

MINIREVIEW

Multi-species relationships in legume roots: From pairwise legume-symbiont interactions to the plant – microbiome – soil continuum

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One sentence summary: Time to integrate bottom-up with top-down approaches: From the groundbreaking understanding of the legume-rhizobium symbiosis to the dynamics and functions of the legume-microbe-soil continuum and the management of sustainable agro-ecosystems.

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ABSTRACT

Mutualistic relationships of legume plants with, either bacteria (like rhizobia) or fungi (like arbuscular mycorrhizal fungi), have been investigated intensively, usually as bi-partite interactions. However, diverse symbiotic interactions take place simultaneously or sequentially under field conditions. Their collective, but not additive, contribution to plant growth and performance remains hard to predict, and appears to be furthermore affected by crop species and genotype, non-symbiotic microbial interactions and environmental variables. The challenge is: (i) to unravel the complex overlapping mechanisms that operate between the microbial symbionts as well as between them, their hosts and the rhizosphere (ii) to understand the dynamics of the respective mechanisms in evolutionary and ecological terms. The target for agriculture, food security and the environment, is to use this insight as a solid basis for developing new integrated technologies, practices and strategies for the efficient use of beneficial microbes in legumes and other plants. We review recent advances in our understanding of the symbiotic interactions in legumes roots brought about with the aid of molecular and bioinformatics tools. We go through single symbiont-host interactions, proceed to tripartite symbiont-host interactions, appraise interactions of symbiotic and associative microbiomes with plants in the root-rhizoplane-soil continuum of habitats and end up by examining attempts to validate community ecology principles in the legume-microbe-soil biosystem.

Keywords: legume; symbiotic; phytobiome; microbiome network analysis; nitrogen cycling

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INTRODUCTION

Interactions of plants with beneficial microbes as rhizobia and endomycorrhizal fungi have been reported since more than a century ago (Frank 1887; Beijerinck 1901). In the last years, interest in mutualistic plant-microbe interactions escalated and expanded into a wide range of microbes in the plant rhizosphere (Philippot et al. 2013). In parallel, evolution and communication with plant hosts were further elucidated (Martin, Uroz and Barker 2017; Clear and Hom 2019). Mutualistic plant-microbe interactions of partners that live together in strong association are viewed as symbiotic. In this review, we use the term 'mutualist' for mutually beneficial microbes that do not necessarily live in close association with their plant partner, and the term 'symbiont' for microbes that live in close association with their plant partner, but do not always result into obvious mutual benefit (see also Glossary). It has become apparent that interactions with multiple symbionts enable plants to receive a wide range of benefits like increased tolerance against biotic and abiotic stresses, biological control of pathogens, enhanced nutrient acquisition and growth enhancement (reviewed by Hardoim et al. 2015; Khare, Mishra and Arora 2018). Symbiotic microorganisms mainly act by altering the plant gene expression, physiology and metabolism (Pozo et al. 2015; Khare, Mishra and Arora 2018), but also the rhizosphere abiotic environment in close proximity to the plant-soil interface (De-la-Peña et al. 2008; Badri and Vivanco 2009), including the root and rhizosphere microbiome (Uroz, Courty and Oger 2019). Direct and indirect changes in the rhizosphere environment facilitate the proliferation of specific microorganisms and trigger feedback mechanisms during an apparent co-evolution process between plants and associated microbes (Lambers et al. 2009; Garcia and Kao-Kniffin 2018). We are now in the position to understand many of the molecular mechanisms underlying the interactions of plants with these different microbes. Current knowledge though, is largely based on pairwise studies, focused on specific types of plant-microbe interactions alone. This is a limiting approach, considering that plants interact in synchrony with a range of microbes in nature, which may be more fruitfully approached as entire microbial consortia, and their effects on plants are far from additive i.e. they do not arise as the sum of the pairwise interactions (Afkhami, Rudgers and Stachowicz 2014). In addition, different symbionts that establish symbioses with the same host interact not only with the host plant, but also with one another (Larimer, Clay and Bever 2014), and induce positive or negative feedbacks on the plant phytobiome and the rhizosphere microbiome (Palakurty, Stinchcombe and Afkhami 2018) (Fig. 1). Overall, microbial symbionts are critical regarding environmental plant adaptation and performance, a role not only related to their direct effects on the plant, but also to the outcomes of microbe-microbe interactions that take place in the plant-microbiome-soil continuum (Hassani, Durán and Hacquard 2018).

Apart of their key role in food production and agroecosystem sustainability worldwide, legumes are a unique model system to study plants and their associated belowground microbial communities. Here, we review current knowledge on the symbiotic interactions that take place in legume roots and discuss recent advances in molecular tools and bioinformatics that allow us for the first time to integrate information from bi-partite, multi-partite and systems approaches, and to explore biotic dynamics in the plant-microbe-soil biosystem as a whole.

The emerging concept of the 'holobiont', the assemblage of host-plant and associated microorganisms considered as a unit,

provides a promising new perspective for understanding the plant-environment interaction, how the plant chooses its symbionts, how it balances the needs for essential nutrients (like nitrogen and phosphorus) and how it responds towards biotic and abiotic stress factors. We aim to show that this perspective, coupled to the recently developed methodological tools will enable us to (a) evaluate the total effect of exogenous microbial application to crops, (b) be cautious of, and avoid, the risks that this practice might convey and (c) design new methodologies which will favor the establishment of an array of mutualistic relations on the same plant in order to improve environmental adaptation and yield. The goal is to develop the tools so to exploit plant microbiome for developing the next generation of nutrition, protection and cultivation solutions for crops and, in parallel, to breed for plants, and potentially for plant communities, that may maximize this benefit.

Glossary

MICROBES

Mutualist: A microbe that presents a mutually beneficial interaction with its host or another microbe. Mutualists do not necessarily live in close association with their plant partner

Symbiont: a microbe that lives in close association with its plant partner. Symbiotic interactions are usually, but not necessarily, beneficial for the partners involved.

Endo-symbiont: a microbe that live inside the tissues of the host or partner.

Ecto-symbiont: a microbe that lives outside, or at the interface with their host or partner

Cheater: a microbe that cheats (cooperates less than its fair share), by providing an inferior benefit to the plant or the microbial community it interacts with, in order to gain fitness advantage over better benefit providers.

Commensal: a microbe that shares the same niche with another microbe or a plant, without affecting its fitness positively or negatively

Parasite: a microbe that creates symbiotic relationship with other organisms, living on, or inside them, and exploiting their nutrient or energy resources with negative results to the host's fitness

Pathogen: a microbe that is capable of causing disease in its host species

COMMUNICATION

Signal: a trait or behavior that has evolved to convey information to a receiver and, as a rule, provides a fitness benefit to both sender and receiver.

Cue: A trait or behavior that benefits the receiver solely, that has evolved to respond to it. Not specifically addressed to the receiver

BIOTIC PLANT ENVIRONMENT

Microbiome: All the microorganisms in a niche, or a set of niches, viewed as a set of interacting entities rather than a list of taxa.

Phytobiome: All the micro- and macro-organisms living in, on, or around the plant and the surrounding soil affected by the plant.

