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**Role of mycelial networks of arbuscular mycorrhizal fungi in mobilizing
symbiotic N-fixing bacteria**

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研余假日，足迹亦尝遍欧陆。既览英法之人文盛景，复赏瑞士之山色湖光；曾沐希腊爱琴海之柔波，亦探东欧捷克波兰之古城遗韵，更于朔方夜空，追寻五彩极光。观人文之璀璨，叹造化之神奇。此番游历，既开阔胸襟，亦启迪思路，于潜心治学之余，亦得人生感悟之乐。

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LIST OF ABBREVIATIONS

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AM fungi	Arbuscular Mycorrhizal Fungi
ATP	Adenosine Triphosphate
cDNA	Complementary Deoxyribonucleic Acid
CFU	Colony-Forming Unit
CMN	Common Mycorrhizal Network
DIP1	DELLA Interacting Protein 1
DMI	Does Not Make Infection
DNA	Deoxyribonucleic Acid
ERM	Extraradical Mycelium
ESI	Electrospray Ionization
HMGR	3-Hydroxy-3-Methylglutaryl-CoA Reductase
MDP	Mycelium Donor Plant
MF	Myc Factor
MS medium	Murashige and Skoog medium
MSR medium	Modified Strullu-Romand medium
NCBI	The National Center for Biotechnology Information
NF	Nod Factor
NPL	Nodulation Pectate Lyase
NSP	Nodulation Signaling Pathway
OTU	Operational Taxonomic Unit
PBS solution	Phosphate-Buffered Saline solution
PCR	Polymerase Chain Reaction
PPF	Photosynthetic Photon Flux
PSB	Phosphate-Solubilizing Bacteria
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RAM1	The Required for Arbuscular Mycorrhization 1
RH	Relative Humidity
Ri T-DNA	Root-Inducing Transfer Deoxyribonucleic Acid
RNA	Ribonucleic Acid
RPG	Rhizobium-Directed Polar Growth
UV	Ultraviolet
YEB	Yeast Extract Broth

GLOSSARY

Arbuscular mycorrhizal fungi: Fungi that belong to the phylum Glomeromycota, forming mutualistic symbioses with approximately 72% of vascular plant species. These fungi evolved over 500 million years ago, during the Ordovician period, and are characterized by the formation of highly branched, tree-like structures called “arbuscules” within the root cortex cells of host plants (Brundrett and Tedersoo, 2018).

Arbuscule: A short-lived, tree-like structure formed by arbuscular mycorrhizal fungi within the root cortical cells of plants. It is the primary site for nutrient exchange, where fungi supply phosphorus and other minerals to the plant in return for carbon energy (Das et al., 2025).

Biofilm: An assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material (Panda and da Silva, 2025).

Chemoattraction: The process by which a cell senses a gradient of chemical signals and moves toward areas of higher concentration. This behavior is driven by receptors on the cell surface that specifically detect these chemical signals. If the gradient is in a soluble form, the movement is referred to as chemotaxis; if it is bound to a surface, it is called “haptotaxis”. Since it is often unclear which mechanism dominates *in vivo*, “chemoattraction” serves as a general term for these directional movements (Rodríguez-Fernández and Cabañas, 2013).

Common mycorrhizal network (CMN): A direct, continuous physical link between plants formed by the mycelium of mycorrhizal fungi. This means that a specific case (involving hyphal continuity) is used to define a much broader phenomenon of hyphae interlinking roots of different plants (Rillig et al., 2024).

Extraradical mycelium (ERM): The network of fungal hyphae developed by arbuscular mycorrhizal fungi in the soil surrounding plant roots. This network extends beyond the root cortex, forming a continuum between the soil and plant, facilitating the uptake, translocation, and transfer of nutrients, ions, and other elements to the host plant (Declerck et al., 2003).

Flavonoids: Flavonoids are polyphenolic secondary metabolites derived from the phenylpropanoid pathway, characterized by a 15-carbon (C6-C3-C6) skeleton comprising two aromatic rings (A and B) linked by a heterocyclic pyran ring (C).

GLOSSARYS

In legume-rhizobia symbiosis, flavonoids serve as critical signaling molecules, inducing the expression of rhizobial nodulation genes (e.g., *nodABC*) via interaction with the NodD transcriptional activator, attracting rhizobia to root surfaces through chemoattraction, and regulating host specificity by ensuring compatibility with specific rhizobial strains. Flavonoids exist as aglycones (e.g., daidzein, genistein) or glycosides (e.g., daidzin, luteolin-7-O-glucoside). Glycosylation involves the enzymatic attachment of sugar moieties (e.g., glucose, galactose, rhamnose) to flavonoid aglycones at positions 3, 7, or 4' by glycosyltransferases (e.g., *GmMaT2*, UGTs), enhancing solubility, stability, and transport within the plant. This process occurs in the cytoplasm, with glycosides often stored in vacuoles. Glycosides are typically hydrolyzed by enzymes like β -glucosidases (e.g., *GmGI*) in the rhizosphere to release active aglycones, which are more effective in activating NodD and initiating symbiotic signaling. Flavonoids also modulate auxin transport to facilitate nodule development, influence quorum sensing in rhizobia, and enhance nutrient acquisition (e.g., phosphorus, iron). They are synthesized via enzymes like chalcone synthase and chalcone isomerase, with isoflavonoids being prominent in legumes (Kotik et al., 2023; Qiu et al., 2024).

Hyphal exudates: Compounds that are actively or passively released into the soil from the fungal hyphae, including sugars, amino acids, carboxylates, polysaccharides, proteins, flavonoids, protons, and other secondary metabolites (Zhang et al., 2022).

Hyphosphere: The hyphosphere is the narrow region of soil immediately surrounding fungal hyphae, representing a critical hotspot for interkingdom interactions between fungi and bacteria. As a key micro-niche within the soil ecosystem, the hyphosphere exhibits distinct physical, chemical, and biochemical properties compared to bulk soil, driven by the influence of hyphal exudates (e.g., enzymes, sugars, and organic acids) and the metabolic activities of hyphae-associated bacteria. These exudates and microbial interactions shape the unique exometabolic chemistry of the hyphosphere, facilitating microbial community restructuring, carbon redistribution, and nutrient cycling (Thomas and Antony-Babu, 2024).

***In silico* study:** A computational approach that utilizes software tools and algorithms to predict and analyze biological interactions and properties, such as the binding affinity between molecules. This method, commonly applied in molecular docking studies, facilitates drug discovery by simulating ligand-receptor interactions and optimizing potential therapeutic compounds. It is cost-

GLOSSARYS

effective, time-saving, and allows for high-throughput screening of molecular targets (Daniel, 2024).

Metabolomics: The large-scale study of all metabolites and low-molecular-weight molecules in a biological sample, such as cells, biofluids, tissues, or organisms. It encompasses a set of techniques and approaches aimed at comprehensively measuring and analyzing these small molecules to understand biological processes and functions (Chin and Slupsky, 2013).

Microbiome: The community of microorganisms, including bacteria, archaea, fungi, protists, and viruses, that inhabit a specific environment. The diversity and functionality of the microbiome are influenced by environmental factors and interactions with the host (Dini-Andreote and van Elsas, 2013).

Myc factors: signaling molecules produced by arbuscular mycorrhizal (AM) fungi, critical for initiating and establishing mutualistic symbiosis with host plants. These molecules, typically with a molecular weight less than 3.5 kDa, consist of two primary types: Myc-LCOs (mycorrhizal lipochitooligosaccharides), which are lipochitooligosaccharides comprising 4-5 N-acetylglucosamine (GlcNAc) units with lipid chain modifications, and Myc-COs (mycorrhizal chitooligosaccharides), which are short-chain chitin oligomers, typically CO4 or CO5, lacking lipid modifications. Myc Factors are perceived by plant LysM receptor-like kinases, such as LYK3 and NFR1 for Myc-LCOs, and *OsCERK1* and *MtLYK9* for Myc-COs, activating the common symbiosis signaling pathway (CSSP). This activation induces nuclear calcium oscillations in root epidermal cells and expression of symbiosis-specific genes, such as early nodulin genes (e.g., *MtENOD11*), even in the absence of direct fungal-root contact. Myc Factors play a pivotal role in mediating fungal recognition and ensuring host compatibility, facilitating the formation of arbuscular structures for nutrient exchange. Structurally distinct from rhizobial Nod factors, Myc Factors elicit specific signaling cascades tailored to AM symbiosis, with variations in perception and response across plant species, including rice, tomato, and *Medicago truncatula*. These differences underscore the adaptability of AM symbiosis to diverse plant hosts (Crosino and Genre, 2022).

Nod factors: A class of lipochitooligosaccharides, signaling molecules produced by rhizobia in response to flavonoids exuded by plants under nitrogen-limited conditions, essential for initiating legume-rhizobia symbiosis by inducing root nodulation and enabling nitrogen fixation (Debellé, 2013).

GLOSSARYS

Nodulation genes: Bacterial genes in rhizobia responsible for synthesizing, modifying, secreting, and regulating lipochitooligosaccharide Nod factors, which serve as signaling molecules to initiate rhizobia-legume symbiosis. These genes induce host plant responses, including root hair curling, cortical cell division, and nodule formation, facilitating nitrogen-fixing symbiosis. Nodulation genes are classified into common nod genes (e.g., *nodA*, *nodB*, *nodC*, for core Nod factor synthesis), host-specific nod genes (e.g., *nodH*, *nodL*, *nodZ*, for structural modifications like sulfation, acetylation, or fucosylation to ensure host compatibility), regulatory nod genes (e.g., *nodD*, for activation in response to plant-derived flavonoids), and secretion-related nod genes (e.g., *nodI*, *nodJ*, for Nod factor transport and secretion) (Ratu et al., 2021; Magne et al., 2024).

Rhizobia: Gram-negative, rod-shaped bacteria (0.5–0.9 µm wide, 1.2–3.0 µm long) capable of motility via flagella. They possess surface lipopolysaccharides and exopolysaccharides essential for host recognition and infection. Rhizobia exhibit phylogenetic diversity, belonging to multiple genera within the Alphaproteobacteria, including *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium* (also known as *Ensifer*), and *Mesorhizobium*, each comprising various species and strains with distinct genomic and symbiotic characteristics. This diversity enables them to form symbiotic relationships with a range of legume species, often displaying host specificity mediated by plant-released flavonoids and rhizobial Nod factors. Within root nodules, they differentiate into nitrogen-fixing bacteroids, enclosed in host-derived symbiosomes, converting atmospheric nitrogen into ammonia to support plant growth. The diversity of rhizobia also encompasses taxonomic variations, symbiotic specificity, genomic structure, and geographic distribution, with certain strains adapted to specific environmental conditions or legume hosts, such as *Bradyrhizobium* being more prevalent in tropical legumes (Chen et al., 2021; Fahde et al., 2023).

SUMMARY

SUMMARY

Legumes rely on nitrogen (N)-fixing bacteria (rhizobia) and arbuscular mycorrhizal (AM) fungi to optimize nutrient acquisition, particularly in nutrient-poor soils. Rhizobia fix atmospheric N₂ by forming root nodules, while AM fungi enhance nutrients uptake (e.g. P and N) and mediate soil microbe interactions. Although rhizobia recruitment is closely linked to the production of flavonoids by plants, this is limited to a few millimeters from the roots. The mechanisms enabling long-distance rhizobial migration and ensuring host-specific recruitment remain poorly understood. Here, we investigated the role of AM fungal extraradical mycelium (ERM) and common mycorrhizal networks (CMNs) in facilitating rhizobial migration and host specific recruitment by legumes.

Using *Medicago truncatula* and *Sinorhizobium meliloti*, we demonstrated that the ERM of *Rhizophagus irregularis* provides a physical pathway for long-distance rhizobial migration. Time-lapse microscopy revealed that the bacterium moved along the surface of the fungal hyphae supported by cytoplasmic flow within the fungal network that can improve the supply of nutrients to the bacterium and signal the presence of a suitable host. Metabolomic analyses identified eight flavonoids unique to ERM connected to *M. truncatula*, which enhanced rhizobial growth and expression of genes involved in nodulation. These findings demonstrate that AM fungal ERM extends rhizobial recruitment beyond the rhizosphere, allowing legumes to attract their symbiotic partners from distant soil niches.

Using *M. truncatula* and *Glycine max* connected together by a common mycorrhizal network of *R. irregularis*, we demonstrated that *S. meliloti* (green fluorescent protein-tagged) and *Bradyrhizobium diazoefficiens* (m-cherry-tagged), migrated towards their specific legume host. Metabolomic profiling revealed distinct flavonoid signals released by each legume species, which guided their respective rhizobia through the CMN. Both *in vitro* and greenhouse experiments confirmed that CMNs not only facilitate rhizobial dispersal but also ensure precise host-specific recruitment, even under competitive conditions.

Our thesis highlighted the dual role of AM fungi in enabling long-distance rhizobial migration and mediating host-specific recruitment through ERM and CMNs. By exploring these mechanisms, we provided novel insights into the molecular and ecological processes underpinning legume-microorganisms interactions. Specifically, our findings demonstrated that AM fungi facilitate host specificity recognition by bacteria by transporting legume-specific flavonoid

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signals through CMNs, which selectively attract compatible rhizobia even in the presence of competing hosts plants and microbial partners. These results pave the way for practical applications of rhizobia and AM fungi in agriculture, in particular by taking advantage of the complementary nature of these two symbionts to improve the efficiency of N₂ fixation in legumes.

INTRODUCTION

In agricultural systems, legumes establish complex symbiotic relationships with two key microbial partners: arbuscular mycorrhizal (AM) fungi and nitrogen-fixing bacteria, known as rhizobia (Saeki, 2011). These interactions are vital for plant nutrition and soil fertility. Rhizobia provide significant amounts of biologically fixed nitrogen (N_2) to agricultural and natural systems – up to 70 million tons per year through root nodule symbiosis (Brockwell et al., 1995) – while AM fungi provide phosphorus and other nutrients to the plant. Both microorganisms thus greatly enhance legume growth, particularly in nutrient-limited soils (Wahbi et al., 2016; Wang et al., 2016b) and form the backbone of legume productivity, particularly in sustainable agricultural practices aimed at reducing chemical fertilizer inputs.

Rhizobia recruitment by legumes is primarily driven by flavonoids secreted into the rhizosphere, which activate rhizobial nodulation (*nod*) gene expression, leading to the production of Nod factors required for root nodule initiation (Zhang et al., 2020). However, this signaling process is limited by distance, as flavonoids are generally perceived only a few millimeters from the root surface (Del Valle et al., 2020). Moreover, the physical properties of soil, such as air-filled gaps between aggregates, and the limited motility of rhizobia in the absence of water flow, further restrict the ability of rhizobia to migrate toward legume roots (Horiuchi et al., 2005; Vicario et al., 2015). While traditional models of rhizobia recruitment focus on mechanisms, localized at the root level, emerging evidence suggest that other soil biotic components, may provide additional pathways for rhizobial dispersal and recruitment over longer distances. For example, a recent study has demonstrated that the facultative biotrophic fungus *Phomopsis liquidambaris* facilitate the migration of rhizobia to legume roots via its hyphae connecting the plant, enhancing nodulation (Zhang et al., 2020).

AM fungi form vast extraradical mycelial (ERM) networks in soil, which have been shown to transport nutrients (Zhang et al., 2018a; Wipf et al., 2019), secondary metabolites (Anckaert et al., 2024; Vieira et al., 2025) and even microorganisms (e.g., *Rahnella aquatilis*) which migrate along fungal hyphae to access nutrient patches, facilitating phosphate mineralization and enhancing nutrient availability (Jiang et al., 2021). This capacity of AM fungal networks presents a potential, yet largely underexplored, avenue for the long-distance migration and selective recruitment of rhizobia. Despite this potential, critical knowledge gaps persist regarding the precise mechanisms by which AM fungi might mediate rhizobial migration, the extent of host specificity in such

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interactions (e.g., discriminating between legumes and non-legumes, or between different legume species), and the specific molecular signals (such as flavonoids) that might be transported by the hyphae to orchestrate these processes.

Therefore, this thesis hypothesizes that AM fungal networks play a multifaceted role in legume-rhizobia interactions, acting as more than just nutrient conduits. Specifically, we propose that these networks can: (1) transport host-derived chemical signals, such as flavonoids, over significant distances, thereby alerting rhizobia to the presence of a compatible host plant; (2) function as physical “highways”, facilitating the long-distance migration of rhizobia through the complex soil matrix to the roots of compatible legumes; and (3) contribute to the host-specific recruitment of rhizobia, enabling them to discriminate between suitable (leguminous) and unsuitable (non-leguminous) hosts, or even between different compatible legume species.

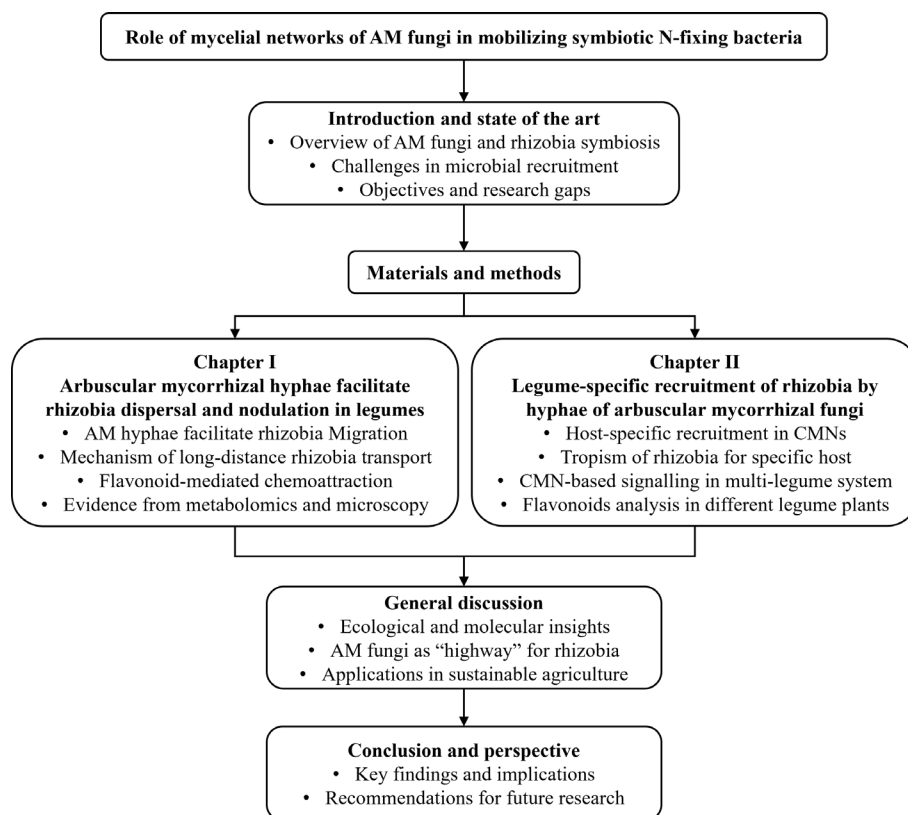
To address the aforementioned research gaps and test these hypotheses, the overall aim of this thesis is to elucidate the role of AM fungal networks in mediating rhizobia recruitment, with a particular focus on long-distance migration, host-specificity, and the underlying signaling mechanisms. This research seeks to fill critical gaps in our understanding of these complex tripartite symbioses. Specifically, the thesis pursued the following four main objectives:

1. To investigate whether and how the extraradical hyphae of the AM fungus *Rhizophagus irregularis* facilitate the long-distance migration of *Sinorhizobium meliloti* and promote nodulation in the model legume species *Medicago truncatula* (Chapter I).
2. To determine if rhizobia recruitment mediated by *R. irregularis* networks exhibits host-plant specificity, particularly by testing whether these networks selectively favor the migration of *S. meliloti* towards its legume host (*M. truncatula*) when compared to non-leguminous plants (*Solanum tuberosum* or *Plantago lanceolata*) (Chapter I).
3. To assess whether common mycorrhizal networks (CMNs) formed by *R. irregularis*, linking two different legume species (*M. truncatula* and *Glycine max*), can mediate the selective recruitment of their respective host-specific rhizobia (*S. meliloti* to *M. truncatula* and *Bradyrhizobium diazoefficiens* to *Glycine max*), thereby examining rhizobial tropism and distribution within such networks (Chapter II).

