

Microbial associations enabling nitrogen acquisition in plants

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Large flows of nitrogen between the atmosphere, terrestrial and aquatic ecosystems contribute to the global cycle on Earth. When balanced, this cycle ensures that life at every level can flourish and diversify. However, in the past 50 years, humans have had a large, negative influence on nitrogen cycle pushing it beyond safe boundaries at the global level. Alternative, wholesome strategies are needed for the agricultural systems to achieve sustainability without compromising crop yields. Decades of research in the field of biological nitrogen fixation in symbiotic root nodules paved the way for ambitious bioengineering projects aiming to meet the nitrogen request in a sustainable manner. Parallel studies of the other microbes that associate with healthy plants in nature unveiled a tremendous, untapped resource for biostimulants. Many of these interactions are now worth investigating in detail to enable understanding at the molecular and ecological level and facile transfer into agricultural settings.

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Introduction

Life on Earth requires large amounts of nitrogen in biologically active forms. Nitrogen is found in abundance, however much of this resource is present in a chemically bound form (dinitrogen-N₂) that makes it difficult for integration in metabolic pathways [1]. Certain lineages of Bacteria and Archea, the diazotrophs, evolved the nitrogenase enzyme complex, enabling these microbes to break the triple bond between nitrogen atoms and to synthesize ammonia. Ammonia is the entry point of dinitrogen in biological systems as it can be directly metabolised into organic molecules or into nitrate via

nitrification. Diazotrophs are therefore powerful engines ensuring a pool of biologically active nitrogen for microbial and eukaryotic life present in terrestrial [2] and aquatic systems [3]. Movement of early plants from aquatic to terrestrial ecosystems in the early Paleozoic time involved adaptation to available nitrogen resources, ammonia, nitrate and organic nitrogen (e.g., free amino acids). Ammonia and organic nitrogen are vital for microbial metabolism and energy production. Plants have therefore evolved multiple strategies for acquiring sufficient nitrogen to support their photosynthetic activities and reproduction. Plants develop large root systems increasing the explored soil area, from where they use primarily nitrate, which even if directly uptaken from soil is energetically costly compared to ammonium. An alternative strategy developed by plants to improve their nitrogen supply, was the evolution of trophic alliances with specially adapted soil microbes, and by this, they gain access to alternative forms of nitrogen [4,5]. Consequently, the nitrogen status in natural ecosystems is now dependent on the metabolic activity of plant-associated microbiota, which in turn is sustained by large carbon supplies derived from plants. In agricultural settings, however, the human need for an increasingly large plant biomass has driven ecosystems outside of their safe boundaries (e.g., atmospheric N₂O emissions have reached their highest levels in the past 650 000 years) threatening their resilience and function [6].

Historically, the importance of microbial associations for nitrogen nutrition in plants has been focused on one, highly efficient symbiotic association, biological nitrogen fixation (BNF) in root nodules [7]. Advances in studies of other binary associations or lately of plant microbiota, uncovered more diverse strategies contributing to nitrogen acquisition and adaptation to environments where direct nitrogen availability for plants is scarce. Here we provide a brief review of various strategies that evolved for nitrogen acquisition through beneficial associations of plants and microbes.

Acquisition of nitrogen through mycorrhizae

The evolution of symbiotic gene networks in plants [8,9] and loss of pathogenicity determinants in fungi, especially of those belonging to *Glomeromycota* order [10] enabled the development of mycorrhiza, the intraradical symbiosis providing partners with increased fitness and ecological advantage. Ecto-mycorrhiza and endo-mycorrhiza allowed plants to increase the surface of explored soil via an extended hyphal network transporting water, phosphorous and nitrogen nutrients. Studies using isotope labelling and differential gene

expression showed that arbuscular mycorrhiza (AM) fungi take up ammonia from the soil and use glutamine synthetase in the extraradical mycelia (ERM) to convert the inorganic nitrogen into aminoacids via glutamine synthetase (GS) and glutamate synthase (glutamine:2-oxoglutarate aminotransferase; GOGAT) pathway (Figure 1a). Arginine is the form of nitrogen transported between ERM and the intraradical mycelia where it is converted back into ammonia by arginases and ureases before being provided to the plant host [11**]. AM fungi are also able to decompose organic matter and to uptake organic nitrogen directly from the soil. A proportion of this form of nitrogen is provided to the plant host [12], but much of it appears to be used by the fungus for increased fitness and ability to colonise secondary hosts [13]. The nitrogen flux from fungus to the host was found to be positively regulated by the carbon flux from the host to the fungus, indicating a tight nutrient regulation between the symbiotic partners [14]. Interestingly, when tested in binary systems, different AM fungi were found to differ in their capacity to transfer nitrogen to the plant, f.ex. *Glomus intraradices* transferring more than *Glomus hoi* [12]. Moreover, microbiota studies of fungal communities associated with plant roots found that in natural environments, plants are colonized by several lineages of the Glomeromycota order [15*,16*,17,18]. It remains to be determined if this diverse AM community has relevance for nitrogen nutrition in plants.

