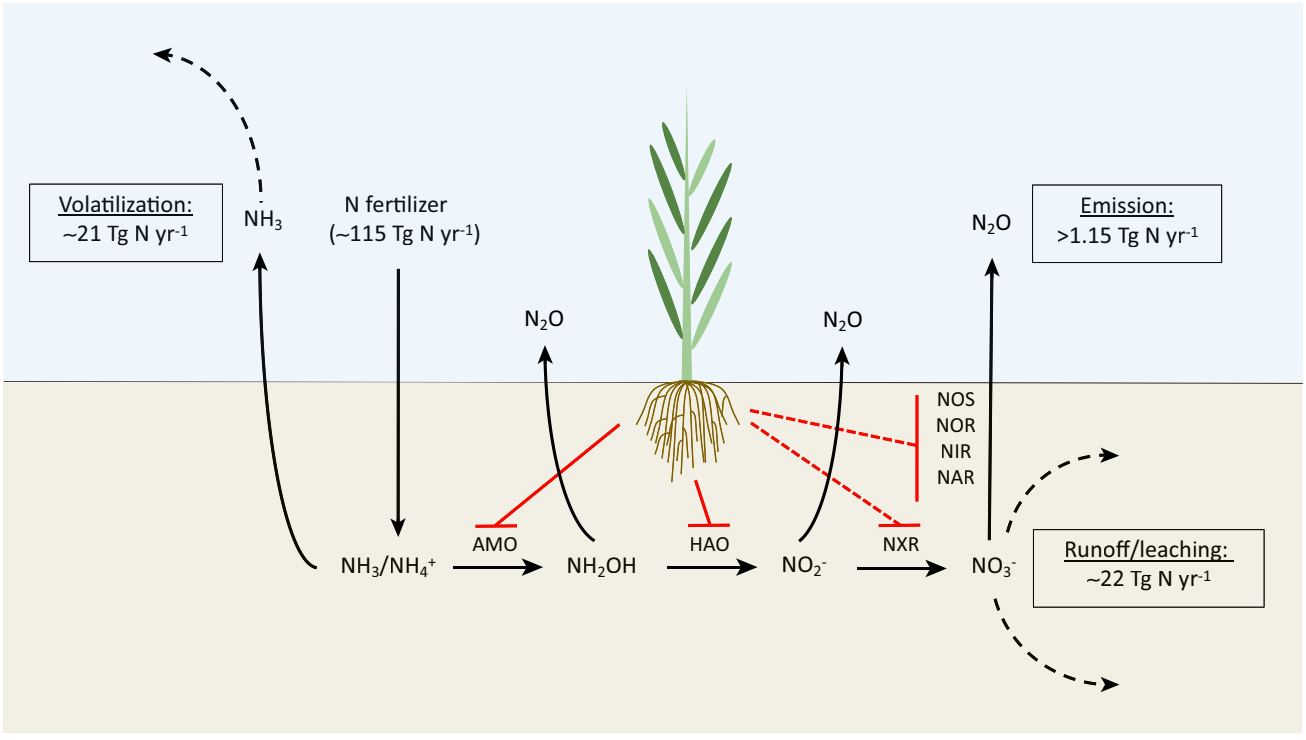

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Figure 2. Root Exudates as a Means To Mitigate Agricultural Nitrogen Losses. The current yearly global N fertilizer application rate is estimated to be ~115 Tg [27], of which, globally, 50–70% is lost from agricultural systems to the environment [28–30]. NO_3^-, the product of nitrification, can be lost via leaching and runoff at global rates estimated to account for 19% of total N-fertilizer application ([34]; see also [124]). Global NH_3 volatilization is estimated to account for ~18% of N-fertilizer application [32]. Direct N_2O emissions, via denitrification and nitrifier denitrification reactions, are estimated globally at 1% of fertilization application [35]; however, including estimates for indirect N_2O emissions (from N leaching, runoff, and atmospheric deposition; broken arrows) can more than double these losses [35,36]. Other potential routes for N loss include NO and N_2 emissions as well as immobilization by other species or soils (not shown). Biological nitrification inhibitors (BNIs) released from root exudates suppress nitrification via AMO and HAO inhibition (text for details). Thus far it is unknown whether root exudates specifically target NXR or denitrification enzymes (but see [64]). Note that the inhibition of nitrification (specifically via inhibition of AMO, which catalyzes the rate-limiting step) can potentially enhance NH_3 volatilization [91] (text for details).