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4. To identify and characterize flavonoids transported by *R. irregularis* hyphae and released in hyphal exudates, by comparing exudates connected to *M. truncatula* versus *S. tuberosum* (Chapter I) and *M. truncatula* versus *G. max* (Chapter II), to elucidate their role in mediating rhizobial recruitment and host specificity.

The successive steps undertaken to address these objectives are schematized in the thesis framework figure.



This thesis is structured as follows:

This Introduction outlines the overall context of legume-microbe symbioses, identifies the key research gaps concerning the role of AM fungi in rhizobia recruitment, presents the central hypotheses and specific objectives of the research, and provides an overview of the thesis structure.

The **State of the Art** section presents a comprehensive literature review covering the core concepts and recent advancements in the study of AM fungi, rhizobia,

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and their interactions with plants. It details the general characteristics and life cycles of these symbionts, their roles in plant nutrient acquisition, the concept of the hyphosphere as a zone of microbial interactions, the critical role of flavonoids as signaling molecules, the common symbiotic pathway shared by AM fungi and rhizobia, and the synergistic and antagonistic interactions within these tripartite systems.

The **Materials and Methods** section describes the biological materials used, including the AM fungus *Rhizophagus irregularis* MUCL 41833, two fluorescently tagged rhizobial strains (*Sinorhizobium meliloti* and *Bradyrhizobium diazoefficiens*), two legume species (*Medicago truncatula* and *Glycine max*), and two non-legume species (*Solanum tuberosum* and *Plantago lanceolata*). It also provides details on the key experimental setups, such as bi- and quadri-compartmented microcosms, and analytical techniques employed, including UPLC-HRMS for metabolomics and fluorescence microscopy.

Chapter I focuses on investigating the interaction between the extraradical hyphae of AM fungi and N-fixing rhizobia, specifically their roles in facilitating rhizobia migration and initiating legume-rhizobia nodulation. Using *in vitro* and greenhouse mesocosm systems, this chapter demonstrates that AM fungal hyphae provide a preferential route for long-distance rhizobia migration, acting as an indirect pathway to establish nodulation. *In silico* studies further support the hypothesis that flavonoids transported by AM fungal hyphae are crucial in attracting rhizobia and activating symbiosis-related gene expression. The findings of this chapter have been published in *The ISME Journal* (2024).

Chapter II explores whether a CMN linking two different legume species can mediate host-specific rhizobia recruitment and aims to identify the host-specific flavonoids driving this process. Using multi-compartmented systems, this chapter demonstrates strong host-specific tropism by rhizobia within the CMN. Comparative metabolomic analysis of hyphal exudates reveals distinct flavonoid profiles associated with each legume host, supporting the role of AM fungi in transporting and releasing host-specific chemoattractants. The findings of this chapter have been published in *The ISME Journal* (2025).

The **General Discussion** section summarizes and critically discusses the major findings of the thesis. It highlights the dual role of AM fungal hyphae as both physical pathways and chemical mediators for rhizobia recruitment and discusses the implications of host-specificity for legume-based cropping systems.

INTRODUCTION

Finally, the **Conclusions and Perspectives** section reviews the major outcomes of the research and proposes avenues for future studies. These include investigating the influence of environmental factors and different AM fungal species on these interactions, as well as exploring the practical applications of these findings in sustainable agriculture.

By addressing these objectives, this thesis aims to provide novel insights into the mechanisms by which AM fungal networks mediate host-specific interactions in complex plant-microbe systems, thereby advancing our understanding of soil microbial ecology and its potential applications.

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1. Arbuscular mycorrhizal fungi

1.1. Generalities

The majority of terrestrial plants form mutualistic associations, called mycorrhizae, with at least one of the seven types of mycorrhizal fungi. Among these, arbuscular mycorrhizal (AM) fungi, belonging to the phylum Glomeromycota (Schüßler et al., 2001; Brundrett and Tedersoo, 2018), are by far the most important, forming symbiotic associations with at least 72% of vascular plant species (Brundrett and Tedersoo, 2018). These fungi are characterized by their obligate nature (they need to be associated to a compatible plant to accomplish their life cycle) and the formation of arbuscules (**Figure 1a**), which are tree-shaped structures within root cells (Parniske, 2008), involved in the exchanges of nutrients and carbon/lipids (Jiang et al., 2017; Vergara and Araujo, 2024) and in some genus (e.g. *Glomus*, *Rhizophagus*) vesicles (**Figure 1b**) which are storage and reproductive structures (Souza, 2015).

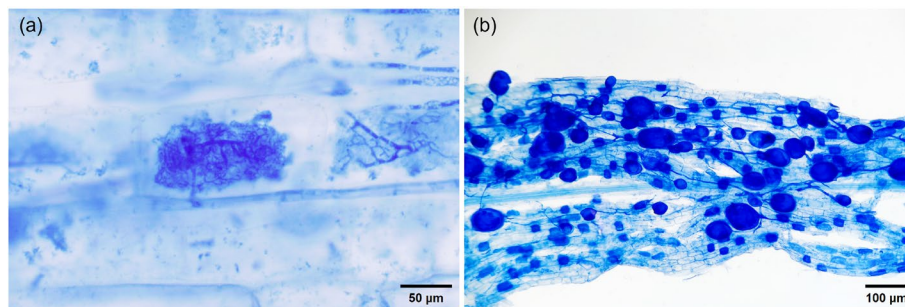


Figure 1. A mature arbuscule **(a)** and vesicles **(b)** of *Rhizophagus irregularis* MUCL 41833.

Kidston and Lang (1921) identified an endophytic structure in the roots of Early Devonian plants through anatomical observations. They observed thick-walled spores at hyphal tips, aseptate hyphae, hyphal coils, and vesicle-like structures resembling those of AM fungi in today's plants. Arbuscules similar to those of modern AM fungi species have also been found in the underground organs of fossil plants dating back 400 million years in the Rhynie Chert formation (Stürmer et al., 2018). Remarkably, fossil evidence indicates that AM fungi may have an even earlier origin, as thick-walled spores resembling those of AM fungi have been discovered in Ordovician sediments, dating back approximately 500 million years (Pirozynski and Dalpe, 1989; Redecker et al., 2000). These spores, along

with associated hyphae, provide strong evidence for the single evolutionary origin of AM fungi. These observations, coupled with the presence of arbuscules in ancient plant roots, support the hypothesis that AM fungi played a critical role in facilitating the transition of plants from aquatic to terrestrial environments (Strullu-Derrien, 2018). The ancient symbiotic relationship likely enabled early plants to acquire essential nutrients in nutrient-poor terrestrial soils, driving the successful colonization of land by plants.

In the association between plants and AM fungi, these later develop hyphae, arbuscules and in some genus vesicles, within plant roots and an extensive hyphal network in the soil. They receive up to 20% of the host plant's photosynthetically fixed carbon (López-Gutiérrez et al., 2008) in exchange for minerals, e.g., nitrogen (N) and phosphorus (P) (Noë and Kiers, 2018; Shi et al., 2023) and water (Xiao et al., 2023). The shift of AM fungi from saprotrophic to symbiotic lifestyles involved genomic adaptations, including the loss of genes for plant cell wall-degrading enzymes (CAZymes), fatty acid synthesis (e.g., absent in *Rhizophagus irregularis* and *Gigaspora* spp.), sucrose hydrolysis, and thiamine synthesis, alongside streamlined secondary metabolism, rendering them obligate biotrophs dependent on host lipids and nutrients (Miyauchi et al., 2020; Duan et al., 2025). Lipids are vital, serving as major carbon storage (triacylglycerols, 40–90% of stored carbon) (Wewer et al., 2014; Jiang et al., 2017), forming nutrient exchange interfaces like peri-arbuscular membranes (Ivanov and Harrison, 2019), enabling carbon transfer (Bravo et al., 2017), signaling development (e.g., hyphal branching, sporulation) (Kameoka et al., 2019), and adapting to stresses like drought or low pH (Feng et al., 2020). Plant fatty acid synthesis genes (*FatM*, *RAM2*) are critical, as their knockout inhibits AM colonization, confirming fungal reliance on host lipids (Bravo et al., 2017; Jiang et al., 2017). This complementary exchange, host lipids for fungal P, and metabolic specialization through gene loss optimizes the mutualistic plant-AM fungal network, enhancing plant nutrient uptake and stress resilience in resource-poor environments.

1.2. Life cycle

The initiation of the AM fungal life cycle begins with the germination of a propagule (generally a spore, a vesicle or a colonized root piece), which can occur independently of a host plant. Although initial growth occurs without a host, the full life cycle is completed only upon successful root colonization.

The AM fungal life cycle is divided into three successive phases (asymbiotic, pre-symbiotic and symbiotic) from the germination of a propagule to the production of new propagules. A detailed examination of each phase is presented below

(considering a spore as propagule), elucidating key events and discussing the signals that influence the successful association between a plant and an AM fungus (**Figure 2**).

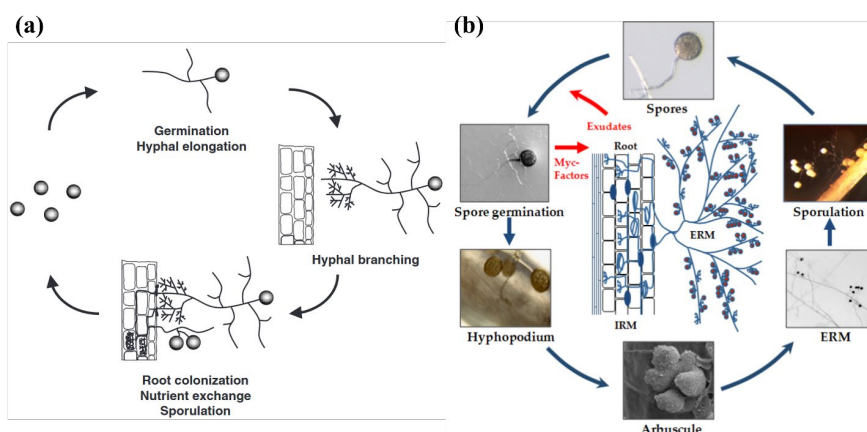


Figure 2. Life cycle of an AM fungus and the different phases during AM development. (a) AM fungal spores germinate independently, but host root-derived strigolactones induce extensive hyphal branching to ensure root contact. Hyphae penetrate the root cortex, forming arbuscules that facilitate nutrient exchange (phosphate for carbohydrates), with fungi relying on hosts due to obligate biotrophy (From Akiyama, 2007). (b) the AM fungal life cycle includes spore germination, hyphopodium formation for root entry, and development of arbuscules or hyphal coils in the cortex. Extraradical mycelium absorbs soil nutrients, intraradical mycelium enables nutrient exchange, and new spores form to complete the cycle (From Bücking et al., 2012). These stages illustrate chemical signaling and infection dynamics in AM symbiosis.

(a) Asymbiotic phase

The asymbiotic phase of AM fungi does not necessitate the presence of a plant. It begins with the germination of a spore under favorable biotic and abiotic conditions, such as suitable soil moisture, pH, temperature, mineral nutrients, and the presence of soil microorganisms (Juge et al., 2002; Bonfante, 2003; Tamasloukht et al., 2003; Bécard et al., 2004; Bianciotto et al., 2004; Bais et al., 2006; Dalpé et al., 2005; Bessier et al., 2006; Verdin et al., 2006; Souza, 2015). These conditions allow the spores to germinate and initiate hyphal growth (Giovannetti, 2000). Spores have sufficient reserves (i.e., lipids) for the development of a germ tube (Dalpé et al., 2005).

During this phase, the volumes of the spore increase slightly due to the absorption of water and nutrients, which also increases the synthesis of RNA, proteins, amino

acids, carbohydrates, organic acids, lipids, and polysaccharides (Siqueira et al., 1985; Spain et al., 2006). This metabolic surge leads to increased nuclear division, vesicles production, and cytoplasmic movement (Bécard et al., 2004). The germ tube protrudes from the spore under the effect of physical pressure exerted on the inside of the spore walls (Oehl and Sieverding, 2004). This stage involves the conversion of lipids, glucose, and fructose into trehalose and amino acids such as arginine and glutamine (Kuga et al., 2008) and into phospholipids (Beilby, 1983).

The asymbiotic mycelium has limited growth (Bécard et al., 2004; Dalpé et al., 2005) and in the absence of a root, undergoes senescence, septum formation, and autolysis (Oehl et al., 2008; Gamper et al., 2009). Conversely, if a connection with plant roots is established, the pre-symbiotic phase is initiated.

(b) Pre-symbiotic phase

The pre-symbiotic phase begins when a germ tube receives signals from a suitable host plant and releases Myc factors (MFs – lipochitooligosaccharides and chitooligosaccharides) in return (Zsögön et al., 2008; Kiriachek et al., 2009). The host plant secretes branching factors mainly strigolactones such as 5-deoxystrigol, which promote the growth of the asymbiotic mycelium towards the root through chemotropism (Buée et al., 2000; Yoneyama et al., 2007; Zsögön et al., 2008). The presence of P also influences this phase depending on the host plant's nutritional status, highlighting the dependency of the pre-symbiotic phase on the host plant (Tawaraya et al., 1998).

Upon the release of strigolactones by the host, the asymbiotic mycelium begins to grow towards the root surface. Strigolactones, when present in high concentrations in the rhizosphere, enhance mitochondrial function which are vital for energizing the mycelium through improved respiration, lipid catabolism, and protein synthesis (Akiyama et al., 2005; Besserer et al., 2006). Proteins such as *Gin1* and *P14-3-3* play significant roles in these processes. *Gin1* interacts with membrane proteins to amplify nuclear signals (Requena et al., 2007), while *P14-3-3* enhances ATPase activity, ion channel function, and increases H⁺ efflux, thus generating substantial energy and creating an electrochemical gradient (Porcel et al., 2006; Ramos et al., 2009).

This electrochemical gradient is crucial for the influx of cations (Ca²⁺, K⁺), glucose, and inorganic P, which facilitate ATP synthesis and the storage of P in vacuoles as polyphosphates, thereby promoting mycelium growth (Requena et al., 2003; Porcel et al., 2006; Ramos et al., 2008, 2009). In response to strigolactones, AM fungal cells produce MFs, initiating hyphopodium formation and

transitioning to the symbiotic phase. MFs production can occur either near the contact point in a DMI (does not make infection—required gene to start symbiotic phase)-dependent manner or away from it through diffusion (DMI-independent) (Tamasloukht et al., 2003; Lanfranco et al., 2005). The DMI-dependent mechanism relies on the common symbiotic signaling pathway (CSSP), involving DMI genes (*DMI1*, *DMI2/SYMRK*, *DMI3*) in legumes, which encode components critical for perceiving fungal Myc factors and initiating calcium spiking at contact points, such as root hairs or epidermal cells (Catoira et al., 2000). Conversely, DMI-independent mechanisms allow residual colonization in mutants lacking functional DMI genes, as observed in tomato *reduced mycorrhizal colonization* (*rmc*) mutants, possibly through diffusion-driven processes or alternative signaling involving GRAS transcription factors like *MtGras4* (Nair and Bhargava, 2012; Hartmann et al., 2019). While the DMI-dependent pathway dominates early infection, the DMI-independent route may support later-stage colonization, though its molecular basis and prevalence across non-legume species remain under investigation.

(c) Symbiotic phase

The symbiotic phase begins when the hyphae emerging from the hyphopodium enters the root via a pre-penetration apparatus, guiding him through the root epidermal cells and colonizing the root cortex (Bücking et al., 2012). This phase is marked by extensive intercellular hyphal growth and the formation of arbuscules, which are highly branched structures surrounded by the plant plasma membrane that serve as the nutrient exchange interface (Genre and Bonfante, 2010). Arbuscules are ephemeral structures, which life span are typically 4–5 days (Bonfante and Genre, 2010).

Within the root cortex, AM fungal colonization develops into two distinct patterns, *Arum*-type and *Paris*-type (**Figure 3**), shaped by the interplay of host plant types, root morphology, and environmental conditions. These patterns are influenced by plant and fungal genetics, particularly the common symbiosis signaling pathway (CSSP) genes like SYMRK and CCaMK, which regulate fungal accommodation, and genes controlling root cortical structure, though specific determinants of each type remain under study (Parniske, 2008; Paszkowski and Gutjahr, 2013). Some plant families or species, such as *Solanum lycopersicum*, form both types of arbuscules depending on fungal partners (e.g., *Rhizophagus irregularis* for *Arum*-type, *Gigaspora margarita* for *Paris*-type) or conditions, reflecting genetic flexibility (Yamato and Iwasaki, 2002; Matekwor Ahulu et al., 2005; Tominaga et al., 2022). Typically, cultivated plants, such as those in Poaceae (e.g., *Zea mays*, *Triticum aestivum*), Solanaceae (e.g., *Solanum tuberosum*), and Asteraceae (e.g.,

Helianthus annuus), display Arum-type colonization, facilitated by well-aerated cortices, while trees and forest herbs, including Aquifoliaceae, Equisetaceae, and Araliaceae, often show Paris-type colonization due to denser cortical structures (Dickson, 2004). Phylogenetic patterns indicate Paris-type is more common across 39 plant families, while Arum-type dominates in 26, with 21 showing both (Smith and Smith, 1997). Agricultural soils, with higher disturbance and nutrient inputs, favor Arum-type colonization by upregulating genes for rapid nutrient exchange, whereas natural soils, with less disturbance and distinct nutrient dynamics, support Paris-type colonization, potentially via epigenetic regulation (Priyadharsini and Muthukumar, 2015). In the Arum-type, the fungus spreads in the root cortex by intercellular hyphae, which extend along well-developed intercellular air spaces, and forms arbuscules in the cortical cells (Smith and Read, 2008). In the Paris-type, the fungus grows intracellularly from one cell to the next within the cortex, forming extensive intracellular hyphal coils, with arbuscules developing intercalary within these coils (Smith and Read, 2008). These patterns have ecological implications: Arum-type enhances nutrient uptake in crops, while Paris-type supports stable nutrient cycling in natural ecosystems (Tominaga et al., 2022).