Root microbiota contributes to nitrogen nutrition

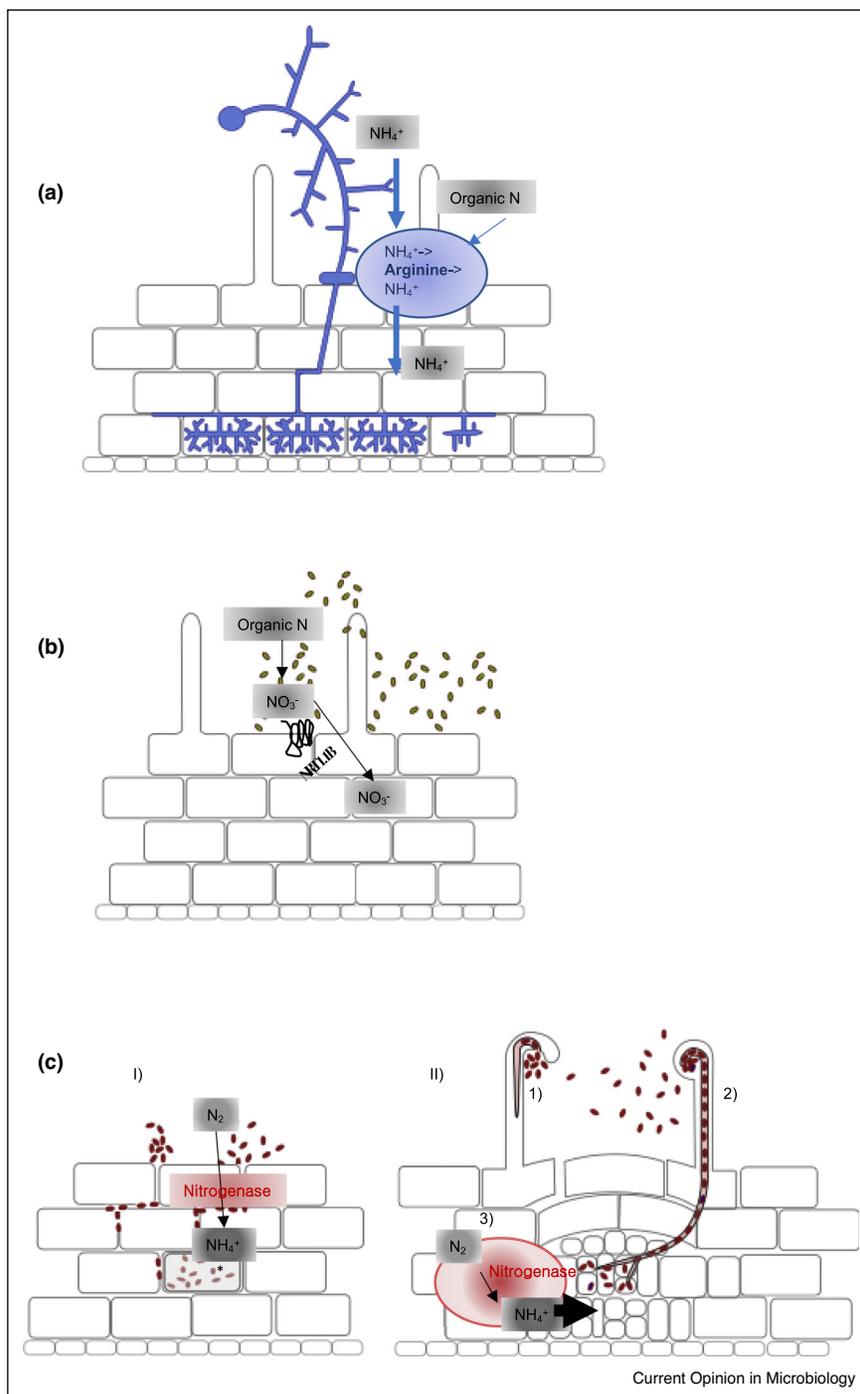
Soil bacteria have a remarkable capacity to decompose organic matter transforming complex organic N-rich sources into ammonia. There is a growing body of evidence, primarily from ecological studies, that plants might have evolved strategies for using this capacity of soil bacteria by priming and/or enriching their associated communities with mineralizing taxa [19**,20]. First evidences for a genetic determinant of this active involvement of the plant host are just emerging. Zhang *et al.* [19**] have found that compared to *japonica*, the *indica* varieties of rice, enrich their associated communities with bacteria having an enhanced nitrogen metabolism function (Figure 1b). Interestingly, this increased capacity is associated with variations in plant nitrogen transporter and sensor, NRT1.1B [21*]. Compared to wild-type *indica* variety, mutants in this transporter have a community with reduced abundance of nitrogen metabolizing bacteria, and the associated communities are depleted of *NapA*, *NapB* and *NirD*, key genes for the ammonification process. Using reconstituted communities of reduced complexity Zhang *et al.*, found that bacteria isolated from *indica* varieties and *japonica* varieties recapitulate the results obtained in the field with *indica*-isolates providing larger shoots and roots in the presence of organic nitrogen. These results indicate that there is great potential in understanding how cooperation between plants and microbes contributes to enhanced nitrogen metabolism in the rhizosphere for mutual benefit. The mechanistic involvement of NRT1.1B transporter in the observed

enrichment of bacterial N-metabolism in *indica* varieties remains to be determined, as does how to extend these findings from the laboratory into the field.