Table 1. BNIs and Related Substances[a,b]

Category	Compound	Source	Comments	Refs
BNIs from root exudates	Sorgoleone	*Sorghum bicolor*	Blocks AMO and HAO; allelopathic compound	[53,56]
	Sakuranetin	*Sorghum bicolor*	Blocks AMO and HAO; non-effective BNI in soil assay; phytoalexin	[53,112]
	Methyl 3-(4-hydroxyphenyl) propionate (MHPP)	*Sorghum bicolor*	Blocks AMO; influences root system architecture	[53,57, 113,114]
	Brachialactone	*Brachiaria humidicola*	Blocks AMO and HAO; reduces field-level nitrification and N_2O emission	[46]
	1,9-Decanediol	*Oryza sativa*	Blocks AMO; release correlated to NUE	[48]
BNIs from tissue extracts	Caffeic acid	Mid- to late-successional species (e.g., *Ambrosia psilostachya*, *Andropogon* spp., *Sorghastrum nutans*, *Quercus* spp., *Pinus ponderosa*)	Complete nitrification inhibition (with 1 μM) in soil suspension (with *Nitrosomonas*)	[52,59]
	Chlorogenic acid	Mid- to late-successional species (e.g., *Ambrosia psilostachya*, *Haplopappus ciliates*, *Andropogon* spp., *Panicum virgatum*, *Sorghastrum nutans*, *Pinus echinata*, *Quercus* spp., *Pinus ponderosa*)	Complete nitrification inhibition (with 100 nM) in soil suspension (with *Nitrosomonas*)	[52,59]
	Ferulic acid	*Pinus echinata*, *Quercus* spp.	Complete nitrification inhibition (with 10 nM) in soil suspension (with *Nitrosomonas*)	[52]
	Methyl ferulate	*B. humidicola* roots	Released via root decomposition	[115]
	Methyl *p*-coumarate	*B. humidicola* roots	Released via root decomposition	[115]
	Linoleic acid	*B. humidicola* shoots	Blocks AMO and HAO; inhibits urease	[116]
	Linolenic acid	*B. humidicola* shoots	Blocks AMO and HAO	[116]
	Methyl linoleate	*B. humidicola* shoots	Most stable BNI of the *B. humidicola* tissue extracts in soils; inhibits urease	[116]
Synthetic nitrification inhibitors (SNIs)	Dicyandiamide (DCD)	Synthetic	Widely used in agriculture; blocks AMO; does not increase yields; risks of degradation, leaching, food and water contamination, increased NH_3 volatilization, indirect N_2O emission	[37,42–45, 116,117]
	3,4-Dimethylpyrazole phosphate (DMPP)	Synthetic	Widely used in agriculture; blocks AMO; more effective than DCD in lowering NH_3 and N_2O emission and NO_3^- leaching; variable effects on yield; risk of increased NH_3 volatilization and indirect N_2O emission	[37,44, 45,103]

Table 1. (continued)