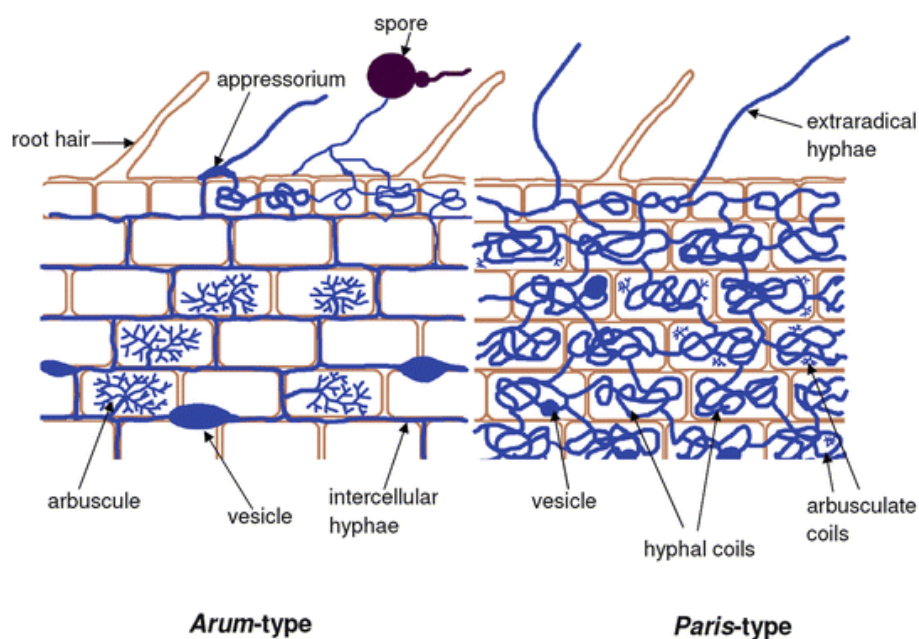


Figure 3. Arbuscular mycorrhizal fungal structures showing *Arum*- and *Paris*-types. *Arum*-type features intercellular hyphae forming intracellular arbuscules, prevalent in Poaceae, Solanaceae, and Asteraceae due to fibrous roots. *Paris*-type involves intracellular hyphal coils with intercalary arbuscules, common in

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Aquifoliaceae, Equisetaceae, and Araliaceae with coarser roots. (From Priyadharsini and Muthukumar, 2015)

Several genera of AM fungi, including *Rhizophagus*, *Glomus*, and *Acaulospora*, form inter- or intracellular vesicles (**Figure 1b**) and spores within the roots, functioning as storage or reproductive structures (Declerck et al., 1998; Smith and Read, 2008). Vesicles are characterized by thin, non-occlusive walls and a balloon-like shape, while intraradical spores are distinguished by their thick, occlusive walls emanating from the subtending hypha (Smith and Read, 2008). Conversely, *Gigaspora* and *Scutellospora* genera do not produce vesicles, and *Acaulospora spp.* do not form spores in roots (Walker et al., 2018).

Following the establishment of the symbiosis, fungal hyphae emerge from the roots and extend into the soil, developing into a dense extraradical mycelium (ERM) network predominantly comprising hyphae and spores. This network plays a crucial role in nutrients uptake and transport to the host root (Bücking et al., 2012). AM fungal spores are formed either individually or in clusters. In certain species, such as *Glomus claviforme*, *Glomus sinuosum*, and *Glomus taiwanensis*, these clusters are enveloped by a hyphal rind, forming a sporocarp, a type of fruiting body (Panna and Highland, 2010). The ERM is coenocytic, exhibiting unlimited growth and developing “branched absorbing structures” on short terminal hyphae which are thought to play a key role in nutrients uptake (Bago et al., 1998).

In species, such as *Gigaspora rosea*, *Cetranspora gilmorei*, *Scutellospora calospora*, and *Dentiscutata reticulata*, the ERM produces auxiliary cells (**Figure 4**), which are clusters of small (approximately 10–20 µm), spiny, knobbed, or smooth structures (Walker et al., 2018). Although the exact functions of auxiliary cells remain unclear, they are hypothesized to act as carbon storage organs or propagules to initiate a novel life cycle (Declerck et al., 2004).

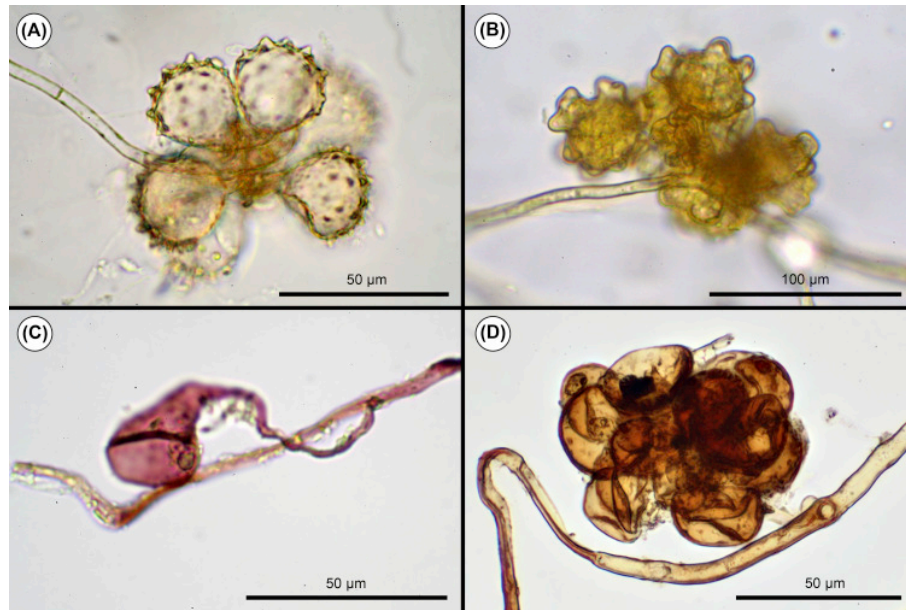


Figure 4. Auxiliary cells. (A) Spiny cells of *Gigaspora rosea*. (B) Knobby cells of *Cetraspora gilmorei*. (C) Single smooth cell of *Scutellospora calospora*. (D) Clustered smooth cells of *Dentiscutata reticulata*. (From Walker et al., 2018)

1.3. Impact of the association between AM fungi and plants on the carbon cycle and plant nutrition

1.3.1. Carbon resources for the AM fungus and soil cycle

AM fungi are obligate symbionts that rely entirely on their host plants for carbon sources, utilizing between 4% and 25% of the photosynthates produced by the plant during photosynthesis (Hobbie, 2006). The primary form of carbon transported from the plant to the AM fungus is sucrose, which is subsequently cleaved into hexose sugars, as AM fungi lack the metabolic capacity to process sucrose directly (Pfeffer et al., 1999; Wipf et al., 2019). This cleavage occurs in the peri-arbuscular membrane, where specific sucrose transporters and monosaccharide transporters facilitate the movement of carbon from root cells to fungal cells (Schüßler et al., 2006; Helber et al., 2011). Once inside fungal cells, hexose sugars provide the necessary energy and carbon skeletons to sustain fungal growth and development of extraradical hyphae. In return, AM fungi play a vital role in improving the nutrient acquisition of their host plants, facilitating the uptake of less mobile soil nutrients like P, zinc, and copper.

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In addition to sugars, recent findings have demonstrated that lipids also serve as critical carbon resources for AM fungi. Unlike plants, AM fungi lack the multi-domain fatty acid synthase complex required for *de novo* lipid synthesis, making them entirely dependent on their host for fatty acids (Wewer et al., 2014; Bravo et al., 2017). During the symbiotic phase, fatty acids synthesized by the plant are transferred to the arbuscules through a highly coordinated process involving lipid biosynthesis enzymes, such as RAM2 and FatM, and transport proteins like STR and STR2 (Wang et al., 2012a; Bravo et al., 2017). These lipids provide an energy-dense source of carbon, supporting the robust growth and metabolic demands of AM fungi, particularly in the development of hyphae and production of spores (Parihar et al., 2020). This lipid-based carbon transfer is hypothesized to explain, at least in part, the obligate biotrophic nature of AM fungi, as lipids provide a more efficient energy source compared to carbohydrates.

AM fungi make a significant contribution to the carbon cycle by introducing large quantities of organic matter into the soil. In particular, extraradical hyphae contribute between 54 and 900 kg·ha⁻¹ of organic carbon to the soil, depending on the ecosystem (Miller et al., 1995). The composition of these hyphae, particularly the presence of recalcitrant chitin in their cell walls, enhances their resistance to microbial decomposition (Zhu and Miller, 2003). The study by Gleixner et al. (2002) indicates that chitin-derived residues can persist in the soil for an average of 49 ± 19 years, thus significantly contributing to the long-term storage of carbon in the soil. In addition to hyphal biomass, AM fungi produce a glycoprotein-like substance (i.e. glomalin) that play an important role in carbon sequestration (Zhu and Miller, 2003). Glomalin also improves soil aggregation by acting as a “biological glue”, stabilizing soil particles into macroaggregates that physically protect organic matter from microbial degradation (Rillig et al., 2003). This stabilization not only slows down the decomposition of carbon compounds, but also encourages their persistence in the soil for long periods, with glomalin-bound soil proteins having residence times ranging from 6 to 42 years (Rillig et al., 2001). The role of AM fungi in carbon dynamics extends to their interactions with other soil organisms. For example, the exudation of carbon compounds by hyphae influence the microbial communities, potentially favoring or inhibiting the decomposition of soil organic matter, depending on the environmental context (Hodge et al., 2001; Cheng et al., 2012).

As a conclusion, by contributing to soil microbial biomass and promoting soil aggregation, AM fungi indirectly regulate carbon fluxes within the soil system, ensuring that carbon is retained in more stable forms, reducing its return to the atmosphere as CO₂. Collectively, these processes underline the multifaceted role of AM fungi in enhancing soil carbon storage and mitigating the effects of climate

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change by supporting long-term carbon sequestration in terrestrial ecosystems (Treseder and Allen, 2000; Rillig, 2004).

1.3.2. Plant nutrition

AM fungi play a crucial role in plant nutrition (Brundrett and Tedersoo, 2018). They assist plants in acquiring essential nutrients, such as P and N, through their extensive extraradical hyphae exploring the soil far beyond the rhizosphere (i.e., the mycorrhizosphere – **Figure 5**) in exchange for sugars and lipids (see above).

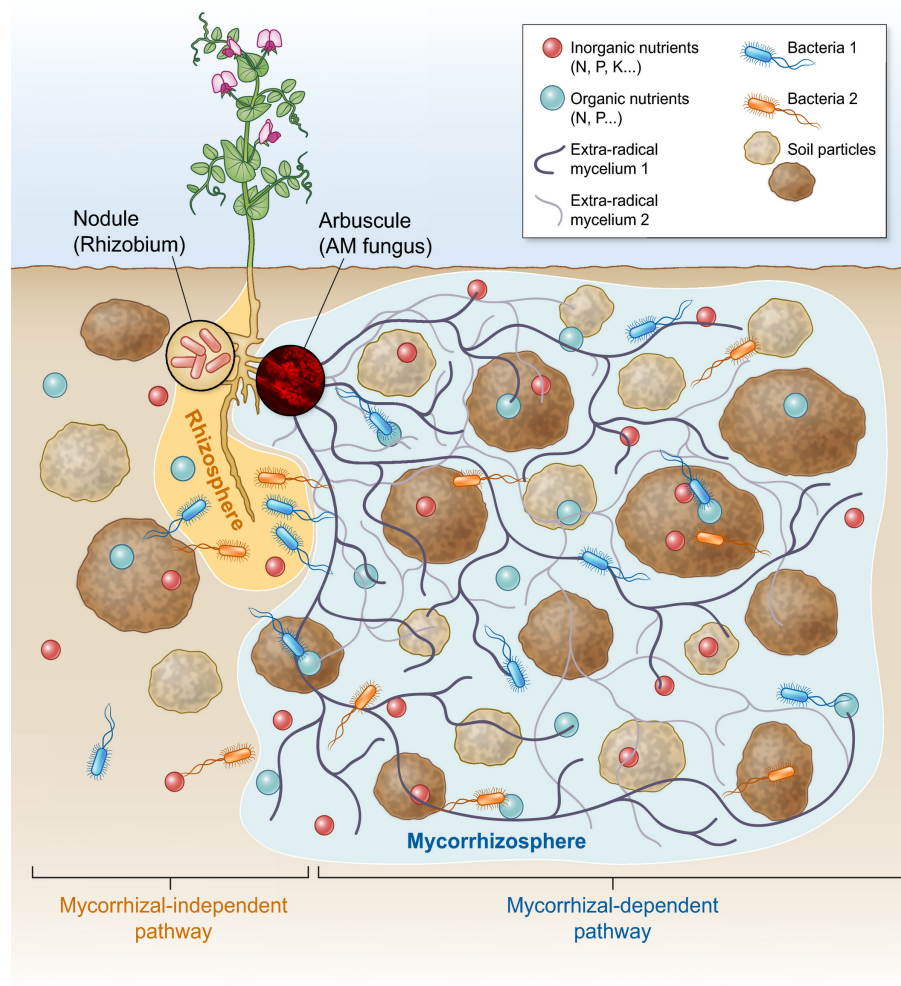


Figure 5. Mycorrhizal-independent pathway versus mycorrhizal-dependent pathway for nutrients uptake and interactions with microflora. The extraradical mycelium 1 and 2 correspond either to two different arbuscular mycorrhizal

fungal species or two isolates from the same species. The extraradical mycelium is an extension of the root system, exploring soil that is not accessible to the root system. Some bacteria interact with the extraradical mycelium to mobilize nutrients. Leguminous plants have two different root symbioses: arbuscular mycorrhizal symbiosis and rhizobia. (From Wipf et al., 2019).

Soil P is generally bound in organic molecules or on mineral surfaces or to poorly soluble salts, making it difficult for plants to access (Rausch and Bucher, 2002). Plants typically absorb inorganic phosphorus (Pi) as orthophosphates from the soil solution through specific phosphate transporter proteins located in their roots. This is named the direct uptake pathway (Wipf et al., 2019). Upon colonization by an AM fungus, a mycorrhizal uptake pathway is initiated, allowing plants to access Pi beyond the depletion zone of roots (Wipf et al., 2019). However, AM fungi lack the capacity to mineralize organic P due to the absence of genes encoding phosphatases or phytases, as revealed by genomic analyses of species such as *R. irregularis* (Tisserant et al., 2013). They rely on microorganisms, such as phosphate-solubilizing bacteria (PSB), living on the surface of their ERM to hydrolyze organic P into inorganic forms, thereby increasing soil organic P turnover (Zhang et al., 2018a). AM fungi may enhance this process by transporting PSB to organic P-rich zones, with the efficacy of this interaction varying across soil types due to differences in pH and mineral composition (Shen et al., 2011; Jiang et al., 2021). While some studies suggest AM fungi may indirectly enhance organic P utilization by stimulating microbial activity (Joner and Jakobsen, 1995), the prevailing view is that they primarily function in the uptake and transport of Pi. Soil pH influences P availability, particularly for Pi, by affecting the solubility of phosphate minerals, such as calcium phosphates in neutral to alkaline soils or aluminum/iron phosphates in acidic soils (Shen et al., 2011). However, acidification alone is insufficient for organic P mineralization, which requires enzymatic hydrolysis by microbial phosphatases (Margalef et al., 2017). AM fungi may secrete organic acids to slightly alter the pH of their hyphal microenvironment, but this primarily enhances Pi solubility rather than organic P mineralization (Bago et al., 2003). The Pi absorbed by the extraradical hyphae is translocated to the intraradical hyphae and then released into the periarbuscular space in arbuscule-containing cells. This transfer is facilitated by specific phosphate transporters such as the Na⁺/Pi transporter *RiPT5*, identified in *Rhizophagus irregularis*, which export Pi from the fungal cytoplasm into the apoplastic space at the arbuscule interface (Garcia et al., 2016). On the plant side, the Pi is taken up through phosphate transporters from the *PHT1* family, such as *MtPT4* in *Medicago truncatula*, located in the periarbuscular membrane. *MtPT4* plays a crucial role in Pi uptake from the periarbuscular space into the plant cortical cells (Javot et al., 2007), a process energized by a proton gradient

generated by H⁺-ATPases. In AM-colonized roots, the plasma membrane H⁺-ATPase gene is co-expressed with these *PHT1* transporters to facilitate Pi uptake (Wipf et al., 2019). This coordinated interplay between fungal and plant transporters underscores the role of AM fungi in delivering Pi, compensating for their inability to mineralize organic P. The proper functioning of these transporters is essential not only for phosphate acquisition but also for maintaining the integrity and lifespan of the arbuscule (Breuillin-Sessoms et al., 2015).

In addition to P, AM fungi considerably improve N acquisition by plants, potentially supplying up to one-third of the protein N in roots (Govindarajulu et al., 2005). The AM fungal hyphae absorb both inorganic N (ammonium and nitrate) and organic N (amino acids and peptides), converting them into arginine for transport to host roots without carbon links (Govindarajulu et al., 2005; Courty et al., 2015). Nitrate uptake is mediated by specific transporters such as those in the NPF, NRT2, and NRT3 families, with GiNT, a high-affinity NRT2 transporter in AM fungi, playing a key role in nitrate acquisition and regulation at the symbiotic interface (Jämtgård et al., 2010; Tian et al., 2010; Koegel et al., 2015). Conversely, ammonium is actively transferred by AM fungi to the periarbuscular space of arbuscules, facilitated by AMT transporters (Kobae et al., 2010; Koegel et al., 2013, 2015, 2017). Among these transporters, *GintAMT1* and *GintAMT2* are crucial for ammonium uptake and retrieval in *R. irregularis*, while *GintAMT3* mediates symbiotic ammonium transfer, a process tightly linked to carbon availability (López-Pedrosa et al., 2006; Pérez-Tienda et al., 2011; Fellbaum et al., 2012; Calabrese et al., 2016). The AM fungi also assimilate organic N from amino acids and small peptides via transporters such as GmosAAP1 and RiPTR2, highlighting their role in symbiotic N transfer and soil nutrient uptake (Jin et al., 2005; Cappellazzo et al., 2008; Casieri et al., 2013; Belmondo et al., 2014).

Beyond nutrient acquisition, AM fungi may enhance plant growth by producing plant hormones. Studies show that *R. irregularis* synthesizes cytokinins (e.g., isopentenyl adenosine), auxins (e.g., indole-3-acetic acid (IAA)), gibberellins (e.g., gibberellin A4), and ethylene, detected in spore exudates via mass spectrometry (Pons et al., 2020). Auxins promote lateral root formation and arbuscule development, increasing colonization sites and nutrient exchange efficiency (Etemadi et al., 2014; Pons et al., 2020). Cytokinins enhance nutrient allocation to colonized roots by stimulating cell division, strengthening the symbiotic sink (Pons et al., 2020). Ethylene, often a negative regulator of mycorrhizal formation, may modulate symbiosis under specific conditions, such as high phosphate levels (Liao et al., 2018). However, genomic analyses suggest AMF lack complete hormone biosynthetic pathways, indicating potential indirect modulation through plant gene regulation or interactions with soil microbes

(Tisserant et al., 2013). AMF also influence stress-related hormones like jasmonic acid, enhancing plant resilience to environmental stresses (Pozo et al., 2015).