Biological nitrogen fixation (BNF) by root-associated and symbiotic diazotrophs

In nitrogen-poor environments, diazotrophic bacteria play a vital role for an increased input of metabolically active nitrogen [2]. Their ability to use the nitrogenase enzyme complex to convert atmospheric dinitrogen into ammonia is key not only for their own survival but also for enriching the pool of biologically active nitrogen in their habitat. Nitrogenase enzyme is extremely sensitive to oxygen, and different strategies have evolved in different biological systems to protect this enzyme from irreversible degradation [22,23]. Studies in the early 20th century revealed that plants and diazotrophic bacteria can establish associations enabling nitrogen fixation [24]. These findings have sparked an increased interest for identifying bacteria able to improve nitrogen nutrition in agricultural settlements. A considerable number of diazotrophs have been identified so far to associate as epiphytes or endophytes, primarily with cereals and grasses (Figure 1c), but only in few instances the actual plant nutrition with bacterial fixed nitrogen was demonstrated [25,26,27*,28]. Pioneer work on *Azospirillum brasiliense*, *Gluconacetobacter diazotrophicus* and *Herbaspirillum seropedicae* in sugar cane [29,30,31] or *Azoarcus* sp. in Kallar grass and rice [32] demonstrated their tight association, while later studies found active bacterial nitrogenase *NifH* promoter in intraradical environments [33]. More recently, Deynze *et al.* [27*] reported the identification of a maize landrace that could benefit from atmospheric nitrogen fixed by associated diazotrophes. When grown in nitrogen-depleted fields of highlands in Mexico, this particular maize landrace produced aerial root mucilage that proved to be an environmental niche enriching for *Nif* gene pools in the associated bacterial communities. These could provide up to 85% of the assimilated nitrogen. In general, plant-microbe associations with BNF capacity appear to be promoted by, and economically efficient in specific natural environments or hosts, but are prone to large variations in efficiency when extended to other conditions [34*,35]. This is most likely due to the complexity of plant-microbe and microbe-microbe interactions established in the rhizosphere, in different environments. Consequently, their use in agriculture is likely below their full biological potential. Understanding the principles that drive the evolution of such mutualistic interactions, their maintenance and efficiency in complex settings holds the key for their success. Such task are not easy though, integrated multidisciplinary research taking advantage of recent developments in plant microbiota studies, plant and bacterial genetics, population genetics, soil ecology, bioinformatics and mathematics might pave the way for efficient use of diazotrophic associations in agriculture.

Figure 1



Nitrogen acquisition through microbial associations.

(a) Arbuscular mycorrhizal fungi take up ammonia (NH_4^+) and organic nitrogen from the soil. Inside fungal hyphae ammonia is converted to arginine that is then converted back to ammonia before being transported into the arbusculated plant cells. **(b)** *Oryza sativa* cv *indica* enrich their associated communities with nitrifying bacteria that convert organic nitrogen from the soil to nitrate (NO_3^-) that is then taken up by the plants via the NRT1.1B transporter. **(c)** Diazotrophic bacteria convert atmospheric dinitrogen (N_2) into ammonia that becomes accessible to plants via root-association (I) or symbiotic interaction (II). It is primarily cereals and grasses that associate with diazotrophic bacteria that can colonise the plants as epiphytes or endophytes. Intraradical colonisation can lead to localised plant cell death (*). Inside the root, these bacteria can have an active nitrogenase enzyme that converts atmospheric dinitrogen into ammonia. Legumes engage in symbiotic association (II) with diazotrophic bacteria that produce compatible Nod factors and, in most cases, initiate an infection thread within root hairs. The plant host will stop the infection if incompatible EPS are detected (1) or enable infection thread elongation into the root cortex and nodule primordia (2). Inside the nodule cells, bacteria are endocytosed into symbiosomes (3) where nitrogenase activity is enabled.

An efficient strategy for the protection of nitrogenase, providing a direct beneficial outcome for the plant, has evolved in nodulating plant species belonging to *Fabales*, *Fagales*, *Cucurbitales* and *Rosales* orders [36]. The nitrogen-fixing bacterium is accommodated intracellularly in symbiotic compartments (symbiosomes or fixation threads) where it benefits from microaerobic and nutrient rich conditions ensured by the plant host in return for ammonia [37,38,39**]. Similar to mutualistic association, the intracellular symbiosis with nitrogen-fixing bacteria has also been under strong selection pressure and, outside of *Fabales*, it was retained in only few plant species belonging to the three other orders [40,41**,42*]. In spite of its limited penetrance among plant species, this symbiotic interaction is a significant contributor to BNF and global nitrogen cycle (up to 25 Tg) [43]. The nitrogen provided by the symbiotic bacteria benefits not only its host, but also nonnodulating plants sharing the same ecological niche, through release of fixed nitrogen into the soil [43]. The establishment of root nodule endosymbiosis is a highly complex, tightly controlled biological process [44]. The most well-understood systems are those where plant and bacterial genetics have been accessible, like legumes and rhizobia and some actinorhizal plants and *Frankia* bacteria.

The efficiency of biological nitrogen fixation in root nodules is influenced by the compatibility between host and bacterial isolate, bacterial infection capacity and ultimately by the efficiency of nitrogen-fixing activity of the symbiont when hosted inside nodules. Establishment of rhizobia-legume symbiosis requires molecular signalling to determine compatibility and initiation of the infection process [45,46]. The symbiotic signalling cascade [47,48**,49] triggered by legume receptors that perceive compatible rhizobial Nod factor leads to nodule organogenesis, and the formation of root hair infection threads that facilitate rhizobial colonisation (Figure 1d). Importantly, these early signalling events do not provide the legume host with information on the rhizobia's nitrogen-fixing ability and therefore, legumes are unable to select for colonising rhizobia on this basis. The host select its infecting symbiont on the basis of the structure of the Nod factors and exopolysaccharides they produce, both signals being suggested to operate as a two-step recognition mechanism [47,48**]. Additional layers of compatibility checks have evolved in the inverted repeat-lacking-clade of legumes through cysteine-rich peptides expressed inside nodules [50*]. As a result of this selection mechanism, rhizobial mutants completely deficient in nitrogen-fixing ability are equally competitive for nodule colonisation as their nitrogen-fixing wild-type parent [51]. The rhizobia's symbiotic competitiveness determines nodule colonisation, and this can lead to inefficient symbiotic interactions resulting in poor levels of fixed nitrogen being provided to the host legume [52,53,54]. However, so far only a limited number of

bacterial genes have been identified relevant for rhizobial competitiveness during root nodule infection [55,56*]. Studies using bacterial population genetics of the same isolate [57*] or highly similar isolates [58**] are prone to provide critical components controlling this stage of symbiosis.