Category	Compound	Source	Comments	Refs
	2-Chloro-6-(trichloromethyl) pyridine (Nitrapyrin)	Synthetic	Blocks AMO; risk of increased NH_3 volatilization and indirect N_2O emission; can also inhibit denitrification	[37,44,45, 116,118, 119]
Biological denitrification inhibitors (BDIs)	Procyanidins	*Fallopia* spp. root extracts	Allosteric inhibitor of nitrate reductase	[63,64]
Synthetic denitrification inhibitors (SDIs)	Acetylene, Azide, Cyanide, Dinitrophenol (DNP), Nitrapyrin, Vapam (pesticide), Dalapon (pesticide), Toluidine (pesticide)	Synthetic	General metabolic inhibitors; mechanisms of action largely unknown	[120]
	Pyrimidone-based and triazinone-based compounds	Synthetic	Inhibitors of copper nitrate reductase (NirK) from *Fusarium oxysporum*	[121]
Urease inhibitors (UIs)	N-(n-butyl) thiophosphoric triamide (NBPT)	Naturally isolated or synthetic	Most widely used; reduces N_2O emissions and increases yield; temperature sensitive	[37,122]

[a]Summary of biological nitrification inhibitors (BNIs) and related substances that influence nitrification and other key steps in the N cycle.
[b]Abbreviations: AMO, ammonia monooxygenase; HAO, hydroxylamine oxidoreductase.

reduce N losses and pollution from agriculture, several strategies have been proposed, including the use of 'enhanced-efficiency fertilizers' (slow-release formulae typically laced with synthetic inhibitors of nitrification and urea hydrolysis via urease; Table 1) and refinements in farming practices (e.g., improvements in fertilizer application rate, source, timing, and placement) [37–39]. Although such strategies have produced variable results across different cropping systems [39–41], it is clear that there should be greater management and policy focus on the improvement of ANUE.

Synthetic nitrification inhibitors have been criticized for their difficulties in application, cost, and environmental safety [42–45]. One alternative that has received much recent attention involves the use of compounds in root exudates that inhibit nitrification, collectively known as **biological nitrification inhibitors** (BNIs [46–50]) (Table 1 and Figure 2). While root exudates have long been postulated to control soil nitrification (e.g., in the context of ecological succession [51,52]), only in the past decade have their presence and function been definitively demonstrated in sorghum [53], *Brachiaria humidicola* [46], rice [48,54], wheat [49], and *Leymus racemosus*, a wild relative of wheat [55]. The recent breakthroughs in the detection and characterization of BNIs are due in large part to technological advances, in particular through the use of a recombinant strain of *Nitrosomonas europaea* that responds bioluminescently to the oxidation of NH_4^+ to NO_2^- [47]. To date, however, only five BNI compounds have been isolated: sorgoleone (a benzoquinone dominant in the hydrophobic fraction of root exudates), sakuranetin (a hydrophilic flavanone), and methyl 3-(4-hydroxyphenyl) propionate (MHPP; a hydrophilic phenylpropanoid) from sorghum [53]; brachialactone (a cyclic diterpene) from *B. humidicola* [46]; and 1,9-decanediol (a fatty alcohol) from rice [48] (Table 1). Given that these BNIs (with the possible exception of brachialactone) are also known to perform roles that are unrelated to nitrogen metabolism (e.g., sorgoleone is a well-known herbicide [56]), the specificity of these compounds has recently been questioned [50]. However, given that in many