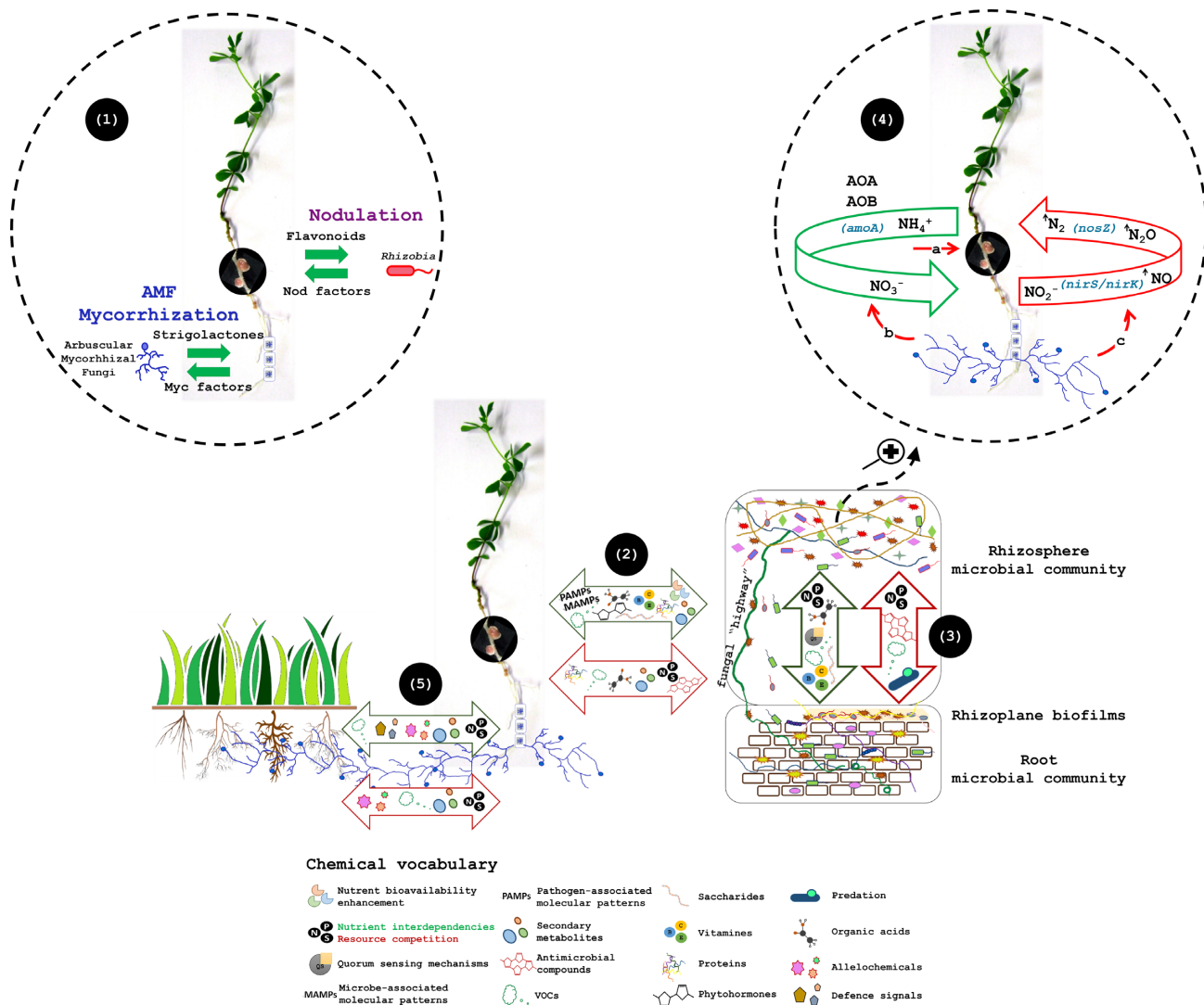


Figure 1. Interactions within the legume—symbiont—microbiome—soil continuum. (1) **Legume-Rhizobia-AMF interactions:** Legumes establish symbioses with two major colonizers, rhizobial and AM fungi (presented in detail in BOX 1 and in BOX 2 respectively). When colonizing the same host plant (tri-partite symbiosis), AM fungi and rhizobia influence each other, their combined effect on their host is not simply cumulative, and the host exercises a regulatory role. (2) **Legume host—microbe interactions:** The plant attracts a range of beneficial microorganisms through root exudation of a variety of molecules, including saccharides, organic acids, proteins, vitamins, volatile organic compounds (VOCs) and other secondary metabolites (e.g. phenolic compounds, terpenes), while it prohibits proliferation of other microorganisms through root exudation of antibiotic or toxic compounds (e.g. organic acids, proteins, secondary metabolites) and through competition for nutrients. The beneficial microorganisms usually possess one or more plant growth promoting traits like the capacity to synthesize phytohormones (e.g. Indoleacetic acid and cytokinin) to enhance nutrient bioavailability for the plant host (e.g. chelating agents for Fe or Cu ions, nutrient solubilization molecules as organic acids, or extracellular lytic enzymes as phosphatases). Moreover beneficiary microorganisms can enhance plant defense through molecular signals (e.g. proteins, glycans and lipids) known as microbe-associated molecular patterns (MAMPs) and pathogen-associated molecular patterns (PAMPs) that activate innate immune responses, protecting the host from infection. The associated microbiota, including the two major host colonizers, induce metabolic changes to the host that alter rhizodeposition by the plant roots. As a result the microbial community assembly in the rhizosphere and in the root endosphere is affected and modified in a feedback loop. (3) **Microbe—microbe interactions within the legume host:** In parallel with plant host—microbe interaction, microbe—microbe interactions take place in the rhizosphere and root endosphere. Cooperative interactions include resource interdependencies (e.g. for specific nutrients or vitamins), the production of molecules that can aid biofilm formation (e.g. saccharides), facilitation of cell movement (e.g. bacterial movement on fungal hyphae called ‘fungal highway’), cell-cell quorum sensing and production of VOC molecules that enhance co-existence. Antagonistic interactions include production of antibiotic or toxic compounds, development of predatory behavior, and competition for available resources and nutrients (e.g. production of siderophores). (4) **Legume host—N cycle interaction:** The best-studied case of the effects of the legume-symbiont system on soil functional microbial guilds refers to the N-cycle. Rhizobial symbiosis is very sensitive to both ammonium and nitrate levels in the soil (4a) while AM fungi are known to compete for ammonium with the nitrifying community (4b). AM fungi have also been found to affect anaerobic denitrifying communities by increasing oxygen levels at the rhizosphere (4c). More detailed information is presented in BOX 3. (5) **Legume host—other plants interaction:** The legume host interacts with neighbouring plants via its symbiotic and associated microbes, rhizobia and AMF in particular. Legume hosts exploit dinitrogen fixation, and increases the nitrogen levels in the soil via rhizodeposition of N-rich compounds that benefit the neighbour plants. Legume plants also directly communicate with neighboring plants by use of their AM fungal external hyphal network. Through that network, they exchange synergetic or antagonistic signals that shape the aboveground plant community. They have been shown to alert neighbour plants for enemies with defense signals, enhance neighbour plant performance, or alert for abiotic stresses, communicating by use of allelochemicals, secondary metabolites or VOCs. At the same time allelochemicals, secondary metabolites or VOCs can act as antagonist molecules to undermine other neighbour plant's performance and reproduction.

Holobiont: The plant and its associated microbiota (the phytobiome), considered as a functional entity.

Mobilome: All mobile genetic elements (mainly plasmids, prophages and transposable elements) of the microbiome.

Core microbiome: The group of microbes commonly found within a host's microbiome. Its particular functional importance, either as a group of microbes or as a gathering of individual parts, remains a hypothesis under investigation.

Rhizosphere: The narrow region of soil that is directly influenced by root secretions and by associated soil microorganisms known as the root microbiome.

Rhizoplane: The external surface of roots together with closely adhering soil particles microbial cells biofilm material and debris. It corresponds to the inner limit of the rhizosphere and it is technically easier to isolate it from bulk soil compared to the rhizosphere.

Rhizodeposition: material lost from plant roots, including exudates, secretions of insoluble materials, lysates, dead root cells and fine roots, and gases such as CO₂ and ethylene. It critically shapes the biotic and abiotic plant root environment.

THE LEGUME SYMBIOSIS MODEL SYSTEM: FROM BI-PARTITE TO MULTI- PARTITE SYMBIOSIS

Legumes are recognized as pioneer plants due to their capacity to initiate nutrient cycling in non-vegetated poor soils via their symbioses with microbes (Graham and Vance 2003). They have a distinctive capacity to form two fundamentally important associations with microbes, producing the root nodule symbiosis and the arbuscular mycorrhiza symbiosis (Parniske 2008), along with a wide range of other endophytic associations (Ikeda et al. 2010). This, together with the availability of well-established legume model-plants, renders legumes excellent biological material for studying different interactions that occur at the same time on the same plant and its rhizosphere.