Inside nodules, the endosymbiont becomes dependent on the host for supplying carbon in the form of dicarboxylic acids, such as malate [59], and other micronutrients, among which sulfate was found to be critical for nitrogenase activity [60*]. A microaerobic environment is created by the expression of plant genes coding for leghemoglobins [61]. This low oxygen environment is critical both for induction of bacterial genes required for nitrogen fixation [62] and for promoting the exchange of nutrients between plant and symbiont [63]. In *Lotus japonicus-Mesorhizobium loti* symbiosis f.ex. the symbiont is dependent on the host for providing homocitrate, a key cofactor for nitrogenase complex [64]. The *Fen1* gene encoding for the homocitrate is induced at 10 days post inoculation when bacteria have already colonized the nodules [64]. In order to promote ammonia secretion from the bacteroids, the host directly interferes with bacterial nitrogen metabolism. Ammonia assimilation in bacteroids is reduced [65], and bacteroids like f.ex. those formed by *Rhizobium leguminosarum* were found to be auxotroph for branched-chain amino acid transport, and dependent on the pea plant for supplying them [66]. In actinorhizal plant-*Frankia* symbiosis, fixed nitrogen is released to the plant in the form of amino acids or amides, that are then broken down to ammonia [67].

Little is known about host and bacterial components that contribute to fine-tune the efficiency of nitrogen fixation when rhizobia are inside nodules, and whether there are possibilities to use plant and bacterial genetics through breeding and inoculum selection for improving the BNF in agricultural settings. Co-inoculation experiments demonstrated that rhizobia can complement each other for missing components resulting in mixed colonisation of nodules [55,68]. In addition, nodules formed on the same root are independent events that can be initiated by compatible but different symbiotic isolates. In natural environments, the development of root-nodule symbiosis occurs in parallel with root microbiota establishment, and non-symbiotic bacteria gain entry to nodules through high-jacking the infection process initiated by symbiotic rhizobia [68]. Consequently, nodules formed on the same plant are found to contain a microbial population dominated by nitrogen-fixing rhizobia [69–71]. In agriculture, commercial coating of legume seeds with efficient nitrogen-fixing rhizobial strains is performed, however, the success of this practice varies widely due to poor persistence and competition of the inoculant strain with the adapted native soil rhizobia populations [72]. The occurrence of these so-called 'cheater' strains that may

represent non-symbiotic rhizobia or a diverse range of bacterial species that take advantage of the carbon sources provided by the plant without providing fixed nitrogen in return, may influence nitrogen-fixing efficiency. To minimise the detrimental effects of cheater strains, legumes are able to sanction against nodules that are not providing sufficient fixed-nitrogen [73] and can do so not only at a whole nodule level but in a cell autonomous manner [74,75]. Sanctioning against ineffective nitrogen-fixers is evolutionary important to maintain the symbiotic association. However, the mechanisms by which legumes sanction against cheater strains, particularly at the cell autonomous level, are not well understood and await discovery.

Conclusions

The growing human population, and the alarming ecological constraints induced by intensive farming constitute great challenges for the agricultural systems. The complex microbial communities that associate with healthy plants in nature have emerged as a tremendous, untapped resource for nitrogen nutrition in sustainable agriculture [76,77]. Plant and bacterial genetics have contributed greatly to our understanding of the complexity and diversity of molecular components that contribute to the efficiency of nitrogen fixation in evolutionary different root nodule symbioses [44,78]. These discoveries form a solid basis for addressing long-term and challenging tasks of engineering BNF in nonlegumes [79,80]. Parallel efforts in understanding, predicting, and controlling plant-microbe interactions at the level of microbial communities hold the key to fully unlock their beneficial potential [81]. Addressing such complex interactions at the level of fundamental and predictive understanding requires integrated systems approaches, where communities are analysed and improved by intertwined empirical and theoretical approaches [82].