The efficiency of nutrient transfer is regulated by internal ammonium and glutamine concentrations, as well as the overall N and P status of both partners, reflecting the intricate interplay between nutrient availability and AM fungal activity (Drechsler et al., 2018). Furthermore, nutrient availability in the soil strongly influences AM fungal colonization and the mutual benefits of the symbiotic relationship. High levels of P and N can suppress AM fungal colonization and reduce nutrient exchange efficiency (Ngwene et al., 2010; Püschel et al., 2017). Conversely, nutrient deficiencies can enhance symbiotic interactions, improving nutrient uptake and promoting plant growth (Bonneau et al., 2013).

1.4. The hyphosphere of AM fungi

1.4.1. Generalities

The hyphosphere is defined as “*the narrow region of soil around the hyphae where the physical, biological and biochemical conditions are different from the bulk soil due to the influence of hyphal exudates*” (Zhang et al., 2022). This zone comprises the endo-hyphosphere (inner hyphosphere) and exo-hyphosphere (outer hyphosphere), with the latter further divided into the hyphoplane (hyphal surface) and ecto-hyphosphere (hyphosphere soil) (Faghihinia et al., 2023 - **Figure 6**). Although these subdivisions exist, it is important to note that they form a continuum without clear demarcation. The hyphosphere is characterized by complex interactions between fungal hyphae, soil minerals (as a reservoir of nutrients), organic compounds, gases, soluble compounds (e.g., ions and small organic molecules derived from hyphal exudates or microbial activity), and active microbial communities. The hyphae of AM fungi release exudates containing various compounds, including sugars and amino acids, as well as AMF-specific compounds such as strigolactones and MFs, which are critical for initiating symbiotic interactions with host plants (Maillet et al., 2011; Genre et al., 2013). These exudates attract and stimulate soil microorganisms such as bacteria and micro-eukaryotes (Jiang et al., 2021; Luthfiana et al., 2021). Notably, the composition of these exudates varies depending on environmental conditions (e.g., P availability) and AMF species, influencing the hyphosphere’s ecological functions (Kikuchi et al., 2014; Emmett et al., 2021). These hyphae provide a high-energy microhabitat for these microorganisms and facilitate their dispersal throughout the soil due to their ability to penetrate soil micropores and forage for

spatially and temporally heterogeneous nutrient resources (Jansa and Hodge, 2021; Jiang et al., 2021).

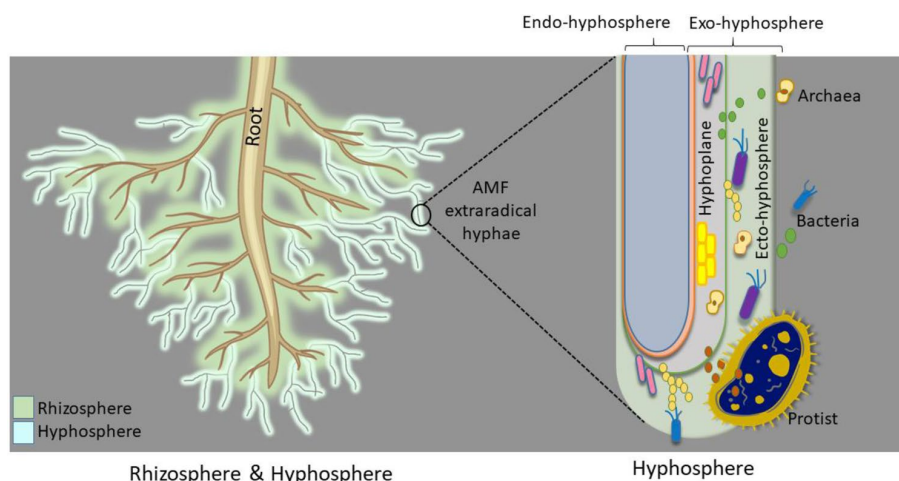


Figure 6. A schematic representation of the arbuscular mycorrhizal fungus hyphosphere, which can be separated into endo-hyphosphere (inner hyphosphere, intracellular space) and exo-hyphosphere (outer or extracellular hyphosphere). Exo-hyphosphere can further be divided into hyphoplane (hyphal surface) and ecto-hyphosphere (i.e., hyphosphere soil) (From Faghihinia et al., 2023)

Scanning electron microscope imaging and experimental studies have shown that some bacterial species (e.g., *Bacillus cereus*, *Bacillus velezensis*, *Paenibacillus brasilensis*, *Pseudomonas fluorescens*, *Rahnella aquatilis*) can firmly attach to the surface of AM fungal hyphae, such as *Rhizophagus intraradices* and *Glomus dussii* (Bianciotto et al., 2001; Artursson and Jansson, 2003; Toljander et al., 2006; Jansa and Hodge, 2021; Anckaert et al., 2024). In addition, some of these bacteria (e.g., *R. aquatilis* and *B. velezensis*) can migrate along the water film formed on the ERM of *R. irregularis*, using them as “fungal highways” (Jiang et al., 2021; Anckaert et al., 2024). This movement is crucial for bacterial ecological competence and competitive success, given the numerous factors that limit their mobility and dispersal in soil (Otto et al., 2017; Jiang et al., 2021; Junier et al., 2021). The fungal highway not only facilitates bacterial movement but also enables contact between prey (e.g., bacteria) and their predators (e.g., protists), thus shaping microbial communities and soil food webs (Otto et al., 2017; Junier et al., 2021). While these observations have been made in some fungal groups using novel tools such as 3D-printed microfluidic devices or controlled microcosm systems, similar studies on AM fungi still require further in-depth exploration (Alekklett et al., 2018; Zengler et al., 2019; Abeyasinghe et al., 2020; Novak et al., 2024).

The AM fungal hyphosphere serves multiple functions in soil processes. It is an energy-rich habitat for microorganisms, facilitates microbial dispersal through the soil matrix, enables contact between prey and predators, selectively recruits beneficial microorganisms, and stimulates bacterial metabolic activities to degrade soil organic matter (Faghihinia et al., 2023). For instance, some microorganisms associated to AM fungal hyphae produce extracellular lytic enzymes that degrade soil organic matter, releasing nutrients that the AM fungi can then exploit for its own needs (Zhang et al., 2018a; Rozmoš et al., 2022). This has been reported, under *in vitro* conditions, with a phosphate solubilizing bacterium (PSB – *R. aquatilis*) dwelling at the surface of hyphae, releasing high amounts of phosphatases to mineralize organic P into inorganic P accessible for the AM fungus, forming a reciprocal interaction that supports mutual nutrient exchange (Zhang et al., 2018a). Furthermore, evidence suggests that AM fungi can alter the microorganism's physicochemical environment by efficiently acquiring and exporting nutrients such as N and P from rich patches and importing fresh carbon into the microorganism's microhabitats (Nuccio et al., 2013; Zhang et al., 2014; Bukovská et al., 2018; Wang et al., 2019). These functions allow the hyphosphere and its associated microbial community to play a disproportionately large role in soil processes relative to their physical size, particularly in nutrient cycling and organic matter decomposition.

1.4.2. Impact of hyphosphere on microbial communities

The hyphosphere of AM fungi hosts a microbiome that differs significantly from the one of the mycorrhizosphere, rhizosphere, and bulk soil (**Figure 7**) (Gahan and Schmalenberger, 2015; Zhang et al., 2018b; Veresoglou et al., 2019; Zhou et al., 2020). A recent study has suggested the existence of a “core AM fungal hyphosphere microbiome” that remains relatively constant across AM fungal species and soil conditions (Emmett et al., 2021). This microbiome is predominantly composed of bacteria both in terms of species diversity and biomass (Bukovská et al., 2021). Four phyla are typically dominating this core microbiome: Pseudomonadota (synonym Proteobacteria), Actinomycetota (or Actinobacteria), Gemmatimonadota, and Bacteroidota (synonym Bacteroidetes). Despite this consistency at the highest taxonomic level, substantial variation exists at genus and species level, possibly reflecting the environmental context or functional redundancy in the interactions between AM fungi and microorganisms.

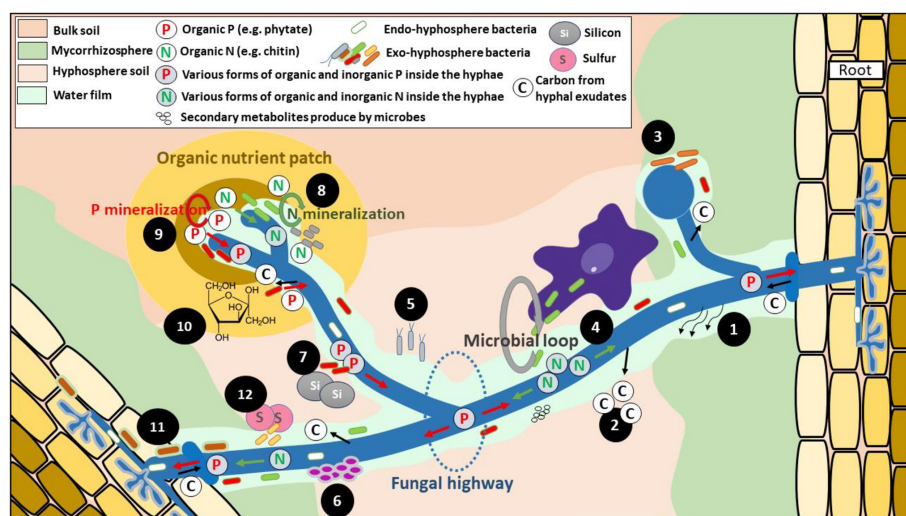


Figure 7. Interactions between AM fungal hyphae and microorganisms in the hyphosphere. Adapted from the review paper of Faghihinia et al. (2023), this figure outlines known and hypothesized interactions within the AM fungal hyphosphere microbiome. The numbered points below describe key processes occurring in this dynamic environment: **(1) Carbon transfer:** AM fungal hyphae deliver plant-derived carbon (e.g., fructose, glucose, trehalose) to soil microorganisms, shaping microbial communities. **(2) Selective enrichment of bacteria:** AM fungi selectively enrich bacterial taxa (e.g., *Bradyrhizobium*, *Pseudomonas*, *Burkholderia*) and other specific groups. **(3) Support for fungal growth:** Bacteria (e.g., *Rhizobiales*, *Bacillales*) aid AM fungal spore germination and hyphal growth. **(4) Nitrogen cycling via protists:** Protists enhance nitrogen release from bacterial biomass, influencing microbial communities. **(5) Bacterial movement:** Motile bacteria migrate along nutrient-enriched AM fungal hyphal networks, often termed “fungal highways”. **(6) Bacterial association with hyphae:** Certain bacteria responds positively to AM fungal hyphae, either attaching to their surfaces or swimming in the surrounding water film. **(7) Silicium-mediated P solubilization:** Silicium-supported bacterial interactions boost P availability for AM fungi. **(8) Organic nitrogen acquisition:** AM fungi acquire organic N with microbial assistance. Such interactions can be cooperative or antagonistic, depending on nutrient patch quality. **(9) Phosphorus mineralization by alkaline P-producing bacteria:** AM fungi promote the enrichment of bacteria with alkaline phosphatase activity, facilitating the mineralization of organic P and increasing P turnover in the hyphosphere. **(10) Fructose-driven phosphorus release:** Fructose exuded by AM fungi serves as both an energy source and a signaling molecule for phosphate-solubilizing bacteria, promoting P release and mineralization. **(11) Bacterial transfer via common mycorrhizal networks (CMNs):** CMNs, which interconnect plants of

the same or different species, enable bacterial transfer between plants. For example, *Bradyrhizobium diazoefficiens*, an N-fixing rhizobial strain, can migrate into legume roots via rhizodermal cracks along AM fungal hyphae. **(12) Sulfur mobilization:** Certain hyphosphere bacteria, including members of *Gammaproteobacteria* and *Actinobacteria*, facilitate the mobilization of organically bound sulfur, enhancing sulfur uptake by AM fungi and plants.

The composition of the hyphosphere microbiome is influenced by various factors, including AM fungal species, soil physico-chemical characteristics, nutrient availability, and plant root exudates, which recruit beneficial microorganisms, enhance nutrient cycling, and modulate microbial activity (Agnolucci et al., 2015; Bukovská et al., 2016; Guyonnet et al., 2018; Vives-Peris et al., 2020; Zhou et al., 2020; Steinauer et al., 2023). Nutrients availability, particularly P and N, plays a crucial role in this process. Changes in P supply can alter the composition of hyphal exudates, influencing bacterial recruitment and microbial community dynamics (Luthfiana et al., 2021). Organic P has been shown to increase bacterial abundance and AM fungal hyphal biomass (Jiang et al., 2021). Nitrogen availability also affects microbial populations, with AM fungal hyphae reducing certain microbial groups, such as ammonia-oxidizing bacteria, through competition for ammonium ions (Nuccio et al., 2013; Bukovská et al., 2018). These interactions, determined by the availability of nutrients, are highly context-dependent and are shaped by environmental conditions and the nutrient status of the hyphae of the AM fungus and host plants. For example, Wang et al. (2019) reported significant changes in the alkaline phosphatase-hosting bacterial community in response to different forms of P, with a higher relative abundance of *Pseudomonas* species in phytin treatments compared to KH_2PO_4 and control treatments.

Different AM fungal species connected to the same plant recruit distinct microbial communities in their hyphosphere, reflecting both specificity and systematic patterns in microbial recruitment. For example, Zhou et al. (2020) demonstrated that in a split-root rhizobox system on cotton (*Gossypium hirsutum* L.), *F. mosseae*, *G. margarita*, and *R. intraradices* recruited different microbial communities. These differences were attributed to variations in hyphal density, ^{13}C abundance, and bacterial OTU richness, and predicted microbial functions, highlighting the selective influence of each fungal species. Such selective recruitment patterns were likely attributed to variations in the composition of hyphal exudates and developmental traits among AM fungal species, as well as to the influence of plant-derived root exudates, which play a crucial role in shaping the microbial community in the hyphosphere (Zhalnina et al., 2018; Luthfiana et al., 2021; Wang et al., 2022).

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Building on this, Emmett et al. (2021) demonstrated that while *R. irregularis* and *G. versiforme* recruited distinct microbial communities, certain bacterial taxa, such as *Betaproteobacteriales* and *Myxococcales*, were consistently enriched across fungal species, suggesting that AM fungi systematically recruit specific microbial groups under the same plant background. Jin et al. (2024) further corroborated this by highlighting that *Streptomyces* species, along with other bacterial taxa, were systematically present in the hyphosphere of different AM fungi under the same plant background. These bacteria not only enhance phosphorus solubilization and organic carbon cycling but also benefit from fungal exudates, creating a mutualistic relationship that supports fungal growth and nutrient acquisition. Together, these findings reveal a unifying pattern in microbial recruitment, where AM fungi selectively shape the hyphosphere microbiome while maintaining consistent enrichment of functionally important bacterial groups.

These studies collectively illustrate that AM fungi influence hyphosphere microbial communities through a dual mechanism: recruiting unique microbial taxa via fungal-specific traits and hyphal exudates, while simultaneously fostering systematic enrichment of certain bacterial groups, such as *Streptomyces*. This interplay between fungal specificity, systematic recruitment, and root-derived exudates highlights the ecological significance of AM fungi in structuring and sustaining hyphosphere microbial communities.

1.4.3. Impact of hyphosphere on interplant communication

The AM fungal hyphae form common mycorrhizal networks (CMNs) that link plants, acting as conduits for the efficient migration of microorganisms, such as plant growth-promoting rhizobacteria (PGPR), across soil gaps (Wang et al., 2022). These networks enhance microbial colonization of new root systems, further improving nutrient acquisition and disease resistance of plants (Jiang et al., 2021; Anckaert et al., 2024). For instance, *B. velezensis* migrates along AM fungal hyphae, such as *R. irregularis*, via chemotaxis, forming biofilms that boost bacterial colonization of the fungal hyphae, contributing to the host plant's systemic resistance against above-ground pathogens (i.e. *Botrytis cinerea*), and protect the hyphae from microbial competitors (Anckaert et al., 2024).

Beyond facilitating microbial dispersal, CMNs facilitate interplant signaling, enabling the transfer of defense signals from the jasmonic acid, salicylic acid, and ethylene pathways during stress events (Johnson and Gilbert, 2015). This signaling activates defense genes and produces secondary metabolites, creating a “warning system” that enhances plant community resilience. Cytoplasmic

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streaming within AM fungal hyphae and water films on the hyphal surface are the primary pathways for signal transmission, complemented by mechanisms like inter-hyphal space diffusion of Ca^{2+} and reactive oxygen species, albeit with limited efficiency (Toyota et al., 2018; Oelmüller, 2019).

CMNs also mediate the dispersal of pathogens (Song et al., 2010; Gilbert and Johnson, 2017), reflecting the ecological significance of CMNs in regulating microbial dynamics and maintaining soil ecosystem balance (Alaux et al., 2020).

In addition, AM fungal hyphae exudates specific molecules, such as carbohydrates (e.g., glucose) and carboxylates, that selectively attract microbial taxa (e.g., *R. aquatilis*), improving nutrient cycling, such as P solubilization and N_2 fixation (Jiang et al., 2021). AM fungi preferentially support plants providing higher photosynthetic carbon, a behavior consistent with the “fair exchange” principle (Babikova et al., 2014; Pickles et al., 2017) and well-explained within the framework of the biological market theory proposed by Noë and Kiers (2018). This theory explains that mutualistic interactions, such as those between AM fungi and plants, are governed by partner choice and dynamic resource allocation strategies. Through this market-like mechanism, AM fungi selectively allocate more resources to plants that supply greater carbon input, thereby optimizing trade for mutual benefit and maintaining the stability of the mutualistic relationship.

Overall, the AM fungal CMN serves as a hub for microbial dispersal and interplant communication, improving nutrients uptake, stress resilience, and community-wide defense. These networks play a critical role in sustaining plant health and ecosystem stability (Wang et al., 2022).

2. Rhizobia

2.1. Generalities

The rhizosphere, the soil zone immediately surrounding plant roots, serves as a dynamic interface for plant-microbe interactions, hosting diverse rhizobacterial communities that profoundly influence plant growth, soil health, and ecosystem functionality. Rhizobacteria encompass a broad range of taxa, including N-fixing bacteria (e.g., *Rhizobium*, *Azotobacter*, *Clostridium*), PGPR (e.g., *Pseudomonas*, *Bacillus*, *Azospirillum*), and PSB (e.g., *Pseudomonas fluorescens*), each contributing distinct mechanisms to enhance plant performance (Glick, 2012; de Andrade et al., 2023a). PGPR are categorized by their functional roles: biofertilizers fix atmospheric nitrogen via nitrogenase or solubilize phosphates

through organic acid secretion; phytostimulators produce phytohormones such as IAA, cytokinins, or gibberellins to stimulate root development; biocontrol agents synthesize antibiotics (e.g., 2,4-diacetylphloroglucinol), lytic enzymes, or volatile organic compounds to suppress pathogens or induce systemic resistance; and stress mitigators employ enzymes like 1-aminocyclopropane-1-carboxylate deaminase to reduce ethylene levels, alleviating abiotic stresses such as drought, salinity, or heavy metal toxicity (Pieterse et al., 2014; Kumar et al., 2024). Recent studies highlight the agricultural potential of PGPR, with strains like *Kosakonia radicincitans* reducing chemical fertilizer use while increasing maize yield under field conditions (de Andrade et al., 2023b). Additionally, rhizobacteria modulate rhizosphere microbial communities, enriching beneficial taxa (e.g., *Bacteroidetes*, *Proteobacteria*) to improve soil structure and nutrient cycling (Bulgarelli et al., 2013; Trivedi et al., 2020). Within this diverse group, rhizobia represent a specialized subset that form N-fixing nodules with legumes, a symbiotic process central to this review and interconnected with AM fungal symbiosis through shared signaling pathways.