The legume—rhizobium symbiosis

The symbiosis of legumes with the soil bacteria *rhizobia* represents one of the most celebrated mutualistic plant-microbe interactions, because of its contribution to the sustainability of agricultural systems and to human nutrition (Peoples et al. 2009; Foyer et al. 2016; Stagnari et al. 2017). During the symbiosis, the rhizobia fix atmospheric nitrogen which they provide to the plant. The amount of nitrogen fixed can often meet a major part of the plant needs. Moreover, nitrogen is left in the soil, available for the following crops, mainly in the form of non-recalcitrant plant residues rich in N. Therefore, by the application of rotation, intercropping and agroforestry techniques, the legume-rhizobia symbiosis may also improve N acquisition by non-leguminous crops in the agro-ecosystem (Giller and Cadisch 1995; Giller 2001). Interestingly, the reverse was also demonstrated recently, as maize exudates were shown to have a highly positive role on functional nitrogen fixation in intercropped faba beans (Li et al. 2016).

The Symbiotic Nitrogen Fixation (SNF) can partially replace the use of synthetic nitrogen fertilizers, which are expensive for the farmer and their production involves wasteful energy

processes. Moreover, a positive feedback mechanism may be expected following reduction of nitrogen fertilizer inputs, since their long-term application appears to induce predominance of less efficient rhizobial strains in the agroecosystem (Weese et al. 2015). Rhizobial inocula are inexpensive to produce and easy to use, therefore they have been developed and applied worldwide. However, the effects of external rhizobial application on legume's performance differ widely and appear to depend on both environmental limitations and cultivation history (Bruin et al. 2010; van Heerwaarden et al. 2018). The advantages of breeding legumes for N₂ fixation in parallel to developing elite rhizobial inocula, and of rotations vs intercropping for grain legumes in smallholder agriculture in Africa were recently highlighted (Vanlauwe et al. 2019).

The signalling pathway and the genetic factors that control the rhizobial infection as well as the developmental stages and physiology of the mature nodule have been lucidly described (Oldroyd et al. 2011) and recent advances have been presented (see for example Liu et al. 2019). A basic description is given in BOX 1.

BOX1: Basics of the legume - rhizobium symbiosis

Formation of functional nodules, the centres of nitrogen fixation in legume roots, requires two distinct but tightly coordinated developmental processes: infection by rhizobia and nodule organogenesis (Oldroyd and Downie 2008). The symbiotic signalling process is initiated when rhizobia secrete nodulation (Nod) factors (lipo-chito-oligosaccharides, LCOs) upon sensing flavonoids released by the plant root (Redmond et al. 1986). Nod factor receptors, like NFR1 and NFR5 in *Lotus japonicus* (Madsen et al. 2003; Radutoiu et al. 2003), are crucial for the perception of rhizobial Nod factors (NF). A range of transcription factors have been shown to control rhizobial infection (Liu et al. 2019). Rhizobia may produce decorated LCOs (Nod factors) and co-evolution with their receptors in legume plants is probably related to the observed host-rhizobial specificity (Moling and Bisseling 2015). Rhizobia enter into the roots through infection threads that in most cases develop in epidermal root hair cells and progress to inner root tissues (reviewed in (Oldroyd et al. 2011). Following nodule organogenesis, rhizobia within nodule cells get differentiated into bacteroids that fix atmospheric di-nitrogen in exchange for plant carbohydrates.

Since SNF is an energy demanding process, the development of nitrogen fixing root nodules is a dynamic process tightly controlled by the host plant. The systemic feedback control mechanism, called autoregulation of nodulation (AON), ensures adjustment of the number of root nodules to the overall plant growth and development (Pierce and Bauer 1983; Kossalak and Bohloul 1984). Mutant plants defective in this mechanism, like *Lotus japonicus har1* (Krusell et al. 2002) and *tml* (Magori et al. 2009), are unable to control the number of nodules that will be formed and thus, such mutations result in overproduction of nodules. More recently, novel regulatory elements, were described to play a role in AON. For example, the post-transcriptional regulation of the symbiosis suppressor *tml* in host roots (Tsikou et al. 2018) and post-infection modulation of Nod factors by host chitinases (Malolepszy et al. 2018) were shown to control nodule numbers and functionality respectively.

The establishment of the legume-rhizobium symbiosis induces global changes to the plant at the molecular level. Relevant plant transcriptomic responses to rhizobia are available for the model legumes *L. japonicus* (Verdier et al. 2013; Kelly et al. 2018) and *Medicago truncatula* (Benedito et al. 2008; Breakspear et al. 2014). Nodule primordia and mature nitrogen-fixing nodules were found to exhibit large transcriptional reprogramming in *Lotus* (Takanashi et al. 2012; Kelly et al. 2018). Such far reaching molecular changes result in modified phenotype and physiological procedures in the host plant (Desbrosses and Stougaard 2011), which may cause altered interactions with other symbionts and endophytes and changes at the plant-soil interface.

From the viewpoint of evolutionary ecology, open questions remain as rhizobial populations would be expected to evolve towards maximized exploitation of host resources and minimized service costs (Porter and Simms 2014; Gano-Cohen et al. 2020). Plant hosts appear to exercise some control on this. Host sanctions against rhizobial cheaters have been demonstrated (Kiers et al. 2003; Sachs et al. 2010) as well as preferential nodule formation for beneficial strains (Heath and Tiffin 2009), suppression of parasitic strains (Regus et al. 2015) and correlation between symbiotic effectiveness and mean nodule mass (Gano-Cohen et al. 2020). On the other hand, selection for rhizobial cheaters has also been demonstrated (Porter and Simms 2014; Gano-Cohen et al. 2019), and coercive interaction of rhizobia with legume plants has been presented, where rhizobial strains manipulate their host biochemically; they induce hypernodulation via rhizobitoxine production and thus exploit carbohydrates at the cost of plant growth (Ratcliff and Denison 2009). Moreover, stabilizing mechanisms that inhibit low-quality partners from proliferating, coupled to maintenance of variation, essential for pursuing partner quality, have not been clearly exhibited (Heath and Stinchcombe 2014).

The legume—AM fungi symbiosis

The association formed between plants and fungi of the phylum Glomeromycota, leading to the establishment of the arbuscular mycorrhizal (AM) symbiosis, is the most ancient and widespread (Corradi and Bonfante 2012; Rimington et al. 2018). AM symbiosis plays a critical role in nutrient acquisition, by providing access to phosphorus in particular, but also to nitrogen and other mineral nutrients. AM fungi were found to possess high-affinity transporters of inorganic phosphate (Pi) (Harrison and Buuren 1995), which accumulates as polyphosphate and is then rapidly translocated to the host plant (Hijikata et al. 2010). Nitrogen is also taken up by AM fungi and genes involved in the transfer of ammonium and aminoacids have been identified (López-Pedrosa et al. 2006; Cappellazzo et al. 2008). In addition to nutrition, AM symbiosis benefits the plant by enhancing tolerance to abiotic stress via a range of mechanisms (Smith et al. 2010; Porcel, Aroca and Ruiz-Lozano 2012; Bárzana et al. 2014; López-Ráez 2016), as well as disease resistance, mainly via induced systemic resistance responses (Bedini et al. 2018), and pest control (Schausberger et al. 2012). Interestingly, it was recently shown, that apart of sugars, AM fungi depend on their host plants for lipid supply (Keymer and Gutjahr 2018) suggesting long-lasting co-evolution between AM fungi and their host plants.

The establishment of legume—AM fungi symbiosis and the relevant signaling processes have been extensively reviewed (see for example Parniske 2008 and Sun et al. 2015 respectively). A basic description is given in BOX 2.

BOX2: Basics of the legume – AMF symbiosis

By contrast to root nodule formation, needed for legume – rhizobia symbiosis, the association with arbuscular mycorrhiza fungi (AMF) does not lead to the formation of new plant organs. Entry into the root is achieved through fungal appressoria that develop on the plant epidermal cell surface (Nagahashi and Douds 1997). Subsequently, the symbiotic organelles of the AM symbiosis, the arbuscules, are developed. They are highly-branched exchange structures formed within the root cortex and surrounded by the invagination of the host cell plasmalemma (Bonfante and Genre 2010). Arbuscules have a short lifetime, then they collapse, and the plant cell returns to its original state and can be re-colonized by a new arbuscule (reviewed by Gutjahr and Parniske 2017).