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Fowler D, Coyle M, Skiba U, Sutton MA, Cape JN, Reis S, Sheppard LJ, Jenkins A, Grizzetti B, Galloway JN *et al.*: **The global nitrogen cycle in the twenty-first century.** *Philos Trans R Soc Lond Ser B Biol Sci* 2013, **368**:20130164.
2. Reed SC, Cleveland CC, Townsend AR: **Functional ecology of free-living nitrogen fixation: a contemporary perspective.** *Annu Rev Ecol Syst* 2011, **42**:489.
3. Martínez-Pérez C, Mohr W, Löscher CR, Dekaezemacker J, Littmann S, Yilmaz P, Lehnen N, Fuchs BM, Lavik G, Schmitz RA *et al.*: **The small unicellular diazotrophic symbiont, UCYN-A, is a key player in the marine nitrogen cycle.** *Nat Microbiol* 2016, **1**:16163
- This paper identifies the UCYN-A cyanobacteria that lives in symbioses with specific eukaryotic algae as large contributors to nitrogen fixation in the tropical North Atlantic oceanic ecosystem.
4. Pirozynski KA, Malloch DW: **The origin of land plants: a matter of mycotrophism.** *BioSystems* 1975, **6**.
5. Lutzoni F, Nowak MD, Alfaro ME, Reeb V, Miadlikowska J, Krug M, Arnold AE, Lewis LA, Swofford DL, Hibbett D *et al.*: **Contemporaneous radiations of fungi and plants linked to symbiosis.** *Nat Commun* 2018, **9**:5451.
6. Rockström J, Steffen W, Noone K, Persson Å, Chapin III FS, Lambin EF, Lenton TM, Scheffer M, Folke C, Schellnhuber HJ *et al.*: **A safe operating space for humanity.** *Nature* 2009, **461**:472.
7. Long SR: **Rhizobium-legume nodulation: life together in the underground.** *Cell* 1989, **56**:203.
8. Venkateshwaran M, Volkening JD, Sussman MR, Ané J-M: **Symbiosis and the social network of higher plants.** *Curr Opin Plant Biol* 2013, **16**:118.
9. Delaux P-M: **Comparative phylogenomics of symbiotic associations.** *New Phytol* 2017, **213**:89.
10. Tisserant E, Malbreil M, Kuo A, Kohler A, Symeonidi A, Balestrini R, Charron P, Duensing N, Frei dit Frey N, Gianinazzi-Pearson V *et al.*: **Genome of an arbuscular mycorrhizal fungus provides insight into the oldest plant symbiosis.** *Proc Natl Acad Sci U S A* 2013, **110**:20117.
11. Govindarajulu M, Pfeffer PE, Jin H, Abubaker J, Douds DD, Allen JW, Bucking H, Lammers PJ, Shachar-Hill Y: **Nitrogen transfer in the arbuscular mycorrhizal symbiosis.** *Nature* 2005, **435**:819
- The authors use stable isotope labelling to identify the metabolic pathway used by AM fungi to uptake, transport and export nitrogen from the soil into plant root cells.
12. Leigh J, Hodge A, Fitter AH: **Arbuscular mycorrhizal fungi can transfer substantial amounts of nitrogen to their host plant from organic material.** *New Phytol* 2009, **181**:199.
13. Hodge A, Fitter AH: **Substantial nitrogen acquisition by arbuscular mycorrhizal fungi from organic material has implications for N cycling.** *Proc Natl Acad Sci U S A* 2010, **107**:13754.
14. Fellbaum CR, Gachomo EW, Beesetty Y, Choudhari S, Strahan GD, Pfeffer PE, Kiers ET, Bucking H: **Carbon availability triggers fungal nitrogen uptake and transport in arbuscular mycorrhizal symbiosis.** *Proc Natl Acad Sci U S A* 2012, **109**:2666.
15. Xue L, Almario J, Fabiańska I, Saridis G, Bucher M: **Dysfunction in the arbuscular mycorrhizal symbiosis has consistent but small effects on the establishment of the fungal microbiota in *Lotus japonicus*.** *New Phytol* 2019, **224**:409-420
- An interesting insight into the role of plant symbiotic genes have on the overall fungal community of legumes. The authors found that AM-impaired mutant plants assemble different root-associated fungal microbiota with a depletion of Glomeromycota and enrichment of Ascomycota.
16. Thierygart T, Zgadzaj R, Bozsóki Z, Garrido-Oter R, Radutoiu S, Schulze-Lefert P: ***Lotus japonicus* symbiosis genes impact microbial interactions between symbionts and multikingdom commensal communities.** *bioRxiv* 2019:547687
- Identifies the effect of legume symbiotic gene mutations on both bacterial and fungal communities, highlighting the effect of these mutations on interkingdom interactions.
17. Orgiazzi A, Lumini E, Nilsson RH, Giralanda M, Vizzini A, Bonfante P, Bianciotto V: **Unravelling soil fungal communities from different Mediterranean land-use backgrounds.** *PLoS One* 2012, **7**:e34847.