cases their release appears to be a tightly regulated process (e.g., stimulated solely by external exposure of roots to NH_4^+ [55,57,58]), it is likely that these compounds also possess roles specific to nitrification inhibition. In addition, given the widespread occurrence of nitrification inhibitors found in plant tissues (although not necessarily exuded by roots; Table 1) [47,59,60], we may reasonably expect many more to be discovered in the near future. Of those identified, all have been demonstrated to effectively inhibit ammonia monooxygenase (AMO; which catalyzes NH_3 oxidation to NH_2OH, the first and rate-limiting step of nitrification), whereas only sorgoleone, sakuranetin, and brachialactone inhibit hydroxylamine oxidoreductase (HAO; which catalyzes the second step, i.e., oxidation of NH_2OH to NO_2^-) (Figure 2). Moreover, in a recent comprehensive study of 96 landraces of wheat, 26 displayed significant BNI activity in their root exudates, including one modern commercial cultivar (cv. Janz) [49]. Although specific BNI compounds have yet to be isolated from root exudates in this species, there appears to be considerable promise in breeding biological nitrification inhibition into modern, elite, wheat cultivars, particularly given the successful transfer of this trait from *L. racemosus* to cultivated wheat via chromosome addition [48]. Similarly, a screening of 19 rice genotypes indicated strong BNI potential in both *indica* and *japonica* subspecies, and, importantly, the strength of inhibition was shown to be positively correlated with both ammonium-use efficiency and ammonium preference [48], suggesting a specific functional relationship (and a genetic link) between BNIs and ANUE. Surprisingly, to our knowledge, biological nitrification inhibition has not yet been demonstrated in maize, the third most important crop species after rice and wheat in terms of global fertilizer consumption and crop output [29].

Of the BNIs, sorgoleone has been the most thoroughly characterized thus far (mostly in its context as an allelochemical [56]). It is produced solely in root hairs and exuded as golden-brown oily droplets from root-hair tips [61]. It is not as clear where other BNIs are exuded along the root axis. The molecular transport mechanisms mediating BNI efflux across plasma membranes into the rhizosphere are also not well understood, although several mechanisms have been proposed. ATP-binding cassette (ABC) transporters, for example, appear to mediate the release of flavonoids [61], but whether they are involved in the release of BNIs has yet to be determined. Other possible mechanisms include MATE (multidrug and toxic compound extrusion) transporters, which have been implicated in the efflux of various root exudates, including flavonoids, as well as simple diffusion, and vesicular trafficking (i.e., exocytosis), which has been postulated as a release mechanism for cytotoxic compounds such as sorgoleone [61,62].

How root exudates influence denitrification is currently unknown. However, **biological denitrification inhibitor** (BDI) activity has recently been demonstrated in root extracts from *Fallopia* spp., an invasive weed associated with low denitrification potential in soils [63]. Here, enzyme-kinetic analysis showed that procyanidins (a class of flavonoid compounds) could specifically inhibit nitrate reductase allosterically in the model strain *Pseudomonas brassicacearum* NFM 421, likely by affecting membrane stability [64]. Similar kinetic analyses are currently lacking for BNIs, but will be important to determine specific mechanisms of inhibition. In the case of BDIs, future studies will be necessary to determine whether root exudates are involved, as opposed to root-tissue extracts. Moreover, it is important to recognize the relative contributions of nitrification and denitrification reactions to N losses among various ecosystems. For example, nitrifier denitrification can account for up to 97% of N_2O emissions in a sugarcane cropping soil, 70% in cereal-cropping and dairy-pasture soils, and only 20% in a vegetable-producing soil [65]. Knowledge of such variable contributions of nitrification and denitrification reactions to N_2O release will help to determine the need for crop rotation, intercropping, or breeding of plants with variable amounts of BNIs and/or BDIs.