The chemical communication between AM fungi and the plant involves: (1) strigolactones released by the plant root (Akiyama, Matsuzaki and Hayashi 2005) and regulated by the plant in order to control colonization (Müller et al. 2019), and (2) a mixture of the signaling molecules, chitoooligosaccharides (COs) and lipochitoooligosaccharides (LCOs), produced (in turn) by the AM fungi called the mycorrhization (Myc) factors" (Maillet et al. 2011; Genre et al. 2013) which were recently shown to act in synergy (Feng et al. 2019). These molecules are structurally similar to the Nod factors in legumes but their receptor complexes are comparatively less well defined. Interestingly, a mycorrhizal LCO receptor is likely the ancestor of rhizobial LCO receptors in legumes (De Mita et al. 2014).

A shared symbiotic signal transduction pathway, carried out by a set of conserved plant proteins, has been shown to operate for both AMF and rhizobia, called common symbiotic pathway (Genre and Russo 2016). It is shown to act downstream of both fungal and rhizobial signal perception, and it appears to be differentiated depending on the molecular and cellular context in order to activate distinct sets of downstream responses.

Converging evidence suggests that the legume-rhizobia symbiosis evolved from the much more ancient symbiotic association of plants with AMF (Parniske 2008; Markmann and Parniske 2009). This theory is supported by the findings that components of the SNF signaling pathway are also required for mycorrhizal signaling (Oldroyd and Downie 2004). Several genes that are required for both the AM symbiosis and the root-nodule symbiosis with rhizobia have been identified in legumes (Kistner et al. 2005), indicating that AMF and rhizobia can activate a common symbiosis signaling pathway (see Genre and Russo 2016 or Lace and Ott 2018 for reviews on the subject). Regarding the regulation of the AMF root colonization by the host plant, a mechanism similar to AON has been put forward, called autoregulation of mycorrhiza or AOM (Meixner et al. 2005), and was shown to share common elements with AON (Wang, Reid and Foo 2018; Müller et al. 2019). However, much less is still understood about the AOM mechanism and key components remain to be identified.

At a transcriptomic level, RNA-seq analysis identified 3641 genes differentially expressed during AM development in the roots of the model legume *L. japonicus*, while 275 genes were co-regulated in both AM and rhizobial symbioses (Handa et al. 2015). Most of the differentially expressed genes in *Lotus*-AM symbiosis were up-regulated and many of those encode for secreted

proteins and transporters like phosphate, ammonium, nitrate and potassium transporters, aquaporins, peptide transporters and cation exchangers (Handa et al. 2015). The expression of such genes is in line with current experimental reports, and denotes changes at the plant-soil interface, the soil physico-chemical properties, and the root and rhizosphere properties and microbial composition in the presence of AM fungi.

Regarding the dynamics of the legume-AMF symbiosis in evolutionary terms, reciprocal rewards by both partners in the form of carbohydrates and nutrient transfer respectively, have been presented and put forward as a mechanism to control cheaters (Kiers et al. 2011).

Rhizobia—AMF tripartite interactions in legume roots

Legumes can form tripartite symbiotic associations with rhizobia and AM fungi, which may improve both nitrogen (N) and phosphorus (P) use efficiency respectively, and enhance plant growth and yield (presented as type 1 interactions in Fig. 1). Synergistic effects between rhizobia and AMF on plant performance have been reported for many legumes, like the model legume *M. truncatula* (Afkhani and Stinchcombe 2016; Kafle et al. 2019) and soybean (Wang et al. 2011), while spectacular increase in seedling establishment and productivity was shown for four grassland legumes (van der Heijden et al. 2016). Co-inoculation studies have indicated that, when simultaneously present, AM fungi and rhizobia affect one another, but in a context dependent way (i.e. depending on the conditions or experimental setup). Different effects of co-inoculation have been reported for different strains and crops. For example, inoculation with *Rhizobium leguminosarum* and a mixture of four AMF species significantly increased the number and mass of nodules, nitrogenase activity, and also the level of mycorrhizal colonization of faba beans grown in an alkaline soil (Abd-Alla et al. 2014). However, in the prairie legume *A. canescens*, AMF and rhizobia responses were each influenced by the other, but not in the same direction: Inoculation with AMF increased nodule number and mass, while inoculation with rhizobia decreased AMF colonization of roots (Larimer, Clay and Bever 2014). Positive effects of AMF on biological nitrogen fixation have often been linked to improved phosphorous acquisition provided by the fungus (Püschel et al. 2017). The interactions between AMF and rhizobial symbionts may partly explain the shortfalls of using single legume-microbe effectiveness indicators to make inferences directly applicable to the agronomic level. To achieve the later, symbiotic effectiveness evaluation must encompass a broad range of traits, including drought tolerance, phosphorus use efficiency and disease resistance (Hohmann and Messmer 2017), but also multi-symbiont complementarity, a trait that emerges as the *sine qua non* for agronomic relevance in legume research. This is a great challenge, and the 'one size fits all' would be the wrong way to approach it regarding exploitation of symbiosis in legumes. Applied research for customized solutions under local conditions is needed, that should also take social and economic constraints under consideration. This discussion goes beyond the scope of this review, but the reader is referred to the concept of the 'socio-ecological niche' and the 'from best bet to best fit' approach, discussed by Ojiem et al. (2006) and Vanlauwe et al. (2019) respectively, in their effort to achieve sustainable integration of legumes in smallholder agroecosystems in Africa.

We still lack a rigorous understanding of how AM fungi and rhizobia will influence each other, although their interaction with their common host during co-inoculations is now witnessed at the molecular level (Afkhani and Stinchcombe 2016).

Interesting observations on gene expression were made on *M. truncatula* plants co-inoculated with AM and rhizobia. RNA-seq analysis showed that 70% of the differentially expressed host genes were affected by the fungi, revealing a strong effect of mycorrhizal fungi on the expression of plant genes, whereas only 10% of the differentially expressed genes were affected by both AM and rhizobia. Moreover, the direction of expression of some genes was even reversed in co-inoculated plants compared to the expression observed in single inoculations, while no significant interaction effects were observed on the expression of the common symbiosis pathway (Afkhani and Stinchcombe 2016). However, as pointed out by the authors, further work in a finer scale of root topology, stage of mutualism and environmental conditions is needed, before concluding dominance of endomycorrhizal effects on host gene expression. In a study on gene co-expression networks performed on the same system (Palakurty, Stinchcombe and Afkhani 2018), it was observed that inoculation with AM fungi changed the co-expression of certain plant genes significantly, but the presence of rhizobia reversed co-expression patterns to that observed without the fungi. The presence of rhizobia caused significant changes in mycorrhizal gene co-expression, indicating that a third-party mutualist can cause major rewiring of another microbe's molecular network (Palakurty, Stinchcombe and Afkhani 2018). In all, the detection of the molecular mechanisms underlying the combined responses of the host may eventually provide a basis to decipher the dynamics of this tripartite system (presented as type 1 interactions in Fig. 1). Rather than indicating that rhizobia and AMF act independently these new insights point towards the need to identify critical gene expression modifications linked to characteristics of agronomic relevance. We elaborate on new tools and methodologies for achieving this goal in the last section of this review.