18. Schlaeppi K, Bender SF, Mascher F, Russo G, Patrignani A, Camenzind T, Hempel S, Rillig MC, van der Heijden MGA: **High-resolution community profiling of arbuscular mycorrhizal fungi**. *New Phytol* 2016, **212**:780.
19. Zhang J, Liu YX, Zhang N, Hu B, Jin T, Xu H, Qin Y, Yan P, Zhang X, ●● Guo X *et al.*: **NRT1.1B is associated with root microbiota composition and nitrogen use in field-grown rice**. *Nat Biotechnol* 2019, **37**:676
- The authors compared the microbiomes of rice varieties that differ in their nitrogen-use efficiency and demonstrated that this microbiome is associated with a plant gene NRT1.1B.
20. Zhu B, Gutknecht JLM, Herman DJ, Keck DC, Firestone MK, Cheng W: **Rhizosphere priming effects on soil carbon and nitrogen mineralization**. *Soil Biol Biochem* 2014, **76**:183.
21. Hu B, Wang W, Ou S, Tang J, Li H, Che R, Zhang Z, Chai X, ● Wang H, Wang Y *et al.*: **Variation in NRT1.1B contributes to nitrate-use divergence between rice subspecies**. *Nat Genet* 2015, **47**:834
- The authors show that genetic variation in the NRT1.1B among rice subspecies leads to differences in their ability to use nitrate from the soil.
22. Ureta A, Nordlund S: **Evidence for conformational protection of nitrogenase against oxygen in *Gluconacetobacter diazotrophicus* by a putative FeSII protein**. *J Bacteriol* 2002, **184**:5805.
23. Gallon JR: **Reconciling the incompatible: N₂ fixation And O₂**. *New Phytol* 1992, **122**:571.
24. Baldani JI, Baldani VLD: **History on the biological nitrogen fixation research in graminaceous plants: special emphasis on the Brazilian experience**. *Anais da Academia Brasileira de Ciências* 2005, **77**:549.
25. Pankiewicz VC, do Amaral FP, Santos KF, Agtuca B, Xu Y, Schueller MJ, Arisi AC, Steffens MB, de Souza EM, Pedrosa FO *et al.*: **Robust biological nitrogen fixation in a model grass-bacterial association**. *Plant J* 2015, **81**:907.
26. Hurek T, Handley LL, Reinhold-Hurek B, Piché Y: **Azoarcus grass endophytes contribute fixed nitrogen to the plant in an unculturable state**. *Mol Plant Microbe Interact* 2002, **15**:233.
27. Van Deynze A, Zamora P, Delaux PM, Heitmann C, Jayaraman D, ● Rajasekar S, Graham D, Maeda J, Gibson D, Schwartz KD *et al.*: **Nitrogen fixation in a landrace of maize is supported by a mucilage-associated diazotrophic microbiota**. *PLoS Biol* 2018, **16**:e2006352
- This article presents the identification of a novel biological nitrogen fixation system between particular maize varieties and diazotrophs within mucilage associated with aerial roots grown in humid climates.
28. Boddey RM, de Oliveira OC, Urquiaga S, Reis VM, de Olivares FL, Baldani VLD, Döbereiner J: **Biological nitrogen fixation associated with sugar cane and rice: contributions and prospects for improvement**. In *Management of Biological Nitrogen Fixation for the Development of More Productive and Sustainable Agricultural Systems: Extended versions of papers presented at the Symposium on Biological Nitrogen Fixation for Sustainable Agriculture at the 15th Congress of Soil Science, Acapulco, Mexico, 1994*. Edited by Ladha JK, Peoples MB. Netherlands: Springer; 1995:195.
29. Gillis M, Kersters K, Hoste B, Janssens D, Kroppenstedt RM, Stephan MP, Teixeira KRS, Döbereiner J, Deley J: ***Acetobacter diazotrophicus* sp. nov., a nitrogen-fixing acetic acid bacterium associated with sugarcane**. *Int J Syst Evol Microbiol* 1989, **39**.
30. James EK, Reis VM, Olivares FL, Baldani JI, Döbereiner J: **Infection of sugar cane by the nitrogen-fixing bacterium *Acetobacter diazotrophicus***. *J Exp Bot* 1994, **45**:757.
31. James EK, Olivares FL: **Infection and colonization of sugar cane and other graminaceous plants by endophytic diazotrophs**. *Crit Rev Plant Sci* 1998, **17**:77.
32. Hurek T, Reinhold-Hurek B, Turner GL, Bergersen FJ: **Augmented rates of respiration and efficient nitrogen fixation at nanomolar concentrations of dissolved O₂ in hyperinduced *Azoarcus* sp. strain BH72**. *J Bacteriol* 1994, **176**:4726.
33. Egner T, Hurek T, Reinhold-Hurek B: **Endophytic expression of nif genes of *Azoarcus* sp. strain BH72 in rice roots**. *Mol Plant Microbe Interact* 1999, **12**:813.