Nitrogen is an essential element for all living organisms as it forms a fundamental component of amino acids, proteins, and nucleic acids like DNA. Although atmospheric N₂ constitutes 78% of the atmosphere, most organisms, including plants, cannot directly use it. Only specific microorganisms, such as rhizobia, actinomycetes, cyanobacteria, and some other prokaryotes, can convert atmospheric N₂ into bioavailable forms through biological N₂ fixation. Industrial processes, such as the Haber-Bosch process ($\text{H}_2 + \text{N}_2 \rightarrow \text{NH}_3$), are used to produce synthetic N fertilizers. However, they contribute significantly to global warming because of the high temperature (400–500°C) and pressure (100–200 atm) required for the chemical reactions.

Biological N₂ fixation can be categorized into three types: symbiotic, free-living, and associative. Symbiotic N₂ fixation, particularly the legume-rhizobia symbiosis, is the most emblematic. This mutualistic relationship involves the formation of a specialized structure called a nodule, where the rhizobia fix atmospheric N₂ into ammonia, providing essential nutrients to the host plant, while the plant supplies carbohydrates to the bacteria (Pawlowski and Demchenko, 2012). The classical model plants for this symbiosis are *Lotus japonicus*, which forms determinate nodules with *Mesorhizobium loti*, and *M. truncatula*, which forms indeterminate nodules with *Sinorhizobium meliloti*. Free-living N₂ fixation is performed by independent microorganisms such as *Azotobacter* and *Glucenobacter*, which fix N₂ in the soil without associating to a plant, contributing significantly to N availability in ecosystems (Santi et al., 2013). Associative N₂ fixation occurs when bacteria like *Azospirillum* form loose, non-nodulating

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associations with plant roots, enhancing N_2 availability and promoting plant growth (Kennedy et al., 1997).

The evolution of rhizobium symbiosis is closely associated with ancient AM symbiosis. Both are sharing a conserved symbiotic signaling pathway known as the common symbiotic signaling pathway (Martin et al., 2017). AM symbiosis, which origin dates back approximately 500 million years, during the colonization of land by plants, relies on calcium signaling pathways (e.g., CCaMK) and transcriptional regulators adapted for nutrient exchange (Vigneron et al., 2018). Today, an approximate of 72% of vascular plants form AM symbiosis, though it has been independently and irreversibly lost in some lineages due to factors like nutrient-rich environments and pathogen pressures (Brundrett and Tedersoo, 2018; Vigneron et al., 2018). Nodule symbiosis evolved around 110 million years ago and is limited to the N_2 -fixing clade (FaFaCuRo) within the Rosidae group, which includes 10 families in orders such as the Fabales, Rosales, Cucurbitales, and Fagales (Wang et al., 2009; Bell et al., 2010). In the Fabales, the N_2 -fixing bacteria are rhizobia, while in the other orders, they are actinomycetes (**Figure 8**).

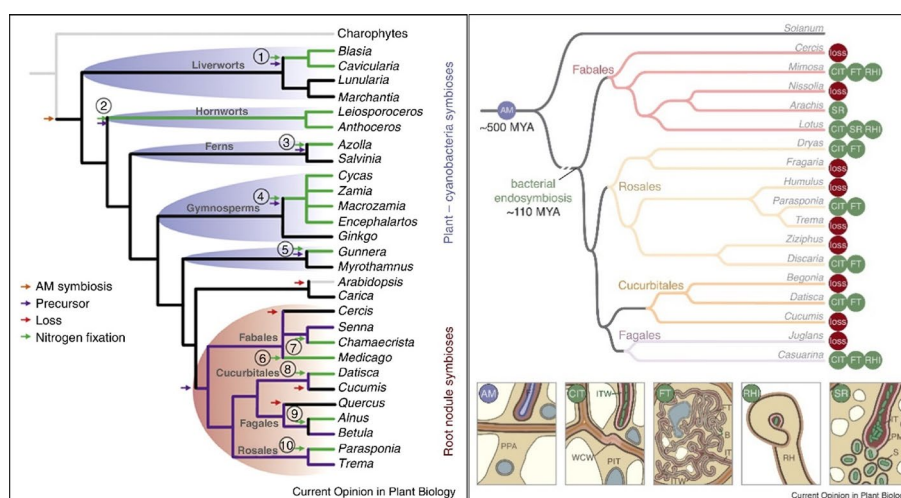


Figure 8. Comparative evolution of AM fungal and nitrogen-fixing symbioses in plants. **(Left)** Phylogenetic distribution of AM fungi and nitrogen-fixing symbioses in land plants. Black branches represent plant clades capable of forming AM fungal symbiosis, while grey branches indicate clades that have lost or never evolved this association. Green branches are nitrogen-fixing clades, with green arrows marking the proposed origins of nitrogen-fixing symbioses. Purple branches highlight predisposed clades with genetic potential for nodulation. Red arrows indicate independent evolutionary losses of the predisposition to form AM symbiosis or nodulation, as observed in *Arabidopsis thaliana* (From Delaux et al.,

2015). **(Right)** Evolution of bacterial uptake mechanisms within the FaFaCuRo clade and related lineages. Key innovations, including cortical cell infection and intracellular bacterial accommodation, are mapped onto a schematic phylogenetic tree. CIT represent the ancestral form, evolving later into RHI and SR in legumes. Diverse infection structures, such as FT and IT, share similar membrane and matrix compositions, suggesting a common evolutionary origin. AM, arbuscular mycorrhiza; PPA, pre-penetration apparatus; PIT, pre-infection thread; IT, infection thread; FT, fixation thread; RHI, root hair infection; SR, symbiosome release into plant cells; CIT, cortical infection thread (From Parniske, 2018).

2.2. Life cycle

The life cycle of *Rhizobium* (**Figure 9**) can be divided into three sequential phases: the asymbiotic, pre-symbiotic, and symbiotic phases.

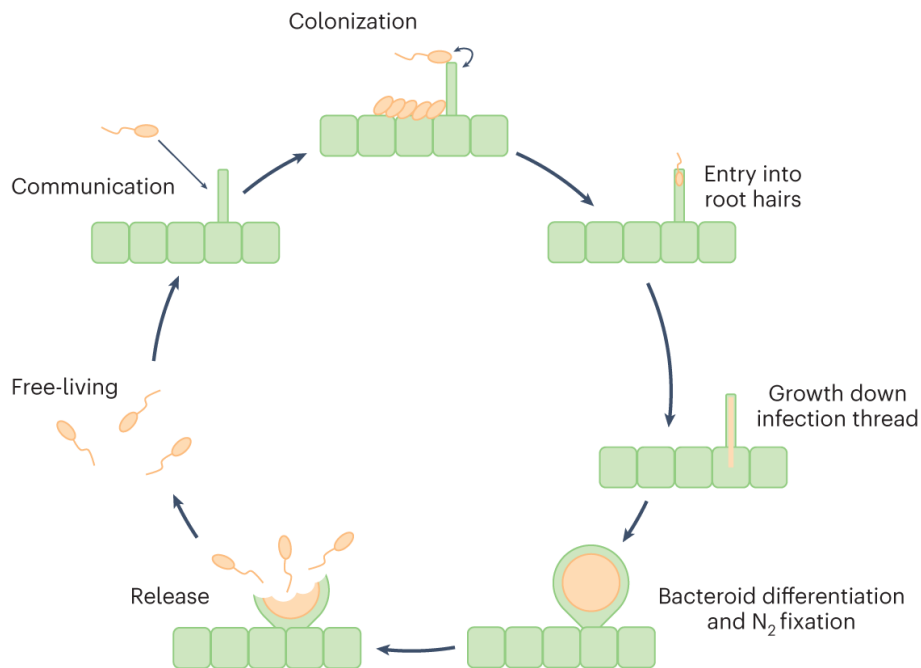


Figure 9. The life cycle of rhizobia. The life cycle of rhizobia illustrates their symbiotic interaction with legume plants, comprising: (1) Free-living: Rhizobia survive in soil, competing with other microbes. (2) Communication: Plants release flavonoids, inducing rhizobia to produce Nod factors for signaling. (3) Colonization: Rhizobia attach to root hairs, causing curling to trap bacteria. (4) Entry into root hairs: Bacteria enter via infection threads. (5) Growth down infection thread: Threads extend into the root cortex. (6) Bacteroid differentiation and N₂ fixation: Rhizobia differentiate into bacteroids within symbiosomes,

fixing nitrogen. (7) Release: Upon nodule senescence, rhizobia are released back into soil, completing the cycle. (From Poole, 2024).

(a) Asymbiotic phase

Rhizobia live in complex soil microenvironments and display remarkable metabolic versatility and genetic plasticity, enabling them to survive in a wide range of conditions (Kajić et al., 2016). They possess sophisticated genetic regulatory mechanisms that allow them to persist as free-living organisms, using a wide range of carbon sources and adapting to fluctuating nutrient availability (Kajić et al., 2016). Their genomic architecture includes multiple replicons, typically a large chromosome and several smaller plasmids, which contribute to their metabolic flexibility and environmental adaptability (Mazur and Koper, 2012). During this asymbiotic phase, the rhizobia maintains active metabolic processes, including carbon metabolism, N assimilation, and stress response mechanisms that prepare them for potential symbiotic interactions (Hawkins and Oresnik, 2022). The bacterial cells actively synthesize various extracellular polymeric substances, develop chemotactic capabilities, and maintain genetic competence, which are critical for their subsequent host recognition and colonization processes (Cangioli et al., 2022). Molecular studies have revealed that even in the free-living state, these bacteria possess latent genetic elements that can be rapidly activated in response to specific plant-derived chemical signals, suggesting an evolutionary optimization for symbiotic potential (Pueppke and Broughton, 1999; Poole et al., 2018).

(b) Pre-Symbiotic Phase

The initiation of the pre-symbiotic phase is characterized by a sophisticated molecular dialogue between rhizobia and the leguminous host plants, featuring a complex chemical communication system that ensures precise symbiotic specificity. The process is initiated by flavonoid compounds exuded by plant roots, activating compatible bacterial species through complex recognition mechanisms (Peck et al., 2006; Subramanian et al., 2007). These plant-derived secondary metabolites interact with bacterial NodD proteins, particularly NodD1, which act as molecular sensors capable of detecting and responding to specific chemical signatures (Peck et al., 2006). Flavonoids bind to NodD proteins in rhizobia, inducing the expression of *nodABC* genes, which are crucial for synthesizing nod factors (NFs) (Redmond et al., 1986; Giraud et al., 2007; Subramanian et al., 2007). These NFs, a class of lipochitooligosaccharides, are essential signaling molecules that initiate symbiosis by triggering further biochemical pathways in both rhizobia and the host legume (Dénarié et al., 1996; Spalink, 2000). NFs have

an oligosaccharide backbone of N-acetyl-D-glucosamine units with a fatty acyl group attached to the non-reducing sugar. A major determinant of host-symbiont specificity is attributed to the different NF substituents attached to the oligosaccharide backbone (Lerouge et al., 1990; Dénarié et al., 1996). NodD1 and NodD2 respond to different types of plant compounds, while NodD3 can activate *nod* gene expression without external inducers, especially under N-limiting conditions (Barnett and Long, 2015). NodD proteins regulate *nod* gene expression by binding to the nod box, ultimately initiating the nodule formation process (Dusha et al., 1999).

This phase represents a critical molecular process, where both bacterial and plant partners engage in a highly discriminative chemical dialogue that ensures the potential for successful symbiotic interaction.

(c) Symbiotic Phase

The symbiotic phase is characterized by a complex series of molecular and cellular events that transform free-living bacteria into symbionts specializing in N₂ fixation. The process begins with root hair infection (Saeki, 2011), a sophisticated mechanism triggered by the recognition of NFs by plant receptor proteins NFR1 and NFR5 (Lévy et al., 2004; Rübsam et al., 2023). This recognition initiates a cascade of cellular responses, including calcium signaling, root hair deformation, and the formation of infection threads (Szczygłowski and Amyot, 2003; Lévy et al., 2004). The infection thread formation involves a dynamic process of bacterial encapsulation within newly deposited cell wall material, creating tubular structures that serve as conduits for bacterial migration through root tissues (Gage, 2004; Gao et al., 2024).

The interaction between NFs and plant receptors triggers mitotic activation of cortical cells, leading to the formation of the nodule primordium (Ferguson et al., 2010). Nodule development emerges as a critical outcome of this symbiotic interaction, manifesting in two distinct morphological types: determinate and indeterminate nodules (**Figure 10**). Determinate nodules, found in species like soybean, develop from ectodermal layer cells and exhibit synchronous bacteroid differentiation with a limited lifespan (Saeki, 2011). Indeterminate nodules, characteristic of alfalfa and clover, originate from endothelial layer cells and possess a persistent meristem, allowing continuous growth and differentiation (Saeki, 2011). The development of nodule primordia is regulated by a complex network of transcription factors, including NSP1 (nodulation signaling pathway 1) and NSP2, and is influenced by hormonal signals, particularly ethylene, that

control the spatial and temporal patterns of cell division (Oldroyd and Long, 2003; Smit et al., 2005; Lohar et al., 2009; Ferguson et al., 2010).

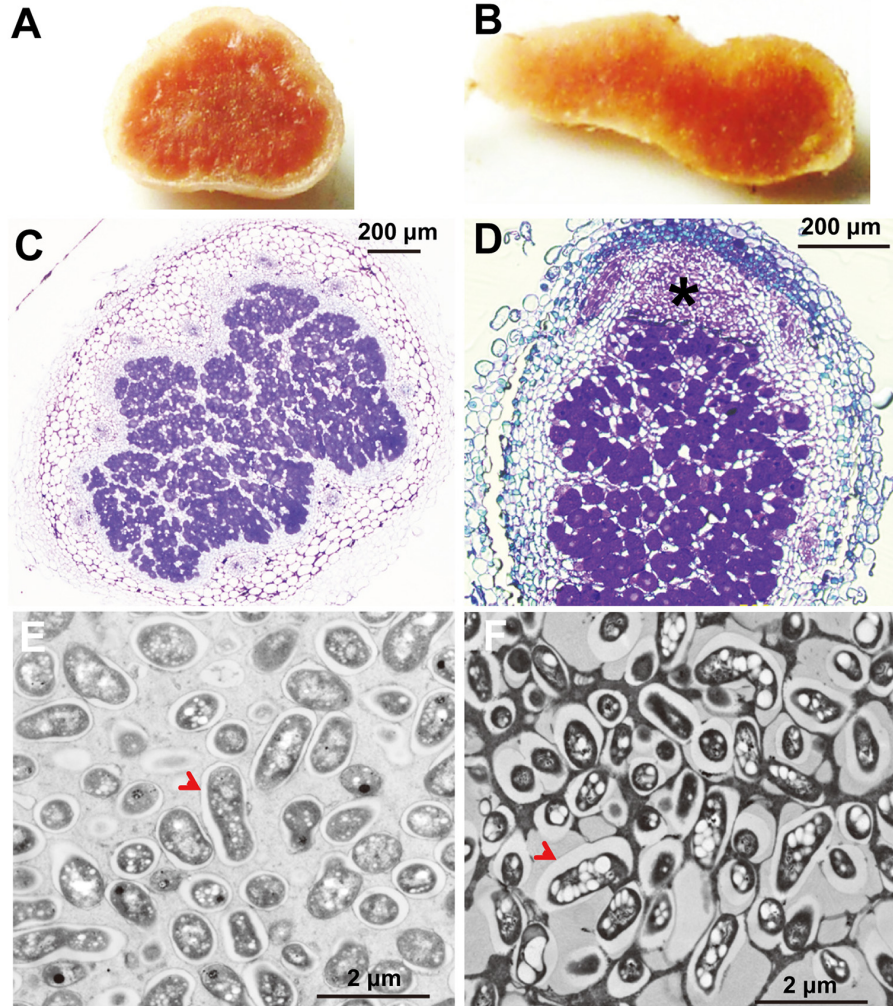


Figure 10. Sections of determinate and indeterminate nodules. N_2 fixing nodules of *Vigna unguiculata* (A) and *Leucaena leucocephala* (B). Semithin sections of nodules from *V. unguiculata* (C) and *L. leucocephala* (D). The asterisk indicates the meristem zone of indeterminate nodules of *L. leucocephala* (D). Ultrathin sections of nodules from *V. unguiculata* (E) and *L. leucocephala* (F), red arrowheads indicate the peribacteroid membrane; and poly-3-hydroxybutyrate granules are seen as electron-transparent droplets in the cytoplasm of bacteroids (From Li et al., 2013).

The formation of symbiosomes represents a pivotal molecular transformation, in which rhizobia are enveloped by plant-derived membranes through a process mediated by genes such as *SYMRK* (Udvardi and Day, 1997). Within the cortical cells, bacteria are released from infection threads and encapsulated by a peribacteroid membrane, initiating their differentiation into bacteroids (Udvardi and Day, 1997). This transformation is regulated by nodule-specific cysteine-rich peptides, which induce terminal differentiation and enhance N₂ fixation efficiency (Ren, 2018).

The ultimate goal of this symbiosis is atmospheric N₂ fixation, achieved through the activation of *nifHDK* genes encoding the nitrogenase enzyme complex (Shen et al., 1989). Bacteroids convert atmospheric N₂ into ammonia, which is then assimilated by plant enzymes such as glutamine synthase and glutamate synthase. The nutrient exchange occurs through sophisticated membrane transport mechanisms, with specialized transporters like *AmtB* facilitating the movement of ammonia, while plant dicarboxylate transporters provide carbon sources to the bacteroids (Day et al., 1998).

2.3. Benefits for plant nutrition

Rhizobia not only provide N to plants through N₂ fixation but also play crucial roles in increasing the availability of other essential nutrients. These bacteria facilitate phosphate solubilization by synthesizing low molecular weight organic acids that convert inorganic P into forms that plants can absorb (Bhattacharyya and Jha, 2012). Effective phosphate solubilizers, such as *Rhizobium leguminosarum* and *S. meliloti*, significantly enhance P availability to plants (Halder et al., 1990; Halder and Chakrabarty, 1993). Additionally, certain microbial enzymes contribute to the mineralization of organic P, further increasing its availability (Richardson and Hadobas, 1997).

Iron, another critical nutrient, is made available to plants through the production of siderophores by rhizobia. These siderophores sequester iron from the soil and facilitate its uptake by plants. Species such as *S. meliloti*, *Rhizobium tropici*, and *Bradyrhizobium* spp. are known to produce siderophores (Antoun et al., 1998; Arora et al., 2001). This ability to enhance iron availability is particularly important in aerobic environments where iron exists as insoluble hydroxides and oxyhydroxides, which are not accessible to plants and microbes (Rajkumar et al., 2010).