Root Exudates, N_2 Fixation, and Symbiotic Relationships

Perhaps the best-understood signaling network involving root exudates is that which orchestrates the endosymbiotic relationship between legumes and the group of diazotrophic bacteria known as rhizobia (e.g., *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* spp.) [66,67] (Figure 3). In particular, flavonoids (e.g., genistein, naringenin, and hesperetin [68,69]) from legume root exudates stimulate the expression of *nod* genes in rhizobia, the products of which synthesize nodulation (Nod) factors. These factors take the form of lipochitooligosaccharides (LCOs) and provide the basis for host–microbe specificity and nodule initiation [67,68,70]. LCOs are perceived by the plant via receptor-like kinases, which are located in the plasma membranes of root epidermal cells, activating a Ca^{2+}-dependent signaling cascade that leads to nodule formation [67]. Interestingly, root exudates also elicit the release of LCOs, called mycorrhizal (Myc) factors, from **arbuscular mycorrhizal fungi** (AMF) [70,71]. While this interaction is mainly initiated by root-exuded compounds known as strigolactones [72], flavonoids also stimulate AMF invasion and arbuscule formation in roots [68] (Figure 3). Like Nod factors, Myc factors are perceived by plants and trigger signaling pathway elements common to those found in the development of rhizobial associations [67,73]. AMF endosymbioses represent the most widespread of terrestrial plant symbioses, and are observed in 70–90% of plants, including cereals and legumes [70,74,75]. Although the primary function of AMF appears to be plant phosphorus (P) acquisition, they have also been shown to promote N nutrition (and subsequently create mulches enriched in N [75]), although the basis of this tendency remains unclear [76]. Currently underway is the challenging feat of engineering BNF in cereals, in part by