34. Terrazas RA, Pietrangelo L, Corral AM, Torres-Cortés G, ● Robertson-Albertyn S, Balbirnie-Cumming K, Morris J, Hedley PE, Barret M, Paterson E *et al.*: **Nitrogen availability modulates the host control of the barley rhizosphere microbiota**. *bioRxiv* 2019:605204
- In this paper, wild and domesticated barley varieties rhizosphere microbiomes were examined and it was identified that nitrogen availability impacts barley rhizosphere microbiome assembly.
35. Pivato B, Bru D, Busset H, Deau F, Matejcek A, Philippot L, Moreau D: **Positive effects of plant association on rhizosphere microbial communities depend on plant species involved and soil nitrogen level**. *Soil Biol Biochem* 2017, **114**:1.
36. Parniske M: **Uptake of bacteria into living plant cells, the unifying and distinct feature of the nitrogen-fixing root nodule symbiosis**. *Curr Opin Plant Biol* 2018, **44**:164.
37. Terpolilli JJ, Masakapalli SK, Karunakaran R, Webb IUC, Green R, Watmough NJ, Kruger NJ, Ratcliffe RG, Poole PS: **Lipogenesis and redox balance in nitrogen-fixing pea bacteroids**. *J Bacteriol* 2016, **198**:2864.
38. Udvardi M, Poole PS: **Transport and metabolism in legume-rhizobia symbioses**. *Ann Rev Plant Biol* 2013, **64**:781.
39. van Velzen R, Holmer R, Bu F, Rutten L, van Zeijl A, Liu W, ●● Santuari L, Cao Q, Sharma T, Shen D *et al.*: **Comparative genomics of the nonlegume *Parasponia* reveals insights into evolution of nitrogen-fixing rhizobium symbioses**. *Proc Natl Acad Sci U S A* 2018, **115**:E4700
- In this paper, the authors identify key symbiotic genes that were retained in *Parasponia* but lost in the sister nonnodulating *Trema*, demonstrating that root nodulation is under selective pressure leading to genetic and phenotypic loss.
40. Pawlowski K, Demchenko KN: *The Diversity of Actinorhizal Symbiosis* 2012, **249**:967-979.
41. Griesmann M, Chang Y, Liu X, Song Y, Haberer G, Crook MB, ●● Billault-Penneteau B, Laressergues D, Keller J, Imanishi L *et al.*: **Phylogenomics reveals multiple losses of nitrogen-fixing root nodule symbiosis**. *Science* 2018, **361**:eaat1743
- In this paper the authors identify that genes also identified by van Velzen *et al.* [39●●] were lost in nonnodulating species belonging to FaFaCuRo orders.
42. Sprent JI, Ardley J, James EK: **Biogeography of nodulated legumes and their nitrogen-fixing symbionts**. *New Phytol* 2017, **215**:40
- This paper presents a comprehensive overview of the nodulated species and their symbiotic partners together with their biogeographical representation.
43. Herridge DF, Peoples MB, Boddey RM: **Global inputs of biological nitrogen fixation in agricultural systems**. *Plant Soil* 2008, **311**:1.
44. Zipfel C, Oldroyd GED: **Plant signalling in symbiosis and immunity**. *Nature* 2017, **543**:328.
45. Kelly S, Radutoiu S, Stougaard J: **Legume LysM receptors mediate symbiotic and pathogenic signalling**. *Curr Opin Plant Biol* 2017, **39**:152.
46. Oldroyd GED, Murray JD, Poole PS, Downie JA: **The rules of engagement in the legume-rhizobial symbiosis**. *Ann Rev Genet* 2011, **45**:119.
47. Radutoiu S, Madsen LH, Madsen EB, Felle HH, Umehara Y, Grönlund M, Sato S, Nakamura Y, Tabata S, Sandal N *et al.*: **Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases**. *Nature* 2003, **425**:585.
48. Kawaharada Y, Kelly S, Nielsen MW, Hjuler CT, Gysel K, ●● Muszyński A, Carlson RW, Thygesen MB, Sandal N, Asmussen MH *et al.*: **Receptor-mediated exopolysaccharide perception controls bacterial infection**. *Nature* 2015, **523**:308
- In this paper the authors demonstrate that Nod factor signalling and exopolysaccharide signalling control bacterial infection in legume roots.
49. Madsen LH, Tirichine L, Jurkiewicz A, Sullivan JT, Heckmann AB, Bek AS, Ronson CW, James EK, Stougaard J: **The molecular network governing nodule organogenesis and infection in the model legume *Lotus japonicus***. *Nat Commun* 2010, **1**:10.
50. Montiel J, Downie JA, Farkas A, Bihari P, Herczeg R, Bálint B, ● Mergaert P, Kereszt A, Kondorosí E: **Morphotype of bacteroids in different legumes correlates with the number and type of**