Rhizobia also produce phytohormones like indole-3-acetic acid, which promotes root and shoot growth, and cytokinins, gibberellins, and abscisic acid, which

regulate various aspects of plant growth and development (Patten and Glick, 1996; Frankenberger and Arshad, 2020). IAA is involved in cell division, differentiation, and vascular bundle formation and is essential for nodule formation. It has been estimated that 80% of bacteria isolated from the rhizosphere can produce IAA (Patten and Glick, 1996). Co-inoculating *Pseudomonas* with *Rhizobium galegae* bv. *orientalis* have been reported to produce IAA that contributes to increases in nodule number, shoot and root growth, and N content. Both environmental stress factors and genetic factors influence the biosynthesis of IAA (Spaepen et al., 2007; Spaepen and Vanderleyden, 2011).

In summary, rhizobia significantly enhance plant nutrition through phosphate solubilization, siderophore production, phytohormone synthesis, and N₂ fixation. They have the ability to persist in the soil and support plant growth under various conditions.

2.4. Flavonoids

Flavonoids, a diverse class of polyphenolic secondary metabolites, are synthesized via the phenylpropanoid pathway and characterized by a C6-C3-C6 backbone, playing pivotal roles in plant physiology, ecology, and agricultural applications (Dixon and Steele, 1999; Kotik et al., 2023; Qiu et al., 2024). Comprising subclasses such as flavonols (e.g., quercetin), flavanones (e.g., naringenin), isoflavones (e.g., genistein), and anthocyanins, flavonoids are initiated by chalcone synthase (CHS), with subsequent modifications like glycosylation, methylation, and hydroxylation yielding over 10,000 structural variants that modulate solubility, antimicrobial activity, and signaling capacity (Winkel-Shirley, 2001). In plants, flavonoids protect against ultraviolet radiation, scavenge reactive oxygen species to mitigate oxidative stress, contribute to flower pigmentation to attract pollinators, and act as phytoalexins to deter pathogens (Kumar et al., 2024). In the rhizosphere, flavonoids serve as antimicrobial agents or chemoattractants, shaping microbial communities by promoting beneficial taxa (e.g., PGPR like *Bacillus*) or inhibiting pathogens (e.g., *Fusarium* sp.) (de Andrade et al., 2023b). For example, quercetin enhances plant resistance to fungal diseases, reducing reliance on chemical pesticides (Chieb and Gachomo, 2023). Recent research underscores flavonoids' role in modulating rhizosphere microbiomes, with specific structures influencing bacterial chemotaxis and community assembly (Kumar et al., 2024). In plant-microbe interactions, flavonoids act as critical signaling molecules, mediating associations with PGPR, rhizobia, and AM fungi, with their structural specificity and concentration determining interaction efficiency.

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The establishment of symbiosis between rhizobia and legumes involves a sophisticated exchange of signals, often described as a “molecular dialogue” (Dong and Song, 2020). Flavonoids are at the heart of this dialogue. These polyphenols are exuded by legume roots into the rhizosphere, acting as chemoattractants for rhizobia and transcriptional activators for *nod* genes, which are essential for nodule development (Brencic and Winans, 2005; Spini et al., 2016).

Flavonoids are an important group of secondary metabolites synthesized by plants. More than 10,000 different structures have been identified, though their production is not uniform across all plant species (Dixon and Steele, 1999). Farag et al. (2007) identified various flavonoids in *M. truncatula*, indicating that these compounds are typical derivatives of the phenylpropanoid pathway, close to the precursors of lignin. They exhibit variations of a fundamental structure characterized by a C6-C3-C6 skeleton, also known as phenylbenzopyran (**Figure 11**) (Marais et al., 2006). Secondary modifications such as glycosylation, methylation, and hydroxylation considerably influence their functionality, solubility and mobility in the plant and soil environment (Dixon and Steele, 1999; Winkel-Shirley, 2001).

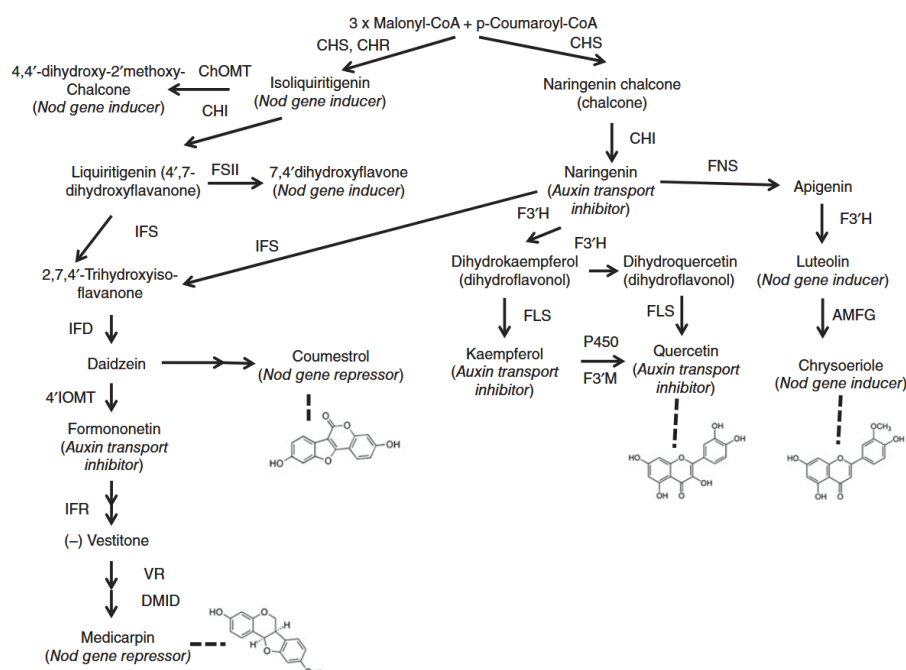


Figure 11. Simplified overview of the flavonoid biosynthesis pathway in *Medicago truncatula* showing bioactive flavonoids. The overview shows the

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major classes of flavonoids that have been reported as *nod* gene activators/repressors or as auxin transport inhibitors during nodulation in *M. truncatula* or *Medicago sativa*. Structures of some of the compounds, including coumestrol (coumestan), medicarpin (pterocarpan), quercetin (flavonol), and chrysoeriol (flavone) are shown. Enzymes are abbreviated as follows: AMFG S-adenosylmethionine:flavonoid 7-O-glucosyltransferase; CHI chalcone isomerase; CHR chalcone reductase; CHS chalcone synthase; ChOMT chalcone-O-methyltransferase; DMID 7,2'-dihydroxy-4'-methoxy-isoflavonol dehydratase; F3'H flavonoid-3'-hydroxylase; FLS flavonol synthase; FNS flavone synthase; FSII Flavone synthase II; IFD 2-hydroxyisoflavanone dehydratase; IFR isoflavone reductase; IFS 2-hydroxyisoflavanone synthase; IOMT isoflavanone-O-methyltransferase; N7OG P450 cytochrome P450; VR vestitone reductase. (From Mathesius, 2020).

In plants, flavonoids play multiple roles, influencing interactions with animals, microorganisms, and the wider environment (Winkel-Shirley, 2001; Taylor and Grotewold, 2005; Naoumkina and Dixon, 2008; Hassan and Mathesius, 2012). They function as UV pigments, contribute to flower coloration attracting pollinators, regulate certain bacterial genes, act as phytoalexins in pathogen responses, and serve as iron chelators in the soil. They also act as chemoattractants or repellents for plant-parasitic nematodes, as developmental regulators, as allelochemicals and as antioxidants (Winkel-Shirley, 2001).

In legumes, flavonoid exudation is mainly limited to the elongating zone of the root hair, where most nodules are formed (Zuanazzi et al., 1998). When flavonoids are released into the rhizosphere, they diffuse into the rhizobial membrane, possibly through porins (Kobayashi et al., 2004; Taylor and Grotewold, 2005; Wang et al., 2012b). Once inside the rhizobia, flavonoids bind to NodD proteins, which are a class of transcriptional activators of the LysR family (Hassan and Mathesius, 2012). This binding activates the NodD proteins, which in turn initiate the transcription of other *nod* genes by binding to conserved 49-bp DNA motifs known as “nod-box” promoters found in the promoter regions of these genes (Gibson et al., 2008). NodD proteins thus act as both sensors of plant signals and transcriptional activators of nodulation loci on symbiotic plasmids (Redmond et al., 1986; Downie, 2010).

The first flavonoids discovered as inducer of a *nod* gene were luteolin, isolated from *Medicago sativa*, and 7,4'-dihydroxyflavone isolated from *Trifolium repens* (Redmond et al., 1986). Since then, approximately 30 *nod* gene-inducing flavonoids, especially flavanones and isoflavonoids, have been identified from various legume species (Limpens and Bisseling, 2009; Gholami et al., 2014).

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These flavonoids are effective as *nod* gene inducers at nanomolar to low micromolar concentrations and stimulate bacterial *nod* gene expression within minutes (Begum et al., 2001; Gholami et al., 2014).

The specificity of flavonoid signaling is partly responsible for the host specificity of the symbiosis. The specific exudation of flavonoid mixtures by legume hosts and the specific perception of these flavonoids by NodD proteins of different rhizobia contribute to this specificity (Gibson et al., 2008; Skorupska et al., 2010; Rose et al., 2012). Point mutations in *nodD* genes can modify flavonoid recognition and broaden the host range (Broughton et al., 2000). Broad host range rhizobia, such as *Rhizobium* sp. NGR234, have NodD proteins that respond to a wider range of flavonoid species, including some that act as inhibitors for other rhizobia, such as vanillin and iso-vanillin (Peck et al., 2006; Wang et al., 2012b).

Different NodD homologues within the same rhizobial strain may have different flavonoid preferences and activation patterns. In *S. meliloti*, for example, NodD1 is activated by a complex extract of plant seeds or by luteolin alone, while NodD2 is only activated by the complex extract and not by purified luteolin. NodD3 modulates the expression of *nod* genes even in the absence of any plant factor (Smit et al., 1992). Structural models of NodD1 have identified residues important for inducer binding, protein multimerization, and interaction with RNA polymerase at *nod* gene promoters, highlighting the complex regulation of these proteins (Peck et al., 2013).

Species-specific flavonoids interact with NodD proteins, which have an N-terminal ligand-binding domain that regulates the activity of the associated C-terminal DNA-binding domain. This interaction is crucial for the initiation of *nod* gene transcription. The perception of flavonoids by rhizobia is linked to an elevation in intracellular calcium concentrations, which subsequently induces NodD proteins for NFs expression (Moscatiello et al., 2010; Hassan and Mathesius, 2012). This process underlines the essential role of flavonoids in activating *nod* genes and facilitating the symbiotic relationship between rhizobia and legumes.

The interaction between flavonoids and NodD proteins is further qualified by the fact that some flavonoids also act as repressors of the *nod* gene in certain rhizobia. For example, isoflavonoids like medicarpin and coumestrol negatively control NF production in *S. meliloti* (Zuanazzi et al., 1998). The dual role of flavonoids in both inducing and inhibiting *nod* gene expression contributes to the specificity and regulation of the symbiotic relationship (Andersen and Markham, 2005).

3. The common symbiotic pathway of AM fungi and *Rhizobia*

3.1. Flavonoids signal

Flavonoids are pivotal in regulating the symbiotic interactions between plants and both AM fungi and rhizobia (**Figure 12**). These secondary metabolites act as crucial signaling molecules in the establishment, maintenance and autoregulation of these symbioses, often modulating the plant's response to its symbiotic partners in a highly specific and concentration-dependent manner. Direct evidence supports their role, particularly in rhizobial symbiosis, where silencing flavonoid biosynthesis genes, such as CHS in *M. truncatula*, significantly inhibits nodule formation, an effect reversible by exogenous application of flavonoids like naringenin or liquiritigenin (Wasson et al., 2006). In AM fungal symbiosis, exogenous application of specific flavonoids, such as chrysin and luteolin, enhances colonization in tomato plants, suggesting a stimulatory role in fungal entry and establishment (Scervino et al., 2007).

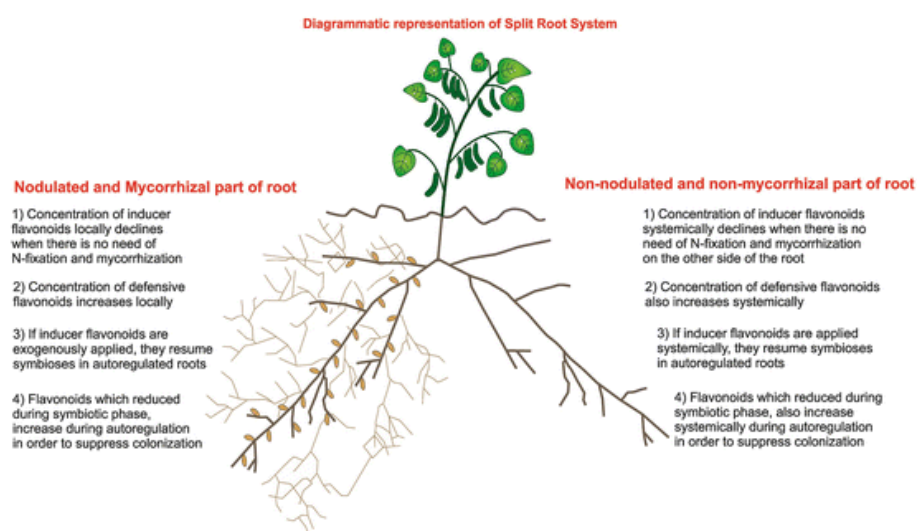


Figure 12. Role of flavonoids in autoregulation of nodulation and mycorrhization. In nodulated and mycorrhizal root parts, flavonoids accumulate to induce rhizobial *nod* genes, promote AM fungal hyphal branching, suppress plant immunity, and inhibit auxin transport, facilitating nodule and arbuscule formation. In non-nodulated and non-mycorrhizal parts, lower flavonoid levels limit microbial colonization, maintain auxin transport, and preserve immune responses, preventing excessive symbiosis. This differential flavonoid distribution regulates symbiosis extent, balancing nutrient exchange costs. (From Singla and Garg, 2017).

Flavonoids play a dual role in AM fungal symbiosis, capable of either promoting or inhibiting fungal colonization depending on their concentration, type, and the specific fungal partner involved (Vierheilig et al., 1998a; Subramanian et al., 2007; Lira et al., 2015). For example, *M. sativa* roots treated with spores and ERM of *R. intraradices* showed increased levels of coumestrol, ononin, and daidzein, and decreased levels of formononetin during the early stages of symbiosis (Larose et al., 2002). This dynamic adjustment of flavonoid levels suggests that the fungal partner signals the plant to promote colonization by upregulating stimulatory flavonoids while reducing inhibitory ones, such as formononetin (Larose et al., 2002). Interestingly, initial accumulation of medicarpin, a defense phytoalexin, suggest that the plant initially perceives the fungus as a potential pathogen. However, as colonization progresses, medicarpin levels normalize, and other flavonoids, such as coumestrol, continue to rise, reinforcing the symbiotic relationship (Larose et al., 2002). Flavonoids also regulate systemic autoregulation of mycorrhization, as demonstrated through split-root experiments. In barley, alfalfa, and soybean, colonization on one side of a split-root system suppresses colonization on the other side, suggesting a systemic feedback mechanism mediated by flavonoids (Vierheilig, 2004; Meixner et al., 2005). For instance, in alfalfa, systemic suppression is associated with changes in isoflavonoid levels, such as reduced formononetin and ononin, and exogenous application of these flavonoids can partially restore mycorrhization, indicating their role in systemic signaling (Catford et al., 2006). In addition, in soybeans, the super-nodulating mutant *nts1007*, which lacks the autoregulatory mechanism for nodulation, also fails to regulate AM fungal colonization, suggesting a shared signaling pathway between AM fungi and rhizobia symbioses (Meixner et al., 2005). Flavonoids are also crucial in the symbiosis with rhizobium. They act as signaling molecules that trigger rhizobia to produce NFs, essential for nodule formation (Scervino et al., 2005a). For example, specific flavonoids such as luteolin, genistein, and naringenin are potent nod gene inducers, while others, like medicarpin and coumestrol, can inhibit nodulation in specific rhizobial strains, highlighting the dual role of flavonoids in promoting or restricting symbiosis depending on the context (Zuanazzi et al., 1998; Subramanian et al., 2007). Moreover, flavones, but not isoflavones, are critical for nodulation in *M. truncatula*, as silencing flavone synthase reduces nodule numbers, underscoring the specificity of flavonoid types in rhizobial signaling (Zhang et al., 2009). Furthermore, studies have suggested that AM fungi can induce mechanisms to reduce inhibitory flavonoids, facilitating their establishment within the root system (Scervino et al., 2005b).

Autoregulation of nodulation is another process mediated by flavonoids. In split-root experiments, the application of NFs or inoculation with *Sinorhizobium*

meliloti altered isoflavonoid levels in roots, with increased daidzein and decreased formononetin, ononin, and medicarpin observed (Catford et al., 2006). These changes suppressed nodulation in non-colonized roots, demonstrating a systemic feedback loop regulated by flavonoids. Moreover, exogenous application of isoflavonoids counteracted this suppression, promoting nodulation even in the presence of autoregulatory signals (Catford et al., 2006). This systemic regulation is likely mediated by specific isoflavonoids, as their exogenous application in alfalfa split-root systems restores nodulation, further supporting their role in autoregulatory signaling (Catford et al., 2006).

Interactions between flavonoids and both symbioses have practical implications. For example, Xie et al. (1995) demonstrated that inoculation with *B. japonicum* increases flavonoid concentrations in root exudates, enhancing AM fungal colonization. This suggests a synergistic relationship where flavonoids help coordinate the establishment of both symbioses. In soybean, Antunes et al. (2006) found that AM fungal colonization increased root concentrations of daidzein and coumestrol, which are involved in bradyrhizobial symbiosis, highlighting their role in the tripartite symbiosis. However, flavonoids may act in concert with other signaling molecules, such as strigolactones, which are critical for AM fungal recognition, suggesting a complementary role in symbiosis establishment (Abdel-Lateif et al., 2012).

3.2. Molecular signal

The common symbiotic pathway in legumes facilitates interactions with both AM fungi and rhizobia, enabling efficient nutrient acquisition (e.g, P) and N₂ fixation. This pathway is initiated by the perception of microbial signals, such as NFs from rhizobia and MFs from AM fungi (**Figure 13**), by plant LysM receptor-like kinases (Oldroyd, 2013). SYMBIOSIS RECEPTOR KINASE (SYMRK/DMI2) is an essential co-receptor in this process, showing autophosphorylation activity *in vitro* (Endre et al., 2002; Stracke et al., 2002; Chen et al., 2012). The receptor-like cytoplasm kinase LjNICK4 interacts with LjNFR5 and shuttles to the nucleus, where it regulates nodule number (Wong et al., 2019). In addition, the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) interacts with *MtDMI2* and is required for calcium spiking (Kevei et al., 2007; Venkateshwaran et al., 2015).