At the ecophysiology level, multiple mutualists are theoretically expected to show synergistic beneficial effects on their host when their roles are functionally distinct and complementary. However, they may lead to negative effects on their shared host, if they compete for the same reward (Afkhani, Rudgers and Stachowicz 2014). In legumes, the mainstream hypothesis is that the autoregulation of nodulation (AON) and of mycorrhization (AOM), recruited by the host plant to control the extent of symbiosis and to prevent potentially parasitic relationships from developing, reflects the plant's need to limit the carbon costs associated with these symbiotic interactions. There are indications that carbon acts as an important regulator for these symbiotic interactions with the plant partner and may determine the extent of symbioses, under environmental constraints. It was recently shown that plants allocated more carbon to rhizobia under low N conditions, whereas, plants that were supplied with N allocated proportionally more C to the AM fungal partner (Kafle et al. 2019). It was, also, shown that under light limiting growth conditions (a treatment that aims to put photosynthesis under stress), double inoculations of the lima bean by both AMF and rhizobial symbionts resulted in additional strain on plant fitness (Ballhorn et al. 2016). A clear decline in colonization of *M. truncatula* by AMF alone was, also, shown under low light intensities (Konvalinková et al. 2015).

Nevertheless, there is evidence that competition for carbohydrates is not the main regulatory mechanism of the tripartite symbiosis: Following an extensive metadata analysis Kaschuk et al. (2009) showed that in a range of experimental cases of combined inoculations, the rate of photosynthesis increased substantially more than the C costs of the rhizobial and AM symbioses, due to sink stimulation of photosynthesis. In line with

the above, the application of Nod factors in a split-root system of *Medicago sativa* systemically suppressed both nodulation and colonization by AMF, but did not influence the allocation of ^{14}C (Catford et al. 2003). In the same study, it was shown that not only Nod factors and existing nodules may suppress AMF colonization, but also that pre-colonization of roots with AMF may systemically inhibit nodule formation (Catford et al. 2003). Similarly, increased nodulation resulted in reduced AMF colonization and spore forming, although this was only observed under controlled environmental conditions in the greenhouse and not in natural populations in the field, highlighting the importance of context-dependent phenotypes (Ossler et al. 2015). These results suggest a putative overlap between AON and AOM. More early evidence for the existence of common elements in these regulatory mechanisms comes from the study of AON mutants, which display not only hyper-nodulation but also hyper-mycorrhizal colonization (Morandi et al. 2000; Shrihari et al. 2000). Future studies on putative links and overlaps between AON and AOM in parallel to agronomic evaluation under specific environmental conditions (both biotic and abiotic) will contribute to our better understanding of how plants choose and control their microsymbionts and how one partner influences the other and the tripartite system as a whole (type 1 interactions in Fig. 1).

Overall, it is clear that (1) applied research on the two major symbionts of legumes, rhizobia and AMF, should be interlinked and (2) It should encompass breeding for multi-symbiont legume hosts and growth under abiotic and biotic stresses in order to exploit performance of symbiont-legume combinations at the agroecosystem level. The later, together with consideration of socio-economical limitations when introducing new technologies and practices, is of paramount importance for meeting the challenges of smallholder agriculture and for expanding to marginal or reclaimed agricultural land.

Multiple microbial interactions in legume roots

Besides studies on co-inoculation with rhizobia and AMF, the effects of co-inoculation with other endophytes have also been reported. For example, co-inoculation with rhizobia and *Bacillus thuringiensis* strain KR₁, a plant-growth-promoting bacterium, resulted in increased numbers of nodules and total biomass compared to rhizobial inoculation alone, in pea and lentil (Mishra et al. 2009). Co-inoculations with *B. japonicum* and three *Bacillus* strains, which were isolated from inside the nodules, also resulted in enhanced nodulation and growth in soybean under low temperature conditions (Bai, Zhou and Smith 2003). Similarly, in peanut, co-inoculations with *Bradyrhizobium* and nodule-associated species of *Pseudomonas*, *Klebsiella* and *Enterobacter* increased the shoot dry weight and the number of nodules (Ibáñez et al. 2009), while more recently *Paenibacillus polymyxa* and *Bacillus megaterium* strains, isolated from common bean nodules, showed synergistic effects with rhizobia on bean growth (Korir et al. 2017). Indeed, the existence of bacterial endophytes inside nodules has been reported for many legumes (reviewed in Martínez-Hidalgo and Hirsch 2017), and co-inoculation experiments with rhizobia suggest that a range of nodule-associated endophytes have growth-promoting properties and could be safe and efficient partners. The finding that the plant growth-promoting actinobacterium *Micromonospora lupini* co-localizes with rhizobia inside the same nodule cell suggests a putative tripartite interaction, but it is not yet known if the presence of the rhizobium is necessary for the entrance of the actinobacterium in the nodule tissues (Benito et al. 2017).

Little is known about the mechanisms controlling the endophytic infection by bacteria. It was shown, however, that infection threads initiated by *Mesorhizobium loti* (the natural symbiont of *L. japonicus*) can selectively guide endophytic bacteria towards nodule primordia. Interestingly, the endophytic nodule infection *per se* was found to depend on functional and efficient *M. loti*-driven Nod factor signaling (Zgadzaj et al. 2016).

A tripartite symbiotic association between legumes, rhizobia and endophytic fungi was recently described. Inoculation with the endophytic fungus *Phomopsis liquidambaris* resulted in enhanced nodulation and N_2 fixation in peanut plants (Xie et al. 2019), an effect attributed to root exudate changes caused by the fungus that led to decreased rhizosphere soil nitrate. Moreover, *L. japonicus* and *M. truncatula* were recently reported to serve as hosts for the endophytic, non-pathogenic, *Fusarium solani* strain K (FsK) which apparently employs at least partly similar signaling pathways to rhizobia and AM fungi (Skiada et al. 2019, 2020). It was also shown that perception of chitin oligomers produced by fungal pathogens are perceived by lysin-motif (LysM)-receptor-like kinases in legume roots, similar to the receptors of Nod factors produced by rhizobia (Bozsoki et al. 2017).

It is becoming clear that evolution, and potentially co-evolution derived complexity of plant–microbe interactions makes it often hard to distinguish who is driving whom in multiple microbial interactions in legume roots, an indication that multiple control and feedback mechanisms operate (Fig. 1). For instance, a *Pseudomonas fluorescence* strain, initially shown to preferentially proliferate in the roots and rhizosphere of mycorrhizal legumes (Viollet et al. 2011), was recently shown to promote legume mycorrhization (Viollet et al. 2017).

SYMBIOSIS-LINKED SHIFTS IN THE MICROBIAL COMMUNITIES OF THE ROOT AND THE RHIZOSPHERE

Plant symbionts can influence the whole phytobiome. Antagonistic/synergistic interactions between legume microsymbionts, plant gene expression reprogramming and direct or plant mediated changes in the physico-chemical characteristics of the rhizosphere may be involved. Studies analyzing the effect of the presence/absence of symbionts on the microbial phytobiome (including legumes and non-legumes) were recently reviewed and summarized by Uroz, Courty and Oger (2019). They used the term ‘symbiosis cascade’ in order to denote the large and multistep joint-impact of the plant and its microbial symbionts on the microbial phytobiome (type 2, 3 and 4 interactions in Fig. 1). In the rhizosphere, this is often a rhizodeposition mediated outcome. A conservatively estimate is that between 10–20% of net photosynthetically fixed carbon is transferred to the rhizosphere, through root exudation (Lynch and Whipps 1990; Kuzyakov and Domanski 2000; Cheng and Gershenson 2007; Nguyen 2009). Root exudates contribute to the synthesis of the microbial community in the rhizosphere (Broeckling et al. 2008; Badri and Vivanco 2009; Tian et al. 2020), while in parallel symbiotic associations dynamically alter the composition of root exudates. Jones, Hodge and Kuzyakov (2004) for example reviewed differences in rhizodeposition between AMF inoculated and non-inoculated plants. These differences have been linked to a range of effects that AM colonization induces like a reduction in membrane permeability in the roots and carbon allocation redistribution, and they appear to include reduction in exudation of sugars altered release of amino acids and increased release of phenolics. However, as recently pointed out

by Canarini *et al.*, (2019) we still lack good understanding on how AMF affect root exudation. Despite improved understanding of resource mediated microbial dynamics in the rhizosphere, reports on the effects of symbionts on rhizodeposition under field conditions are still scarce. This is potentially due to the technical difficulties in discriminating changes in rhizodeposition from other dynamic processes occurring in parallel, as microbial metabolism responses, sorption processes, and their feedbacks.