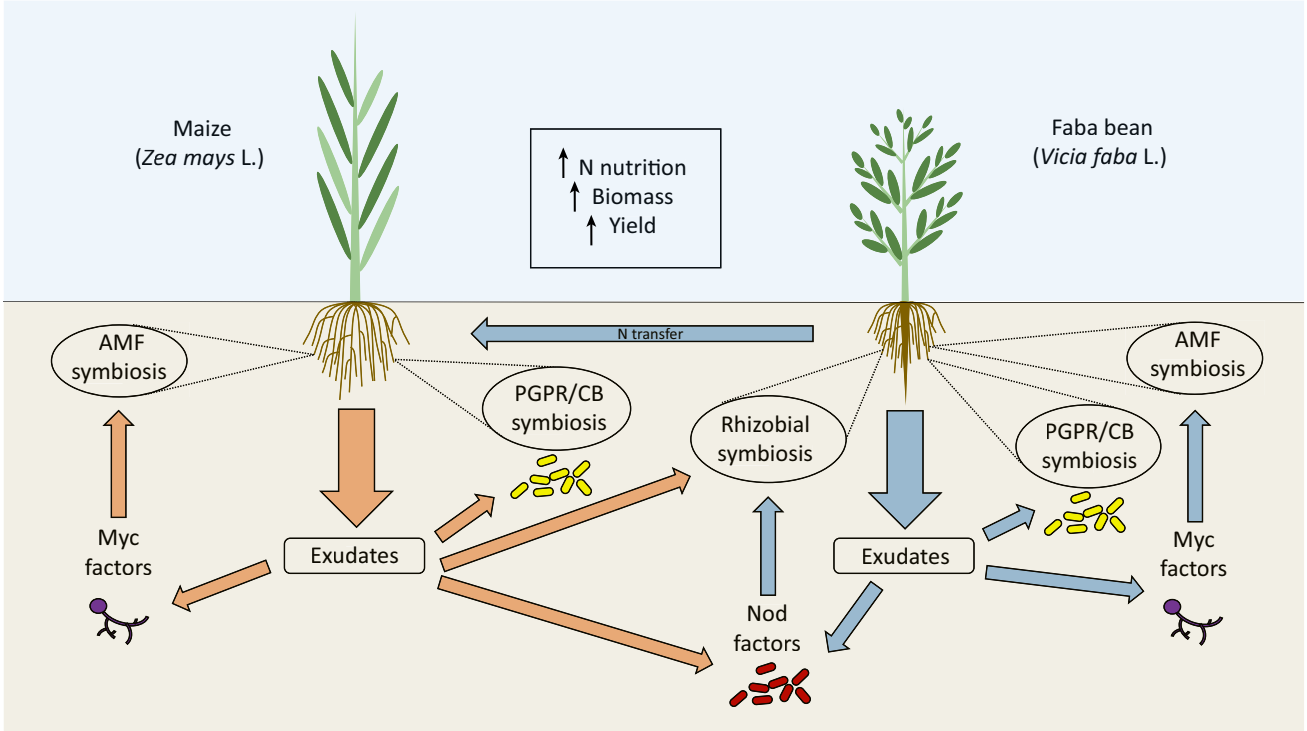

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Figure 3. TThe Influence of Root Exudates on Symbiotic Relationships in an Intercropping System. In a maize–faba bean intercropping system, root exudates from maize (e.g., flavonoids such as genistein) can stimulate rhizobial Nod factors, as well as nodulation and biological N_2 fixation (BNF) in faba bean roots, thereby enhancing N nutrition, biomass, and yield. In exchange, root exudates containing fixed N (e.g., NH_4^+, amino acids, etc.) can be transferred from faba bean to maize, thus also benefiting N nutrition, growth, and yield of maize. Root exudates (e.g., strigolactones, flavonoids) from both species can stimulate Myc factors from arbuscular mycorrhizal fungi (AMF), stimulating AMF symbiosis which can improve plant N nutrition. Root exudates can also recruit diazotrophic plant growth-promoting rhizobacteria (PGPR) and cyanobacteria (CB), which can colonize roots and improve plant N nutrition.

exploiting the similarities in signaling networks between rhizobial and AMF endosymbioses [77,78]. Such a development could greatly benefit agriculture, particularly in subsistence farming systems, and reduce the global reliance on synthetic N fertilizers and their environmental impact.

Nodulation and BNF in non-legumes are also influenced by root exudates [79]. Symbioses with the actinobacterial genus *Frankia* occur in >200 species of actinorhizal plants, spanning eight families, all of which are dicotyledonous, and all of which, except for the herbaceous genus *Datisca*, are trees and shrubs [80]. Here, flavonoids in root exudates may play a role in host–microbe specificity, although the molecular mechanisms underlying symbiotic development (e.g., the involvement of canonical *nod* genes) remain unknown [26,68,81]. However, isoflavonoids in root exudates of the actinorhizal tree *Casuarina cunninghamiana* have been shown to promote growth and alter the surface properties of an associated strain of *Frankia*, and facilitate the infection and nodulation process [82–84]. It is anticipated that recent advances in RNAi technology and the complete genome sequencing of *Frankia* (strain Ccl3) will greatly improve our understanding of such processes [73,80].

Chemoattractants found in root exudates are also involved in cyanobacteria–plant symbioses, as observed, for example, in *Nostoc* attraction to its natural hosts, cycads, liverworts, and *Gunnera*, and to the non-hosts rice, wheat, and *Arabidopsis* [79,85,86]. Hormogonia, an infectious and highly motile form of filamentous cyanobacteria, can be induced by hormogonia-inducing factors (HIFs) in host and non-host root exudates, typically in response to low-nitrogen conditions [79]. However, to our knowledge, no signaling or attractant compounds involved in hormogonia recruitment have been identified.

In addition, some free-living diazotrophs form ‘associative’ (i.e., non-nodulating) interactions with plants, residing on root surfaces and in extracellular spaces (e.g., by penetrating openings at sites of lateral root emergence), and contributing to increases in biomass and yield in important cereals including rice, wheat, and maize [79,87] (Figure 3). Known as **plant growth-promoting rhizobacteria** (PGPR), these organisms are found in many genera of alpha- and betaproteobacteria, and include *Azoarcus*, *Azospirillum*, *Azotobacter*, *Burkholderia*, *Enterobacter*, *Herbasprillum*, *Glucenobacter*, and *Pseudomonas* [79]. As in endosymbioses between legumes and rhizobia, and between actinorhizal plants and *Frankia*, flavonoids in root exudates appear to be important plant signals for PGPR interactions [79]. For example, the flavonoid naringenin was shown to significantly stimulate wheat root colonization by diazotrophic rhizobacteria, including *Azospirillum brasilense* and *Azorhizobium caulinodans* [88]. However, like actinorhizal associations, the mechanistic underpinnings of this process are as yet poorly understood.