symbiotic NCR peptides. *Proc Natl Acad Sci U S A* 2017, **114**:5041

In this paper the authors present the identification and expression of NCR peptides from different legumes belonging to IRLC clade. Interestingly, the NCR effect was found to be affected by the rhizobial surface polysaccharides.

51. Westhoek A, Field E, Rehling F, Mulley G, Webb I, Poole PS, Turnbull LA: **Policing the Legume-Rhizobium symbiosis: a critical test of partner choice.** *Sci Rep* 2017, **7**:1419.
52. Triplett EW, Sadowsky MJ: **Genetics of competition for nodulation of legumes.** *Ann Rev Microbiol* 1992, **46**:399.
53. Lardi M, de Campos SB, Purtschert G, Eberl L, Pessi G: **Competition experiments for legume infection identify *Burkholderia phymatum* as a highly competitive β -Rhizobium.** *Front Microbiol* 2017, **8**.
54. Pahua VJ, Stokes PJN, Hollowell AC, Regus JU, Gano-Cohen KA, Wendlandt CE, Quides KW, Lyu JY, Sachs JL: **Fitness variation among host species and the paradox of ineffective rhizobia.** *J Evol Biol* 2018, **31**:599.
55. Kelly SJ, Muszyński A, Kawaharada Y, Hubber AM, Sullivan JT, Sandal N, Carlson RW, Stougaard J, Ronson CW: **Conditional requirement for exopolysaccharide in the *Mesorhizobium-Lotus* symbiosis.** *Mol Plant Microbe Interact* 2013, **26**:319-329.
56. de Campos SB, Lardi M, Gandolfi A, Eberl L, Pessi G: **Mutations in two *Paraburkholderia phymatum* type VI secretion systems cause reduced fitness in interbacterial competition.** *Front Microbiol* 2017, **8**
- In this paper the authors present the identification of typeVI secretion system as a major contributor to *Paraburkholderia phymatum* bacterial fitness when in competition with other bacteria present in the inoculum.
57. Perry BJ, Akter MS, Yost CK: **The use of transposon insertion sequencing to interrogate the core functional genome of the legume symbiont *Rhizobium leguminosarum*.** *Front Microbiol* 2016, **7**
- In this paper the authors present the development of a transposon insertion sequencing approach that can be adapted to nitrogen-fixing rhizobia.
58. Karasov TL, Almario J, Friedemann C, Ding W, Giolai M, Heavens D, Kersten S, Lundberg DS, Neumann M, Regalado J *et al.*: ***Arabidopsis thaliana* and *Pseudomonas* pathogens exhibit stable associations over evolutionary timescales.** *Cell Host Microbe* 2018, **24**:168-179.e164
- The authors present a detailed analysis of *Pseudomonas* population associated with *Arabidopsis thaliana* in natural environments and identify that despite large differences in gene content and disease phenotype when tested in binary systems, their individual fitness at the population level is under strong selective pressure from the wild-host *Arabidopsis*.
59. Udvardi MK, Price GD, Gresshoff PM, Day DA: **A dicarboxylate transporter on the peribacteroid membrane of soybean nodules.** *FEBS Lett* 1988, **231**:36.
60. Schneider S, Schintlmeister A, Becana M, Wagner M, Woebken D, Wienkoop S: **Sulfate is transported at significant rates through the symbiosome membrane and is crucial for nitrogenase biosynthesis.** *Plant Cell Environ* 2019, **42**:1180
- The authors demonstrate that symbiosomes are a sulfate sink and require a plant host gene for transporting this nutrient into the symbiotic space.
61. Ott T, van Dongen JT, Gunther C, Krusell L, Desbrosses G, Vigeolas H, Bock V, Czechowski T, Geigenberger P, Udvardi MK: **Symbiotic leghemoglobins are crucial for nitrogen fixation in legume root nodules but not for general plant growth and development.** *Curr Biol* 2005, **15**:531.
62. Martinez-Argudo I, Little R, Shearer N, Johnson P, Dixon R: **The NifL-NifA system: a multidomain transcriptional regulatory complex that integrates environmental signals.** *J Bacteriol* 2004, **186**:601.
63. Ott T, Sullivan J, James EK, Flemetakis E, Günther C, Gibon Y, Ronson C, Udvardi M: **Absence of symbiotic leghemoglobins alters bacteroid and plant cell differentiation during development of *Lotus japonicus* root nodules.** *Mol Plant Microbe Interact* 2009, **22**:800.
64. Hakoyama T, Niimi K, Watanabe H, Tabata R, Matsubara J, Sato S, Nakamura Y, Tabata S, Jichun L, Matsumoto T *et al.*: **Host plant genome overcomes the lack of a bacterial gene for symbiotic nitrogen fixation.** *Nature* 2009, **462**:514.
65. Patriarca EJ, Tate R, Iaccarino M: **Key role of bacterial NH₄(⁺) metabolism in *Rhizobium*-plant symbiosis.** *Microbiol Mol Biol Rev* 2002, **66**:203.
66. Lodwig EM, Hosie AHF, Bourdès A, Findlay K, Allaway D, Karunakaran R, Downie JA, Poole PS: **Amino-acid cycling drives nitrogen fixation in the Legume-Rhizobium symbiosis.** *Nature* 2003, **422**:722.
67. Valverde C, Huss-Danell K: **Carbon and nitrogen metabolism in Actinorhizal nodules.** In *Nitrogen-Fixing Actinorhizal Symbioses*. Edited by Pawlowski K, Newton WE. Netherlands: Springer; 2008:
68. Zgadzaj R, James EK, Kelly S, Kawaharada Y, de Jonge N, Jensen DB, Madsen LH, Radutoiu S: **A legume genetic framework controls infection of nodules by symbiotic and endophytic bacteria.** *PLoS Genet* 2015, **11**:e1005280.
69. Martínez-Hidalgo P, Hirsch AM: **The nodule microbiome: N₂-fixing rhizobia do not live alone.** *Phytobiomes J* 2017, **1**:70.
70. Zgadzaj R, Garrido-Oter R, Jensen DB, Koprivova A, Schulze-Lefert P, Radutoiu S: **Root nodule symbiosis in *Lotus japonicus* drives the establishment of distinctive rhizosphere, root, and nodule bacterial communities.** *Proc Natl Acad Sci U S A* 2016, **113**:E7996.
71. Checcucci A, Azzarello E, Bazzicalupo M, Galardini M, Lagomarsino A, Mancuso S, Marti L, Marzano MC, Mocali S, Squartini A *et al.*: **Mixed nodule infection in *Sinorhizobium melliloti-Medicago sativa* symbiosis suggest the presence of cheating behavior.** *Front Plant Sci* 2016, **7**:835.
72. Wakelin S, Tillard G, van Ham R, Ballard R, Farquharson E, Gerard E, Geurts R, Brown M, Ridgway H, O'Callaghan M: **High spatial variation in population size and symbiotic performance of *Rhizobium leguminosarum* bv. *trifolii* with white clover in New Zealand pasture soils.** *PLoS One* 2018, **13**:e0192607.
73. Kiers ET, Rousseau RA, West SA, Denison RF: **Host sanctions and the legume-Rhizobium mutualism.** *Nature* 2003, **425**:78.
74. Regus JU, Quides KW, O'Neill MR, Suzuki R, Savory EA, Chang JH, Sachs JL: **Cell autonomous sanctions in legumes target ineffective rhizobia in nodules with mixed infections.** *Am J Bot* 2017, **104**:1299.
75. Daubech B, Remigi P, Doin de Moura G, Marchetti M, Pouzet C, Auriac M-C, Gokhale CS, Masson-Boivin C, Capela D: **Spatio-temporal control of mutualism in legumes helps spread symbiotic nitrogen fixation.** *eLife* 2017, **6**:e28683
- In this paper the authors provide evidence of cell-autonomy of legume sanctions in indeterminate nodules.
76. Bulgarelli D, Schlaeppi K, Spaepen S, Themaat EVL, Schulze-Lefert P: **Structure and functions of the bacterial microbiota of plants.** *Ann Rev Plant Biol* 2013, **64**:807.
77. Finkel OM, Castrillo G, Herrera Paredes S, Salas González I, Dangl JL: **Understanding and exploiting plant beneficial microbes.** *Curr Opin Plant Biol* 2017, **38**:155.
78. Sachs JL, Quides KW, Wendlandt CE: **Legumes versus rhizobia: a model for ongoing conflict in symbiosis.** *New Phytol* 2018, **219**:1199.
79. Burén S, López-Torrejón G, Rubio LM: **Extreme bioengineering to meet the nitrogen challenge.** *Proc Natl Acad Sci U S A* 2018, **115**:8849.
80. Rogers C, Oldroyd GED: **Synthetic biology approaches to engineering the nitrogen symbiosis in cereals.** *J Exp Bot* 2014, **65**:1939.
81. Busby PE, Soman C, Wagner MR, Friesen ML, Kremer J, Bennett A, Morsy M, Eisen JA, Leach JE, Dangl JL: **Research priorities for harnessing plant microbiomes in sustainable agriculture.** *PLoS Biol* 2017, **15**.
82. Widder S, Allen RJ, Pfeiffer T, Curtis TP, Wiuf C, Sloan WT, Cordero OX, Brown SP, Momeni B, Shou WY *et al.*: **Challenges in microbial ecology: building predictive understanding of community function and dynamics.** *ISME J* 2016, **10**:2557.