Symbiont-linked shifts in microbial communities in legume roots and rhizosphere are expected to contribute to legume growth and ecological fitness. Studies on non-nodulated, wild-type nodulated, and hyper-nodulated soybean genotypes showed a correlation of the endophytic microbial community structure with the extent of nodulation (Ikeda *et al.* 2008; Okubo *et al.* 2009). In the same line, a community profiling analysis of *L. japonicus* wild type and mutants impaired in root nodule symbiosis, showed that several bacterial orders were almost entirely depleted from the mutant roots, and that rhizobium symbiosis is needed for the establishment of distinctive bacterial communities in the root and rhizosphere, including members of Burkholderiales, Flavobacteriales and Rhizobiales (Zgadzaj *et al.* 2016). The establishment of AM fungi symbiosis in roots has also been reported to cause changes in the respective bacterial communities. For example, the structures of bacterial communities in *M. truncatula* roots and the rhizosphere soil, were found to differ significantly between the AMF-colonized wild-type plants and an AM-symbiosis deficient mutant (Offre *et al.* 2007).

A recent community profiling study suggests that shifts in microbial communities may be regulated by the induced symbiotic signaling pathways in the host plants (Thiergart *et al.* 2019). This study used *L. japonicus* plants growing in natural soil to show that key host genes needed for root nodule and AM symbioses determine the composition of bacterial and fungal communities, respectively, in plant roots and rhizosphere. *n symRK-3* and *ccamk-13* mutant plants in particular the simultaneous impairment of root nodule and AM symbioses resulted in dramatic changes in the composition of both bacterial and fungal communities (Thiergart *et al.* 2019). It was recently shown that genes of the common symbiosis pathway of rhizobia and AMF may be shared by a wider range of symbionts (Skiada *et al.* 2020), indicating a direct role for this pathway in determining root associated microbiota. The significant impact of the CCaMK gene on the diversity of root-associated bacteria observed in rice (Ikeda *et al.* 2011) is also in line with this. It should be noted, however, that as we move from single interactions to community level effects, distinguishing signals from cues is becoming increasingly challenging.

Microbially driven soil processes have been related to microbial community composition (Balser and Firestone 2005). Links of functional bio-processes of rhizosphere soil with marker genes and specific microbial taxa do emerge as genomic and metagenomic data on soil microbiomes accumulate, particularly marker genes related to N-cycle processes like nitrification (Philippot *et al.* 2013; Fierer 2017). The links of legume symbionts with microbial processes of the nitrogen cycle (type 4 interactions in Fig. 1) are presented in BOX 3.

BOX3: The case of N cycling microbes - most studied relationship between legume root microbiome and soil functional guilds

The Nitrogen cycle (N-cycle) is one of best studied biogeochemical nutrient cycles due to its importance in ecosystem

primary production. Main processes of agronomic importance within the soil N cycle are: (i) autotrophic nitrification, the conversion of ammonia to nitrate by ammonia-oxidizing bacteria and archaea (AOB and AOA), (ii) denitrification, the subsequent conversion of nitrates to N_2O and N_2 by denitrifying bacteria, and (iii) N_2 -fixation by both symbiotic and free-living soil bacteria.

Legume-rhizobium symbiosis, and the relevant capacity for symbiotic nitrogen fixation, depends on the entire nitrogen turnover in the rhizosphere (the N-cycle) and reciprocally, legumes introduce changes in the respective soil microbial communities and affect N-cycle processes (group 4 interactions in Figure 1). Ecological studies show that legumes, alter the structure of ammonia oxidizing bacteria (AOB) communities in the rhizosphere, enhance potential nitrification (Malchair *et al.* 2010), and positively influence the soil nitrifying and denitrifying enzyme activities (Le Roux *et al.* 2013). Due to the importance of legume-rhizobium symbiosis for legume crop production, inoculation of legumes with rhizobial strains at seed or field level, was historically one of the first agronomic applications of specific microbial inocula. Although microbial inocula are used over many decades, however, only a few studies investigate how these inocula affect the functional soil microbial guilds involved in soil nitrogen cycling and availability. The inoculation of *Medicago sativa* seeds with two contrastingly effective *Sinorhizobium meliloti* strains influenced the abundance of N-related functional genes in the rhizosphere, though plant developmental stage was found to have a greater impact on the gene abundance patterns than the application of the inocula (Babic *et al.* 2008). Effects on microbial guilds of the N-cycle, have also been attributed to legume plant inoculation with non-rhizobial bacterial consortia. For example, the co-inoculation of *Cajanus cajan* seeds with *Bacillus megaterium*, *Pseudomonas fluorescens*, and *Trichoderma harzianum* strains increased nodulation and nitrogen-fixer abundance and in general affected the abundance of N-functional microbial guilds in the rhizosphere (Gupta *et al.* 2012).

In the case of AMF-plant associations, the role of AMF in plant N acquisition had been overlooked for many years. It is, however, now well recognized that AMF not only provide an important route for improved N uptake by the host plant, but they also induce changes in the N cycling processes (reviewed by Veresoglou, Chen and Rillig 2012 and by Hodge and Storer 2015). Veresoglou, Chen and Rillig (2012) suggested several pathways for this, including: changes in substrate availability as that of organic-N, NH_4^+ and NO_3^- ; modification of abiotic soil environment variables as pH, water and O_2 availability; composition and relative abundance shifts on the microbial community; effects on the host plant metabolism; and AMF-linked changes on neighboring plants.

A negative relationship between AMF root colonization and potential nitrification rates was reported in low-fertility soils, where the presence of AM-colonized plants resulted in low potential nitrification rates (Veresoglou *et al.* 2011). This relationship was undetectable under conditions of high fertility, still a moderate shift in the community of ammonia oxidizers was observed in the presence of AM fungi (Veresoglou *et al.* 2019). Under high fertility conditions, AM fungi led to decreased AOA and AOB abundances, while inducing particular changes in the AOA community structure, which seems to be more sensitive than the AOB

community to AM fungi (Chen et al. 2013). Overall, a probable main mechanism underlying the interaction observed between AM fungi and ammonia oxidizers appears to be a competition for soil NH_4^+ .

The decrease in N_2O emissions under AMF inoculation has attracted more interest. Storer et al. (2018) and Teutscherova et al. (2019) applied different experimental strategies and they both proposed that the higher soil N_2O emission observed in the presence of non-inoculated plants was through nitrifier-mediated denitrification. Many AOA and AOB have *nirK* genes and are thus capable of producing N_2O . However, the exact mechanism under which the AMF inoculated plants decreased the emitted N_2O it is not yet clear. It could be the result of substrate competition between AMF and ammonia oxidizers. Additionally, the AMF-plant symbiosis could alter the microbial community by favoring non N_2O producing nitrifiers or N_2O reducing microorganisms. Bender et al. (2014) that also recorded lower N_2O emission from treatments with AMF-inoculated compared to non-inoculated tomatoes, observed negative correlations between AMF root colonization and *nirK* abundances (abundance of NO_2^- reductases), while they also observed positive correlations between AMF root colonization and *nosZ* gene abundance (abundance of N_2O reductases).

On the agronomic level, plant inoculation with beneficial microbial symbiont(s) has been widely used in agriculture to improve productivity. However, little information is available on their non-target effects on microbial communities in the root and rhizosphere and the resulting impact on soil functioning and relevant feedbacks on plant performance. Many reports indicate that the external application of either rhizobial or AMF strains, via soil or seed inoculation, can influence the structure of the indigenous microbial communities (reviewed in Trabelsi and Mhamdi 2013 and Rodríguez-Caballero et al. 2017). Consequently, effects on plant performance, observed after inoculation, could not be directly linked to the inoculum, and might encompass effects on the indigenous microbial populations. The reverse is also observed. For example, Gupta, Bisaria and Sharma (2016) reported stimulatory effects after inoculation with non-symbiotic bioinoculant microorganisms on the indigenous beneficial microbes in the rhizosphere of *Cajanus cajan*, like nitrogen-fixing bacteria and phosphate-solubilizing bacteria.