Interestingly, it is now known that root exudates from non-nodulating plant species can influence root nodulation and N_2 fixation in legumes (Figure 3). In a recent study, the intercropping of maize with faba bean was shown to increase nodulation and N_2 fixation in faba bean [89], and the underlying crosstalk was attributed to maize root exudates. In bean plants, these exudates elicited a nearly twofold increase in genistein exudation, an 11-fold increase in expression of chalcone-flavanone isomerase (a key enzyme involved in flavonoid synthesis), and an upregulation of several key nodulation genes [89]. This study provides an important mechanistic basis for the well-established phenomenon of enhanced ecosystem productivity and overyielding observed in legume/cereal intercropping systems [90,91]. It may prove scientifically and pragmatically worthwhile to further investigate these effects in other cereals, legumes, or cultivars of maize. Moreover, future studies along these lines might benefit from the addition of squash in polyculture, the third member of the ‘three sisters’, an ancient cropping system in the Americas [92]. The increased biomass and yield production in the polyculture,

relative to the respective monocultures, have largely been attributed to a 'complementarity' effect, wherein differences in the root architectures of intercropped species allow niche specialization via unique, but complementary, nutrient-foraging strategies [92]; however, it has recently been suggested that the production of root exudates may in fact be more important here [93]. An improved mechanistic understanding of the role of root exudates in intercropping systems can be highly beneficial, particularly in resource-limited agricultural systems that rely on this practice [91], but also in terms of reducing global reliance on fertilizers and their environmental impact.

The belowground transfer of fixed N from legumes to non-legumes (e.g., from faba bean to maize) represents another fascinating facet of the intercropping relationship (Figure 3). Belowground N transfer can occur through three possible pathways: (i) indirectly, via decomposition of root tissues and subsequent uptake of mineralized N, (ii) directly, via exudation of soluble N compounds by legumes and uptake by non-legumes, or (iii) via mediation by plant-associated mycorrhizal fungi [94]. During the early growth stages of legumes, however, the majority of belowground N transfer appears to occur via root exudates [95]. Roots can release organic forms of N [96,97], primarily through root nodules and root tips [98], and neighboring plants are able to take up these forms of N [99,100]. Among most temperate legumes (e.g., alfalfa), NH_4^+ and amino acids are the most prevalent forms of low molecular weight N-compounds contained in root exudates [95,101]. By contrast, tropical legumes (e.g., soybean) primarily release ureides [102].

The roles of root exudates in influencing crucial symbiotic relationships in the N cycle, such as those between legumes and rhizobia, and between actinorhizal plants and *Frankia*, as well as a wide variety of interactions between plants and diazotrophic PGPR, cyanobacteria, AMF, and even neighboring plants, are only beginning to be elucidated. Only with better mechanistic understanding of these important interactions can efforts be pursued to breed or genetically engineer traits such as increased root exudation to promote both BNF and belowground N transfer.

Concluding Remarks and Future Perspectives

Given the rapid pace with which our understanding of the influence of plant root exudates in nitrification and N_2 fixation is increasing, a case may be made that we are on the verge of a new 'green' revolution, one in which the wasteful and environmentally damaging losses of agricultural N can be curtailed, without reducing crop productivity. In the case of biological nitrification inhibition, a reduction of nitrification via root exudation is expected to not only improve ANUE by reducing N losses via leaching, runoff, and denitrification but also mitigate agriculturally sourced N pollution, which causes eutrophication and climate forcing via N_2O emissions. However, possible trade-offs of BNI-stimulated agriculture must be considered, as with the application of synthetic nitrification inhibitors. Although effective in reducing such N losses and increasing N-use efficiency [103], synthetic inhibitors also stimulate NH_3 volatilization and subsequent indirect N_2O emissions, undermining or even outweighing the benefits of nitrification inhibition [44,45]. Whether this is the case for BNIs has yet to be investigated, but given that BNI exudates may be preferentially released from the root-tip region (see above), it is possible that BNI release occurs mostly in deeper soil layers, thereby minimizing NH_3 volatilization, which occurs predominantly in the surface layers where synthetic nitrification inhibitors are typically applied.