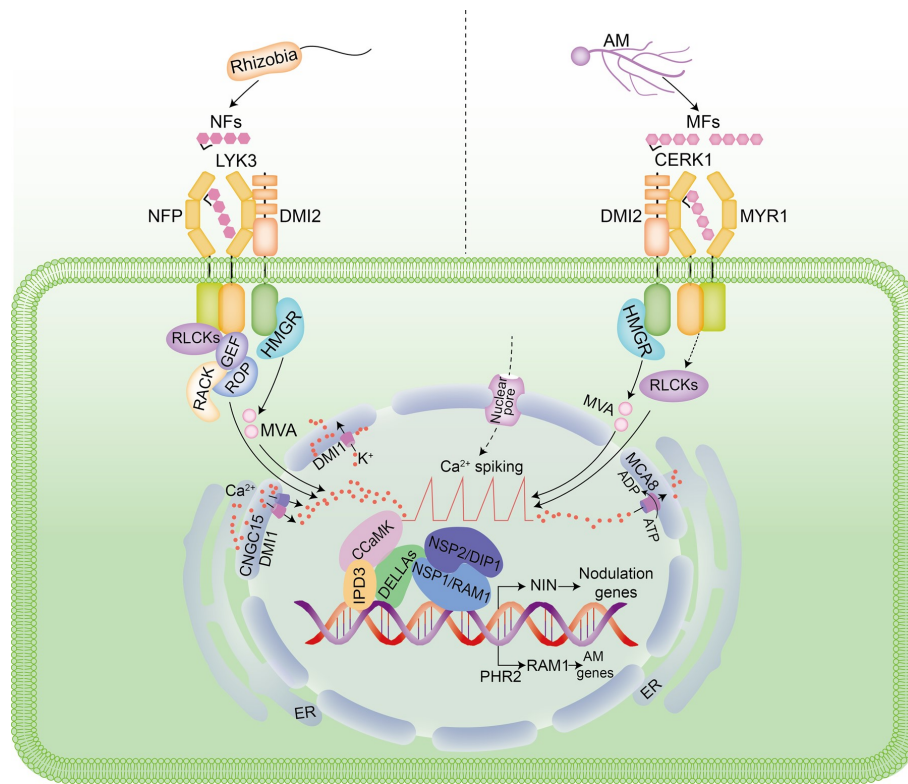


Figure 13. The common symbiotic signaling pathway. In *M. truncatula* and *L. japonicus*, LYK3/NFR1 and NFP/NFR5 form a receptor complex to perceive NFs. In rice, CERK1 (homolog of LYK3/NFR1) and MYR1 (homolog of NFP/NFR5) recognize MFs in a similar manner. Following the recognition of the symbiotic factor, rhizobial and AM symbioses share common signaling components. DMI2/SYMRK and RLCKs interact with the symbiotic receptor complex and contribute to nodulation and AM symbioses. HMGR associates with DMI2/SYMRK to promote the production of the potential second messenger MVA. MVA somehow activates the Ca^{2+} channels DMI1/POLLUX and CASTOR, which may interact with CNGC15 to regulate the influx of Ca^{2+} into the cytoplasm from the ER lumen-perinuclear space. The Ca^{2+} ATPase MCA8 then recaptures Ca^{2+} from the nucleoplasm and nucleus-associated cytoplasm, thereby resetting the system. DMI1, CNGC15 and MCA8 thus form a regulatory loop to maintain continuous Ca^{2+} spiking. The nuclear pore complex is also required for Ca^{2+} oscillations, but its role is unclear. CCaMK decodes Ca^{2+} spikes and phosphorylates the transcriptional activator IPD3/CYCLOPS. DELLA acts as a bridge to connect IPD3 and NSP2/DIP1. NSP2/DIP1 and NSP1/RAM1 form different heterodimers to output nodule or AM signaling through NIN and PHR2, respectively. MCA8 is a sarco/endoplasmic reticulum Ca^{2+} ATPase. (From Wang et al., 2022a)

Signal perception triggers a cascade of events, including the activation of ion channels like DMI1/CASTOR/POLLUX and CNGC15s, which generate calcium oscillations in and around the nucleus (Charpentier et al., 2016). These calcium signals are modulated by nuclear pore complex components NENA, NUP85, and NUP133 (**Figure 13**) (Kanamori et al., 2006; Saito et al., 2007; Groth et al., 2010). The ATPase MCA8, which localizes to the nuclear envelope and endoplasmic reticulum membrane, also contributes to symbiotic calcium spiking (Capoen et al., 2011). The calcium oscillations are decoded by CCaMK/DMI3, which phosphorylates the transcription factor IPD3/CYCLOPS (Yano et al., 2008). Constitutively activated CCaMK induces the spontaneous formation of nodules and the AM-associated pre-penetration apparatus (Gleason et al., 2006; Tirichine et al., 2006; Takeda et al., 2012).

This activation leads to the expression of key symbiosis-related genes, including *NIN* and *ERN1*, which are crucial for both rhizobial infection and nodule organogenesis (Singh et al., 2014; Cerri et al., 2017). *NIN* controls the expression of nodulation-specific genes such as *RHIZOBIUM-DIRECTED POLAR GROWTH (RPG)*, *CBS1*, *NODULATION PECTATE LYASE (NPL)*, and *NF-YA1*, while *ERN1/2* are required for the expression of *ENOD11* and *ENOD12*, which are also induced during mycorrhization (Journet et al., 2001; Garg and Geetanjali, 2007). The pathway also involves GRAS family transcription factors, such as *NODULATION SIGNALING PATHWAY1 (NSP1/NSP2)* for nodulation and *MYCORRHIZATION 1 (RAM1)* for mycorrhization, which are regulated by DELLA proteins (Jin et al., 2016; Pimprikar et al., 2016). DELLA plays a similar role in AM interactions, bridging interactions between *MtIPD3* and the downstream GRAS protein *DELLA INTERACTING PROTEIN1 (DIP1)* to promote AM symbiosis (Yu et al., 2014).

Common structural components like VAPYRIN and exocyst subunits are required for the formation of infection structures in both symbioses (Murray et al., 2011; Liu et al., 2019). The cytoskeleton plays a crucial role in these processes, with ROP-Guanine-nucleotide Exchange Factor signaling modules interfacing with symbiotic signaling to modulate actin dynamics (Ke et al., 2012; Gao et al., 2021). For instance, GmNFR1a phosphorylates GmGEF2a, which promotes its activation of GmROP9, shedding light on how NF signaling directs cytoskeleton remodeling during the re-direction of root hair growth and infection thread formation (Gao et al., 2021).

The establishment of nutrient exchange interfaces - arbuscules for AM fungi and symbiosomes for rhizobia - involves the recruitment of specific transporters, such as *MtPT4* for phosphate uptake in mycorrhizal symbiosis and various transporters

for N₂ fixation in nodules (Javot et al., 2007; Banasiak et al., 2021). In arbuscule-containing cells, the endoplasmic reticulum surrounds the arbuscule branches, the Golgi bodies and peroxisomes relocate alongside the branched hyphae, and the vacuoles become highly invaginated to accommodate arbuscules development (Pumplin and Harrison, 2009). The plastids, the source of lipid biosynthesis, increase in number and form a complex network surrounding the arbuscules (Fester et al., 2001).

The extent of symbiotic colonization is regulated by a systemic feedback mechanism known as autoregulation of nodulation, which involves CLE peptides perceived by shoot receptors like NARK/HAR1/SUNN, triggering the production of shoot-derived inhibitors such as miR2111 and cytokinin (Reid et al., 2011; Sasaki et al., 2014; Gautrat et al., 2020). This regulatory system is further influenced by nutrient availability, with nitrate-dependent CLE expression controlled by NLP transcription factors (Lin et al., 2018; Luo et al., 2021; Nishida et al., 2021). Recent evidence suggests that similar autoregulatory mechanisms may also control mycorrhizal colonization, with PHR2 acting as a key regulator orchestrating AM symbiosis and phosphate nutrient capture (Shi et al., 2021). (Shi et al., 2021)

The evolutionary progression from mycorrhizal to rhizobial symbiosis is reflected in the stepwise adaptation of the symbiotic membrane interface, culminating in the highly specialized bacteroids in legume nodules (Coba de la Peña et al., 2018; van Velzen et al., 2018). This evolutionary legacy is evidenced in the genomes of nodulating nonlegumes by the presence of homologs of *NIN* and *RPG* in the actinorhizal lineages; these genes are required for infection by rhizobia but not by mycorrhizal fungi (Griesmann et al., 2018; Wu et al., 2022).

4. Plant-AM fungi-*Rhizobia*

AM fungi and rhizobia are symbiotic microorganisms that establish symbiotic relationship with leguminous plants. Dual inoculation with the two symbionts is more beneficial for the growth and development of the host plant than single inoculation or no inoculation, due to the synergistic effects between the AM fungi and rhizobia (Smith, 2010), marked by an increase in N and P in the plant, and therefore an increase in plant biomass (Pereira et al., 2019). The interaction between AM fungi and rhizobia plays a crucial role in plant growth, nutrient uptake, and stress resistance, but these interactions can exhibit both synergistic and antagonistic effects depending on environmental and biological factors.

4.1. Synergistic interactions

Dual inoculation of AM fungi with rhizobia has been shown to promote plant growth more effectively than single inoculation, enhancing N₂ fixation, nutrient uptake, and plant productivity. AM fungi infection enriches root nodules, enhancing the N-fixing capacity of host plants, while rhizobia provide N to increase AM fungal colonization. For example, Liu and Yuan (2020) observed improved N₂ fixation and nutrient absorption in *M. sativa* co-inoculated with *R. intraradices* and *S. medicae*. Studies on legumes such as *Glycine max* show that *G. rosea* can upregulate nodulin genes expression, increasing root nodule numbers (Sakamoto et al., 2019).

Dual inoculation improves plant stress and disease resistance. Under salinity stress, it increases proline content and antioxidant activity, enhancing tolerance (Gao et al., 2018). In cadmium-stressed soil, it promotes antioxidant enzyme activity and enriches beneficial bacterial groups (Wang et al., 2021). Furthermore, dual inoculation enhances soil N and P cycles, microbial diversity, and reduces disease incidence while improving contaminated soil quality (Luna et al., 2016; Trivedi et al., 2020). The mutualistic relationship between AM fungi and rhizobia is attributed to several factors: AM fungi provide nutrients, enhance photosynthesis, and improve root systems, creating more infection sites for rhizobia. Rhizobia, in turn, provide N and induce flavonoid exudation, facilitating AM fungal colonization. This interplay optimizes nutrient uptake and N₂ fixation efficiency (Ding et al., 2016; Pereira et al., 2019; Sakamoto et al., 2019).

4.2. Antagonistic interactions

The interplay between AM fungi and rhizobia does not always lead to synergistic outcomes. A key factor driving these mutual constraints is competition for photosynthetically derived carbon, particularly under conditions of limited host carbon availability (Tsimilli-Michael et al., 2000). Under such circumstances, increased colonization by one symbiont may suppress the other. For example, high-density rhizobia inoculation in pea reduces AM fungal colonization, suggesting a negative correlation between extensive nodule formation and fungal infection (Ossler et al., 2015). Similarly, Larimer et al. (2014) observed reduced AM fungal hyphae colonization within plant roots when rhizobia invaded *Amorpha canescens*, a prairie legume species.

In addition, AM fungi and rhizobia share host regulatory pathways, such as *SYM* genes involved in early signal transduction and autoregulatory mechanisms that limit the density of colonization when host resources are constrained (Ossler et

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al., 2015; He et al., 2017). These shared pathways can lead the plant to prioritize colonization by one symbiont over the other, further exacerbating competitive interactions. For example, the autoregulation of nodulation pathways in legumes, which typically limit nodule formation under high N availability (Reid et al., 2011; Sasaki et al., 2014; Gautrat et al., 2020), can indirectly affect AM fungal colonization by altering resource allocation and hormonal signaling. This “carbon trade-off” can lead AM fungi to shift from a mutualistic to a parasitic relationship (Kaschuk et al., 2010).

Antagonistic interactions between AM fungi and rhizobia are highly context-dependent and influenced by factors such as fungal and rhizobial species, host plant genotype, soil nutrient availability, and environmental conditions (Ossler et al., 2015; He et al., 2017). For example, in *M. sativa*, the competitive balance between *R. intraradices* and *S. medicae* varies with soil P levels (Liu and Yuan, 2020). Similarly, when other nutrients, such as N, are abundant but carbon availability is relatively limited, competition for photosynthetic carbon between the two symbionts becomes more pronounced (Kaschuk et al., 2010). Under these conditions, the carbon demand from dual colonization increases disproportionately, intensifying resource competition and reducing the overall efficiency of the symbiosis.

In summary, the interaction between AM fungi and rhizobia is highly dynamic, capable of both enhancing and constraining plant growth and nutrient acquisition. While dual inoculation often demonstrates significant potential to improve plant performance through synergistic interactions, it can also lead to antagonistic effects under certain environmental conditions. This duality highlights the need for more comprehensive studies to determine the specific contexts in which dual inoculation is beneficial or detrimental. Future research should focus on elucidating the molecular mechanisms underlying these interactions, particularly the regulatory pathways and signaling networks that mediate symbiotic compatibility and competition. To achieve this, it is critical to employ advanced experimental approaches, such as molecular biology techniques, real-time imaging, and isotopic tracing, to investigate the micro-scale dynamics of carbon and nutrient exchange between the symbionts and the host. Furthermore, systems-level analyses, including transcriptomics, proteomics, and metabolomics, can provide deeper insights into the metabolic and physiological shifts associated with dual inoculation. By integrating these tools, researchers can validate the mechanisms driving these interactions and develop predictive models to optimize their use in agricultural systems. Ultimately, a more precise understanding of these dynamics will enable the targeted application of AM fungi and rhizobia to

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maximize crop productivity, enhance soil health, and contribute to sustainable agricultural practices.

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1. Biological materials

Three microorganisms and four plants were considered for the experiments.

***Rhizophagus irregularis* MUCL 41833:** The AM fungus *Rhizophagus irregularis* (Blaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler comb. nov. MUCL 41833 was supplied by the *Glomeromycota in vitro* collection (GINCO, Belgium, <https://www.mycorrhiza.be/ginco-bel/index.php>). It was maintained *in vitro* on Ri T-DNA transformed roots of carrot (*Daucus carota* L.) clone G3 in bi-compartmented Petri plates (90 mm in diameter), with the root compartment (RC) filled with sterilized (121°C for 15 min) Modified Strullu-Romand (MSR) medium (Declerck et al., 1998) and the hyphal compartment (HC) containing the same medium without sucrose and vitamins (**Figure 14**). The composition of the MSR medium is given below. The Petri plates were incubated in the dark at 27°C until sufficient spores were produced for the experiments.

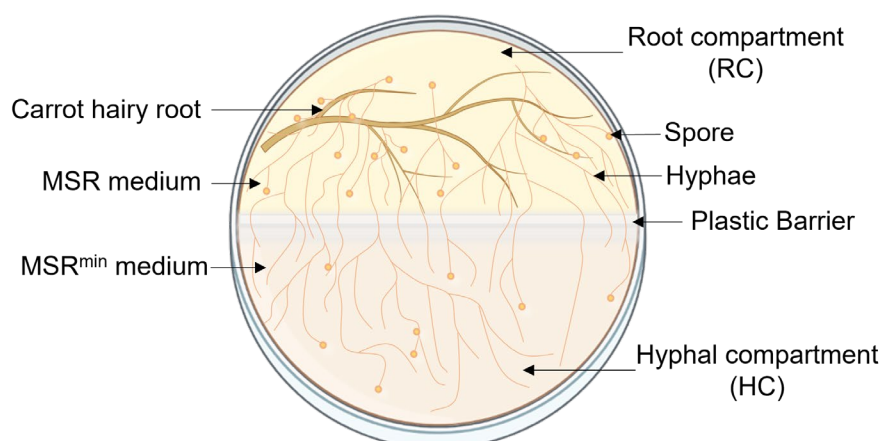


Figure 14. Schematic representation of the root-organ culture (ROC) system.

***Sinorhizobium meliloti* 2011 pHC60-GFP** (Cheng and Walker, 1998): This strain, tagged with green fluorescent protein (GFP) and resistant to tetracycline, was provided by the VIB-UGent Center for Plant Systems Biology (Ghent University, Belgium). The methodology for GFP tagging and use in visualizing symbiotic interactions was adapted from previously described approaches (Cheng and Walker, 1998). In this strain, GFP is constitutively expressed from the plasmid pHC60, ensuring stable fluorescence for tracking during plant interactions. It was cultured in yeast extract broth (YEB) medium containing 5

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g·L⁻¹ peptone, 5 g·L⁻¹ beef extract, 5 g·L⁻¹ sucrose, 1 g·L⁻¹ yeast extract, and 0.4 g·L⁻¹ MgSO₄·7H₂O, adjusted to pH 7.5 and autoclaved at 121°C for 15 min. Tetracycline was added post-autoclaving at 5 µg·mL⁻¹ from a stock solution (5 mg·mL⁻¹ in 50% ethanol, filter-sterilized with a 0.2 µm Acrodisc Syringe Filter, Pall Corporation, New York, USA). The bacterium was grown at 28°C with shaking at 180 rpm until reaching OD₆₀₀ = 0.8-1.0. For inoculation, cultures were centrifuged at 4000 × g for 10 min, resuspended in sterile phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) autoclaved at 121°C for 15 min, and adjusted to 9 × 10⁵ CFU·mL⁻¹ based on OD₆₀₀ calibration (He et al., 2024).

***Bradyrhizobium diazoefficiens* mChe-1** (Ledermann et al., 2015): This strain tagged with mCherry (red fluorescent protein) and resistant to spectinomycin was provided by the VIB-UGent Center for Plant Systems Biology (Ghent University, Belgium). Constitutive expression of mCherry was achieved using stable tagging methods, including plasmid-based expression and chromosomal integration, as previously described (Ledermann et al., 2015). It was cultured in YEB medium supplemented with 50 µg·mL⁻¹ spectinomycin, prepared as a 50 mg·L⁻¹ stock in deionized water, filter-sterilized, and added post-autoclaving. Growth conditions were identical to *S. meliloti*: 28°C, 180 rpm, until OD₆₀₀ = 0.8-1.0. Cultures were harvested and diluted in PBS to 9 × 10⁵ CFU·mL⁻¹ as described above.

The plants *Medicago truncatula* L., cv. **Jemalong A17**, *Glycine max* (L.) Merr. and *Plantago lanceolata* L. were supplied as seeds by the South Australian Research and Development Institute (SARDI, Australia), VIB-UGent Center for Plant Systems Biology, Ghent University (Belgium), and ECOSEM (Corroy-le-Grand, Belgium), respectively. These seeds were surface disinfected by immersion in an 8% active chloride bleach solution for 15 min, followed by three rinses with sterilized deionized water (121°C for 15 min), as per the protocol of Dupré de Boulois et al. (2006). They were then placed in Petri plates (90 mm in diameter, 10 seeds per plate) containing 40 mL of sterilized solid MSR medium, and kept in an incubator (WTB binder-78532 TUTTLINGEN, Germany) at 20°C in the dark until germination. Following germination, the plants were transferred in a growth chamber at 22°C with 16 hours of light per day under a photosynthetic photon flux (PPF) of 100 µmol·m⁻²·s⁻¹, and a relative humidity (RH) of 70%, as detailed in Voets et al. (2005).