Changes in host associated microbial composition may, however, have undesirable effects if important native species are lost. A study on microbial communities in *Arabidopsis thaliana* roots provides evidence that the associated bacterial microbiota is essential for plant health and survival because of their negative interactions with root-associated filamentous eukaryotes (Durán et al. 2018), highlighting the risks of disturbing the host-microbiota balance. Recently, Prudent et al. (2020) demonstrated the role of rhizosphere microbial communities' diversity in drought stress recovery and yield production of two pea (*P. sativum* L.) genotypes. Indications for strong competition of an *R. irregularis* inoculum with native AMF species (Symanczik et al. 2015), of non-predictable site specificity of inoculum establishment (Kokkoris et al. 2019) and of negative effects of allochthonous AMF inocula on plant performance

under salinity (Kavroulakis et al. 2020) were recently presented. In the light of a rapidly developing market for microbial inocula, a comprehensive evaluation of the effects of the artificially-introduced microorganisms on the indigenous microbial populations is urgently needed.

Moreover, cultivated plants have already undergone a long process of domestication. To that end, the need to investigate changes in rhizosphere microbiome assembly and functions during domestication, and to reinstall beneficial plant-microbe associations was highlighted (Pérez-Jaramillo, Mendes and Raaijmakers 2016). This is however, challenging since other system parameters, as plant phenotype, also change throughout domestication. For example, differences in rhizosphere microbiome composition during domestication in *Phaseolus vulgaris* have been recently linked to differences in specific root length (Pérez-Jaramillo et al. 2017). Additionally, certain domestication practices, as breeding legumes for pathogen resistance were shown to result in collateral advantages, as rhizosphere microbiomes were enriched in plant-beneficial microbes and functions (Mendes et al. 2018, 2019).

RECENT ADVANCES IN MOLECULAR TOOLS AND BIOINFORMATICS, APPLIED IN THE LEGUME-MICROBE-SOIL SYSTEM. TOWARDS AN ECOLOGICAL PERSPECTIVE ON LEGUME-MICROBE INTERACTIONS

Outstanding progress has been achieved regarding the phylogenetics, specificity and physiology of the legume-microbe symbiosis in recent years (Fig. 2). With the aid of molecular tools and mutant plant lines, relevant signaling pathways were elucidated at a chemical/molecular level. However, we have come to realize that the barriers between mutualists, commensals, parasites and even pathogens appear increasingly context- rather than simply taxon-related. Facultative mutualists or opportunistic pathogens are not just peculiar exceptions (Pérez-Brocal, Latorre and Moya 2013). Current research, points towards co-evolutionary processes between multiple mutualists, or pathogens, commensals, and their hosts, and towards partly shared signaling pathways (Oldroyd 2013; Skiada et al. 2020), and microbially secreted effectors (Miwa and Okazaki 2017). The sharing and overlap of distinct, already described symbiosis mechanisms is now unraveled under evolutionary terms (Clear and Hom 2019) and aids us to understand how the plant balances its needs and adapts to new environmental conditions, forming together with its associated microbiomes the so called 'extended plant phenotype' (Cantó et al. 2020).

Studying the dynamics of multiple interactions within the phytobiome system, which extends in the interconnected root, rhizoplane and rhizosphere-soil compartments, is however challenging. Next generation sequencing and -omics techniques may now provide relevant datasets, with unprecedented and rather overwhelming information resolution, in a cost-effective way. In parallel, novel customized bioinformatics and statistical tools are constantly developed and refined, since dataset analysis and interpretation often constitute a bottleneck in the exploitation of these technologies. As a result, microbial ecological network analysis on taxon-proxy datasets, that derive from next generation sequencing of community DNA targeting ribosomal marker genes, is getting widely adopted (Layeghi-