Biological N_2 fixation can provide another obvious benefit for ANUE, increasing bioavailable N directly to the plant and decreasing the reliance on synthetic fertilizers; root exudates can also be of major practical importance here. As in the case of BNIs [48,49], there is clearly a need for major screening studies among a wide range of plant species and cultivars to determine how widely distributed BNF-stimulating root exudates might be. This holds for rhizobial and

Outstanding Questions

To what extent can root exudation in major crop species (especially cereal grains) be used as a means of increasing ANUE and agricultural productivity, while limiting greenhouse gas production by soil processes? What might the trade-offs be – for example in terms of resource and energy budgets toward root exudates versus grain yield, or BNI activity resulting in enhanced NH_3 volatilization and deposition?

Do plant roots exude compounds that influence every stage of the N cycle in soils, or only certain steps (e.g., nitrification and N_2 fixation)?

How specific are such compounds? How are they synthesized and transported, and by what mechanism do they act upon other organisms (prokaryotes, fungi, herbivores, and other plants)?

How do root exudates involved in N cycling influence community structure?

How have the complex underground systems of chemical communication evolved?

actinorhizal plants, as well as for plants that recruit diazotrophic PGPR and cyanobacteria; the importance of PGPR and cyanobacteria is illustrated in highly productive rice cropping systems benefiting from endophytic rhizobial associations [104] and the use of *Azolla* as 'green manure' [105,106]. The finding that non-BNF plants (e.g., maize) can stimulate nodulation and N_2 fixation in neighboring legumes highlights the importance of expanding our understanding of the role of root exudates in intercropping and crop-rotation systems, such as in the maize–legume relationship discussed above. This is further underscored by the finding that residual soil BNI activity, following rotation with *B. humidicola*, resulted in improved ANUE in maize, as well as improved yield under low-N conditions [107].

While the use of bacterial test strains such as *Nitrosomonas multiformis*, and, in particular, the bioluminescent recombinant strain of *N. europaea*, has been instrumental in much recent progress in BNI research [46,48,49,54], the field has largely overlooked the complexity and variability of soils, as well as the involvement of a multitude of other microbial players, including other bacterial nitrifiers and also archaea [7]. Although such procedures are undoubtedly important as an early step in the mechanistic tackling of complex problems in chemical ecology, it is also important to be cognizant of the complexities of natural soils and the limitations of transferring results from *in vitro* studies into the field. In one important example, sakuranetin was shown to effectively suppress nitrification *in vitro* with the bioluminescence assay, but was found not to be effective in a soil assay [53].

Indeed, there is a great deal of fascinating physiological work on root exudation that remains to be done in and beyond the context of the N cycle (see Outstanding Questions). These endeavors will take the forms of elucidating, among other things, the pathways and mechanisms involved in the synthesis of exudates, their release from roots, and their interactions with environmental factors (both biotic and abiotic), as well as the genetics and molecular biology of these diverse processes. In addition, an analysis of the specificity of root-exuded compounds to a given biochemical process should be undertaken because some BNIs (e.g., sorgoleone) have been found to also have functions very distinct from nitrification inhibition [49,50,56].

Lastly, it is important to consider the role of root exudates in the N cycle in the context of climate change. Enhanced root exudation has been demonstrated in some ecosystems under elevated atmospheric CO_2, and this can lead to accelerated microbial activity associated with soil organic matter decomposition and rhizosphere N turnover, particularly under N-limiting conditions [108–110]. Similar effects have been seen at elevated temperatures [111]. It remains unclear, however, how enhanced root exudation due to climate change will affect other components of the N cycle, such as BNF, nitrification, and denitrification.

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