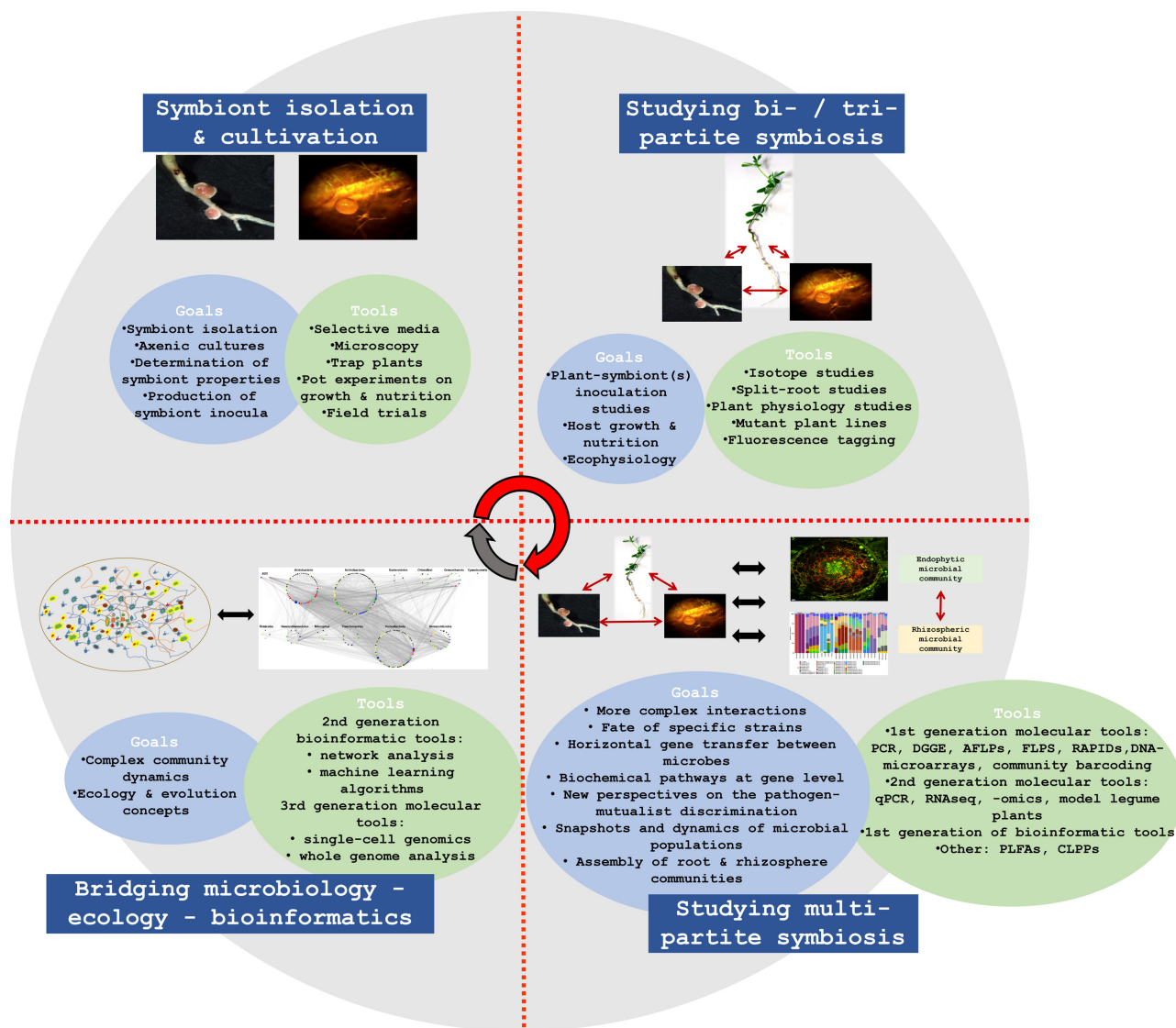


Figure 2. Methodological tools and approaches in studying the legume symbiont—microbiome—soil system. The study of the legume-symbiont interactions started with the successful isolation and cultivation of the rhizobial strains that colonize the legume roots, followed, at a later stage, by the identification and propagation of AMF colonizers. The properties of these symbiotic microorganisms and their effects on host growth and performance were determined, and microbial inocula were produced (A). The development of more advanced tools, like mutant plant lines and sophisticated use of isotopes, led to an advanced understanding of the molecular mechanisms that control specific steps in the legume—symbiont interaction and the ecophysiology of the symbiosis. Besides, we could now shed light on the infection development of the microbial symbiont and evaluate the pros and cons for the plant under specific environmental settings (B). As we moved forward, we entered the era where plants are considered as part of a wider and more complex system that includes all microorganisms that live in close proximity or inside the plant, the phytobiome. First-generation molecular tools, that provide qualitative information regarding the microbial fingerprints of the plant's rhizosphere and endosphere indicated that the establishment of a symbiotic relationship between legumes and microorganisms have further impact on the microbial community structure. With the advent of the second generation molecular tools (the advent of -omics and single-cell genomics) we may get a more detailed qualitative and quantitative view of shifts in the root and rhizosphere microbiomes. RNAseq analysis is a valuable tool in understanding the dynamics of the plant-microbe and microbe-microbe interactions and the effects of abiotic stressors at the gene level while novel metabolic pathways can be discovered and refined by combining proteomic and metabolomic analysis (C). We are now at the 'big data' period. Sequencing data are massively generated at low cost while computer power demands for analyzing these data are getting vastly increased. New more advanced bioinformatics tools are needed, based on the fields of machine learning and artificial intelligence, to extract meaningful information from the generated data-sets. Big data analysis may reveal core microbiomes, rules of microbial community assembly, community level effects and interactions with plants, and taxa or microbial genes of particular importance (D). A significant biological problem arises from the high percentage of proxy microbial taxa, as OTUs, with no cultured representative. To overcome this bottleneck we need to go back to the basics as more effort is needed to enrich microbial culture collections and evaluate the role of specific microbial inocula at the ecosystem level. This is particularly important for symbionts and endophytes.

fard, Hwang and Guttman 2017, 2018). In parallel, shotgun metagenomics approaches that target genes pertinent to functional interpretation, besides relating to taxonomically informative genomic loci (Sharpton 2014), are increasingly applied. For example, Kamutando et al. (2019), showed genes related to the

metabolism of nitrogen, sulphur, carbohydrates, antibiotics and vitamins to be over-represented in the rhizosphere of *Acacia dealbata* and mostly linked to its preferred symbiont *Bradyrhizobium* species, which appear essential for the success of *A. dealbata* as an invasive legume.

The presence of ecologically meaningful properties of mutualistic networks as nestedness and modularity (Fortuna *et al.* 2010) may also be investigated in the networks formed by legume plant communities and their symbionts. Legume-rhizobium interaction networks were shown to be highly modular and not nested, properties that are in line with high rhizobium-host specialization (Le Roux, Mavengere and Ellis 2016). This makes native legume-rhizobia networks rather robust to invading legumes, which appear to need their associated rhizobia in order to succeed; a conclusion remarkably in line with that of Kamutando *et al.* (2019) mentioned above, indicating the potential of amplicon-based and metagenomics-based methodologies to lead to robust deductions of ecological importance in legume-symbiont interactions. Regarding AMF-plant networks, nestedness (Davison *et al.* 2011), as well as both nestedness and high modularity (Montesinos-Navarro, Valiente-Banuet and Verdú 2018) have been shown, a difference attributed to high and low network connectance respectively. Plant communities in these studies did involve legumes, however, to the best of our knowledge, specific studies restricted to legume plant communities alone have not been carried out yet.

As our understanding of the interactions between hosts, microbiomes and abiotic environments grows, the use of synthetic microbial communities (Hartman *et al.* 2017), network theory (Kurtz *et al.* 2015) and systems biology (Rodríguez *et al.* 2019) are among the most promising tools in order to integrate current findings at a community function level, identify key players (hub taxa), functional traits and emergent properties, and also spot missing links and address them (Fig. 2C-D).

Community metatranscriptomics, metabolomics, and metaproteomics techniques also emerge (Jansson and Hofmockel 2018), mainly from the relevant field of human microbiome studies (Mallick *et al.* 2017). In parallel, machine learning methods are used to process the data and assist host microbiome trait prediction (Qu *et al.* 2019; Zhou and Gallins 2019). Their relevance to plant-microbiome systems appears promising: For example, Duvallet *et al.* (2017) recently showed that many bacterial taxa in the gut microbiome are not disease-specific, but rather indicate a shared response to a range of diseases. Identifying such community-level indications in agro-ecosystems, could pioneer a new way to improve plant health.

Targeting genes or taxons should be viewed as rather complementary and as already mentioned, shotgun metagenomic sequencing tools may now combine the two targets (Xu *et al.* 2018). However the high demands in economic cost and analytical skills make their broad use currently hard to implement. A wide range of taxonomically different soil microbiomes shaped by environmental filtering, interspecific competition-mutualism, and stochastic processes, may still accommodate and express similarly functional biochemical pathway toolboxes, despite wide taxon composition differences (Nelson, Martiny and Martiny 2016; Louca *et al.* 2018). It appears that much of the species-level diversity probably reflects functional redundancy (Salles, Le Roux and Poly 2012; Schimel and Schaeffer 2012), but deep evolutionary divergences may be represented by functionally differentiated microbial guilds. Focus in microbial guilds or consortia rather than single taxons may often be the key for unraveling functional microbial relevance (Fierer 2017; Louca *et al.* 2018). On the other hand, despite functional redundancy for a major part of soil microbiota, specific taxons may have unique roles. Still, their higher

taxonomic ranking may point towards shared within rank ecological relevance (Philippot *et al.* 2010), and they may show phylogenetic trait conservatism (the tendency for lineages to retain ancestral functional characteristics through evolution). Symbionts and host associated microbes in particular may have acquired complex co-evolutionary developed traits and need to be identified and their role specifically investigated in relation to their hosts. Moreover, taxon assembly signatures are expected to eventually reveal critical functional information on the host phenotype and the role of plant associated microbial communities, as refined taxonomic and metagenomic data accumulate (Fierer 2017).

CONCLUSION

Starting from the groundbreaking understanding of the legume-rhizobium symbiosis, we have come a long way to now exploit the assembly, dynamics, role and function of the rhizosphere and root microbiota, under different biotic conditions and environmental settings. This is a paradigm change. Roots, rhizosphere and rhizosphere-soil may be viewed as an ecosystem continuum, a landscape characterized by steep trophic and abiotic gradients occupied by microbes, interacting between them and with their host and, at the same time, acting within community clusters as microbial guilds with functional relevance.

In analogy, effects of microbial inocula under agronomically meaningful conditions should be investigated in the context of their combined functions and interactions with each other, and with associated microbial biota, within specific legume/soil habitats.

The big picture has been revealed and we have the tools to acquire unprecedentedly detailed and refined information at both taxon and gene pool levels that describe the relevant bio-communities, and to shed light on their dynamics. Luckily, tailored analytical tools as network analysis and machine learning are gradually employed in order to make sense out of the overwhelming information and complexity derived from these datasets, albeit not always accompanied by relevant theoretical insights (Inkpen *et al.* 2017).

The question of whether functional relevance carries strong phylogenetic signals at a gene, microbe or microbial guild level, or whether mobilome-based genome fluidity, functional redundancy and substitution prevail, is still open and debated. The answer, deeply rooted in evolutionary ecology, is apparently highly context dependent and not resolved in black and white terms.

Indeed, by focusing on accumulating 'context delimited studies' (testing hypotheses or at least comparatively describing legume microbiome relations under specific conditions) and on their meta-analysis, we are starting to distinguish critical from irrelevant characteristics of plant-associated microbiomes, and re-occurring potentially essential taxa, gene families, microbial consortia or core-microbiomes with functional relevance, that determine legume ecological performance. These studies are expected to lead to 'improved context-targeted strategies' at the agronomic level.

The expectation, is to understand the dynamics of the legume-microbe-soil system as a whole. The aim within reach, is to design improved context-targeted strategies for the efficient and environmentally sound use and management of beneficial microbes in legumes and beyond. The safety rule, is to acknowledge that ecology and biotechnology should carry on hand in hand along this journey.

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSEC](https://femsec.oup.com/femsec) online.

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