Solanum tuberosum L. cv Bintje was supplied in the form of *in vitro* propagated plantlets by the Station de Haute Belgique in Libramont (Belgium). Nodal cuttings were placed in sterile culture boxes (80 mm diameter, 110 mm height, 10 cuttings per box), filled with 80 mL of 4.412 g·L⁻¹ Murashige and Skoog (MS)

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medium (**Table 1**) (Murashige and Skoog, 1962), supplemented with 20 g·L⁻¹ sucrose, 3 g·L⁻¹ Phytigel (Sigma-Aldrich Co.) and adjusted to pH 5.9 before sterilization (121 °C for 15 min). Plantlets were kept in a growth chamber at 22 °C and illuminated 16 h·d⁻¹ under a PPF of 100 µmol·m⁻²·s⁻¹ and RH of 70% as described by Voets et al. (2005).

Table 1. Concentration of stock solutions (g·L⁻¹) to prepare the Murashige and skoog (MS) medium

Macro-elements	g·L⁻¹	Vitamins	g·L⁻¹
NH ₄ NO ₃	16.50	Glycine	2 × 10 ⁻²
KNO ₃	19.00	Myo-Inositol	1
CaCl ₂ ·2H ₂ O	4.40	Nicotinique acid	5 × 10 ⁻³
MgSO ₄ ·2H ₂ O or	3.70	Thiamine	1 × 10 ⁻³
MgSO ₄ ·H ₂ O	3.30	Pyridoxine	5 × 10 ⁻³
KH ₂ PO ₄	1.70		
		Fe-EDTA solution	g·L⁻¹
Micro-elements	g·L⁻¹	FeSO ₄ ·7H ₂ O	2.78 × 10 ⁻²
H ₃ BO ₃	6.2 × 10 ⁻²	Na ₂ EDTA·2H ₂ O	3.732 × 10 ⁻²
MnSO ₄ ·7H ₂ O	1.67 × 10 ⁻¹		
ZnSO ₄ ·7H ₂ O	8.6 × 10 ⁻²		
KI	8.3 × 10 ⁻³		
Na ₂ MoO ₄ ·2H ₂ O	2.5 × 10 ⁻³		
CuSO ₄ ·5H ₂ O	2.5 × 10 ⁻⁴		
CoCl ₂ ·6H ₂ O	2.5 × 10 ⁻⁴		

Preparation of the MS medium:

1. Fill a 1000 mL Erlenmeyer flask with 700 mL of distilled water.
2. Add the following elements from the prepared stock solutions:
 - 100 mL of the macro-element stock solution,
 - 1 mL of the micro-element stock solution,
 - 1 mL of the vitamin stock solution,
 - 10 mL of the Fe-EDTA stock solution.
3. Add 20 g of sucrose and stir until fully dissolved.
4. Adjust the final volume to 1000 mL with distilled water.

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5. Adjust the pH to 5.9 using NaOH or KOH and HCl.
6. Add 3 g·L⁻¹ of Phytigel.
7. Autoclave the medium at 121°C for 15 minutes.
8. After autoclaving, the medium can be used immediately or stored for a few hours at 40 – 60 °C until needed.

2. Composition of the different Modified-Strullu-Romand medium

Three culture media were used, all deriving from the MSR medium (Declerck et al., 1998).

(1) The MSR^{min} medium, without sucrose and vitamins, (2) the MSR^{min0N} with 0 mM N (KNO₃ and Ca(NO₃)₂·4H₂O were replaced by 75.2 mM KCl and 152 mM CaCl₂·2H₂O, respectively) and (3) the MSR^{min½N} containing half the N concentration (1.99 mM) of the normal MSR^{min} medium (i.e., 37.6 mM of KNO₃ and 76 mM of Ca(NO₃)₂·4H₂O) (**Table 2**).

Table 2. Concentration of stock solutions (g·L⁻¹) to prepare the MSR, MSR^{min}, MSR^{min½N}, and MSR^{min0N} medium

	MSR	MSR ^{min}	MSR ^{min½N}	MSR ^{min0N}
Macro-elements				
KNO ₃	7.6	7.6	3.8	-
KCl	6.5	6.5	6.05	12.1
KH ₂ PO ₄	0.41	0.41	0.41	0.41
MgSO ₄ ·7H ₂ O	73.9	73.9	73.9	73.9
Calcium Nitrate				
Ca(NO ₃) ₂ ·4H ₂ O	35.9	35.9	17.95	-
CaCl ₂	-	-	8.435	16.87
NaFeEDTA	1.6	1.6	1.6	1.6
Micro-elements				
MnSO ₄ ·4H ₂ O	2.45	2.45	2.45	2.45
ZnSO ₄ ·7H ₂ O	2.8 × 10 ⁻¹	2.8 × 10 ⁻¹	2.8 × 10 ⁻¹	2.8 × 10 ⁻¹
H ₃ BO ₃	1.85	1.85	1.85	1.85
CuSO ₄ ·5H ₂ O	2.2 × 10 ⁻¹	2.2 × 10 ⁻¹	2.2 × 10 ⁻¹	2.2 × 10 ⁻¹
(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	3.4 × 10 ⁻²	3.4 × 10 ⁻²	1.7 × 10 ⁻²	-
Na ₂ MoO ₄ ·2H ₂ O	2.4 × 10 ⁻³	2.4 × 10 ⁻³	2.57 × 10 ⁻²	4.9 × 10 ⁻²

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Vitamins					
Ca panthotenate	1.8×10^{-1}	-	-	-	-
Biotine	1.8×10^{-4}	-	-	-	-
Pyridoxine	1.8×10^{-1}	-	-	-	-
Thiamine	2×10^{-1}	-	-	-	-
Cyanocobalami	8×10^{-2}	-	-	-	-
Nicotinic acid	2×10^{-1}	-	-	-	-

Preparation of culture media:

1. To prepare 1000 mL of MSR, combine the following stock solutions:

- 10 mL of the macro-element stock solution,
- 10 mL of the calcium nitrate stock solution,
- 5 mL of the NaFeEDTA stock solution,
- 1 mL of the micro-element stock solution,
- 5 mL of the vitamin stock solution.

2. Add 10 g of sucrose (not needed for the preparation of MSR^{min}, MSR^{min½N}, and MSR^{min0N}) and stir until fully dissolved.

3. Adjust the final volume to 1000 mL with deionized water.

4. Adjust the pH to 5.5 using NaOH or KOH and HCl.

5. Add 3 g·L⁻¹ of Phytigel as the gelling agent.

6. Autoclave the prepared medium at 121 °C for 15 min under 1 bar of pressure.

7. After autoclaving, the medium can be used immediately or stored at 40 – 60 °C for a few hours until needed.

3. Mycelium donor plant (MDP) *in vitro* culture system

Bi-compartmented Petri plates (90 mm diameter) were used to grow excised Ri T-DNA transformed actively growing carrot (*D. carota* L. clone G3) roots and *R. irregularis* as detailed by St-Arnaud et al. (1996). Briefly, in one compartment (i.e. the root compartment – RC), 25 mL MSR medium containing 3 g·L⁻¹ Phytigel™ was added, while in the second compartment (i.e. the hyphal compartment – HC), 25 mL MSR^{min} medium containing 3 g·L⁻¹ Phytigel™ was added. An excised transformed carrot root associated with *R. irregularis* was transferred in the RC and incubated in a growth chamber at 27°C in the dark. Roots crossing the plastic barrier and growing in the HC were regularly trimmed using sterile forceps. After ~13 weeks, a profuse ERM was observed in the HC. Three holes, each with a diameter of approximately 2 mm, were created at even

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intervals on the side of the HC (**Figure 15**). Three seven-days-old *M. truncatula* or *S. tuberosum* or *G. max* seedlings were transferred to the HC, with the roots on the surface of the medium and the shoot extending outside the system. The systems were then sealed with Parafilm (Pechiney, Plastic Packaging, Chicago, IL 60631, USA) and the openings plastered with sterilized (121°C for 15 min) silicon grease (VWR International, Belgium). The systems were subsequently wrapped with opaque plastic bags to keep the AM fungus and plant roots in the dark, while shoots developed under light conditions. The systems were transferred to a growth chamber at 22/18°C (day/night), 70% relative humidity, a photoperiod of 16 h·day⁻¹ and an average PPF of 225 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. After 2 weeks, the plant were colonized by the AM fungus and used for subsequent experiments.

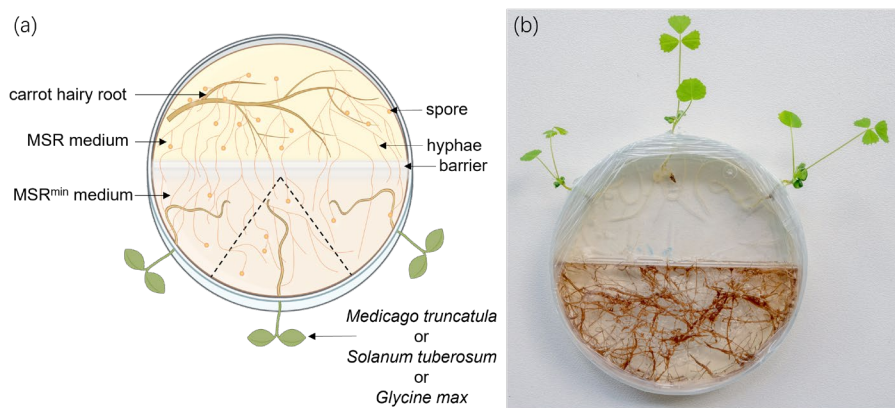


Figure 15. Schematic (a) and picture (b) representation of the mycelium donor plant (MDP) *in vitro* culture system

4. Evaluation of root colonization by AM fungi

Root pigmentation, including tannins and polyphenols, typically obscures the structures of AM fungi within plant roots. To assess AM fungal root colonization, a clearing and staining procedure is essential. Firstly, the roots are subjected to a 30-min treatment with 10% KOH at 70°C. This process facilitates cell wall softening, cytoplasmic evacuation of cortical cells, tannin removal, and enhanced ink penetration.

Secondly, the roots undergo a brief neutralization step using 1% HCl for 1 min at ambient temperature. This step optimizes pH and improves affinity for the staining solution. The staining process involves immersion in a solution of 2% Parker Super Quink Permanent blue ink (United States) in 1% HCl for 30 min at

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70°C. After staining, the roots are preserved in a solution of lactoglycerol (1:1:1 v/v/v ratio of lactic acid, glycerol, and water).

For quantitative analysis of root colonization, we used the method described by McGonigle et al. (1990). This method involves microscopic examination of randomly selected root fragments, focusing on the presence of key AM fungal structures: arbuscules, vesicles, and hyphae. The observed frequency of these structures is used to extrapolate the overall colonization rate of the root system.

In our experiments, 300 intersections per plant root sample were examined under a compound microscope (Olympus BH2, Olympus Optical, GmbH, Germany) at 40 X magnification. This allowed for the estimation of total root colonization percentage (%RC), hyphae percentages (%H), arbuscules percentage (%A), and spores/vesicles percentage (%V) (**Figure 16**).

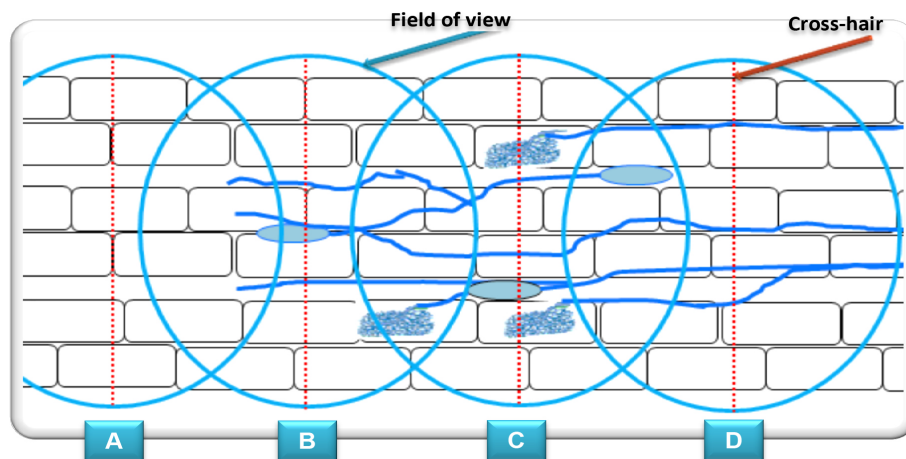


Figure 16. Evaluation of the AM fungal root colonization using the method of McGonigle et al. (1990).

During microscopic analysis, various scenarios may be encountered:

In case of absence of AM fungal structure at cross-hair: note “negative-intersect” (**Figure 16A**).

In case of presence of spores/vesicles or hyphae, but not arbuscules: note “presence” of the observed structure (spores/vesicles, hyphae) (**Figure 16B**).

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When at the same time spores/vesicles, arbuscules and hyphae are observed in the intersection (**Figure 16C**): note “presence” of spores/vesicles and “presence” of arbuscules.

When only hyphae are observed at the intersection (**Figure 16D**): note “presence” of hyphae.

Percentage of hyphae colonization (%HC), percentage of arbuscules colonization (%AC) and percentage of spores/vesicles colonization (%VC) are then calculated by dividing the number of intersections with the presence of hyphae or arbuscules or spores/vesicles, respectively by the total number of intersections examined. Total colonization (%TC) is calculated as the proportion of non-negative intersections.

Formulas:

$\%AC = (\text{number of intersections with the presence of arbuscules} / \text{total number of intersections examined}) * 100$

$\%HC = (\text{number of intersections with the presence of only hyphae} / \text{total number of intersections examined}) * 100$

$\%VC = (\text{number of intersections with the presence of spores/vesicles} / \text{total number of intersections examined}) * 100$

$\%TC = ((\text{total number of intersections examined} - \text{“negative-intersect”}) / \text{total number of intersections examined}) * 100$

5. Determination of soil hyphal density

Soil hyphal density was estimated following the method described by Bethlenfalvai and Ames (1987) with slight modifications. In brief, fresh soil samples, each weighing 5 g, were placed in 50 mL centrifuge tubes and suspended in 10 mL of 0.1 mol·L⁻¹ PBS at pH 7.8. After gentle agitation, 8 mL of the resulting suspension was transferred to a 10 mL centrifuge tube. A staining solution comprising 2% Parker Super Quink Permanent blue ink (United States) in 1% HCl was prepared, and 2 mL of this solution was added to each sample. The mixture was then gently homogenized and incubated at 70°C for 20 min. Following incubation, the samples were allowed to cool to room temperature. For microscopic analysis, 1 mL aliquots were extracted from each sample and

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examined using a compound microscope (Olympus BH2, Olympus Optical, GmbH, Germany) at 10× magnification (**Figure 17**).

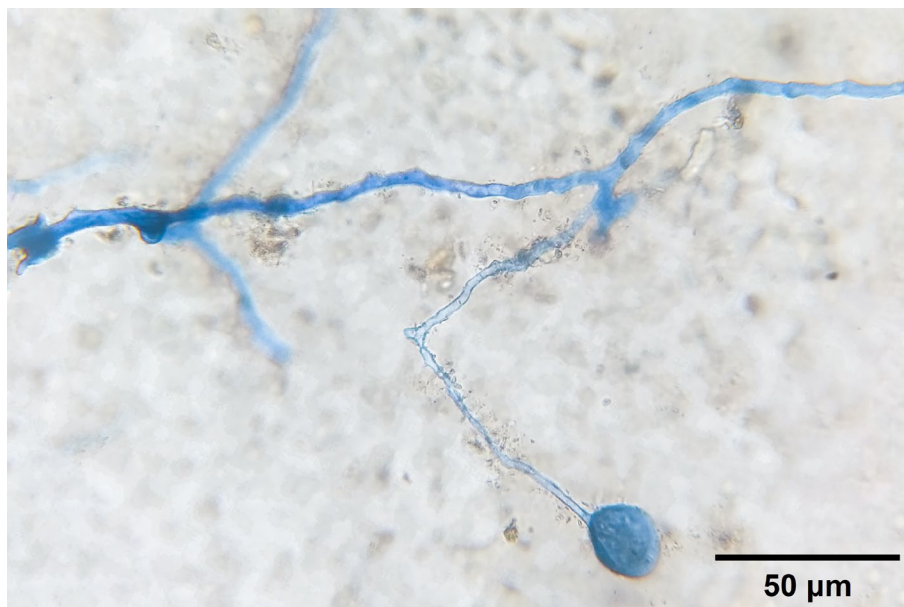


Figure 17. Stained hyphae of *Rhizophagus irregularis* MUCL 41833 in soil.

6. *S. meliloti* migration along AM fungal hyphae and cytoplasmic flow velocity within hyphae

To investigate the interactions between *S. meliloti* and fungal hyphae, we assessed two parameters: the migration velocity of *S. meliloti* along the hyphae surface and the cytoplasmic/protoplasmic flow velocity within the hyphae. Both parameters were measured at multiple time points (e.g., 2, 4, 6, 12, and 24 h) and analyzed based on dynamic observations under a microscope.

6.1. Microscopy and video/image acquisition

Microscopic observations were performed using an Echo Revolve RVL2-K microscope (<https://discover-echo.com/revolve/>), with two magnifications applied for specific purposes:

4× magnification: Used to capture the overall structure of the fungal hyphae and visualize the bacterial migration pathway. Images were stitched together using Fiji's Stitching plugin (Schindelin et al., 2012) to create a complete visualization

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of the hyphal network. These stitched images provided context but were not used for quantitative analysis.

20× magnification: Used for detailed tracking of both *S. meliloti* migration and cytoplasmic/protoplasmic flow velocity. This magnification enabled precise measurements of bacterial movement along the hyphal surface and particle flow within the hyphae.

Videos were recorded continuously at all time points, and static images were captured for subsequent quantitative analysis. Consistent microscope settings (automatic brightness, contrast, and saturation) were applied to ensure reproducibility across all observations.

6.2. Experimental setup and inoculation

Actively growing fungal hyphae exhibiting visible cytoplasmic/protoplasmic flow were selected for the experiments. A 1 μL drop of *S. meliloti* suspension ($9 \times 10^5 \text{ CFU} \cdot \text{mL}^{-1}$, $\text{OD}_{600} = 0.1$) was inoculated onto a predefined starting point on the hyphal surface. The inoculation points were marked for subsequent tracking. Hyphae were visually separated by several millimeters to avoid overlap between replicates.

6.3. Image and video analysis

The analysis of *S. meliloti* migration and cytoplasmic flow velocity relied on Fiji/ImageJ software (Schindelin et al., 2012) for precise measurements. Both parameters were quantified using methodologies, as detailed below.

Distance measurement - Videos recorded at 20× magnification were randomly paused during playback to capture static frames for analysis. Several frames were extracted from each video: an initial frame and a subsequent frame captured after a short, defined time interval (e.g., a few seconds apart). The time interval between these two frames was recorded. Six times intervals were analyzed per plate at each time point, and their average velocity was calculated.

For *S. meliloti* migration, the “Measure” tool in Fiji (Schindelin et al., 2012) was used to mark the position of the furthest observed bacteria along the hyphal surface in the initial frame and the corresponding position in the subsequent frame (**Figure 18**).

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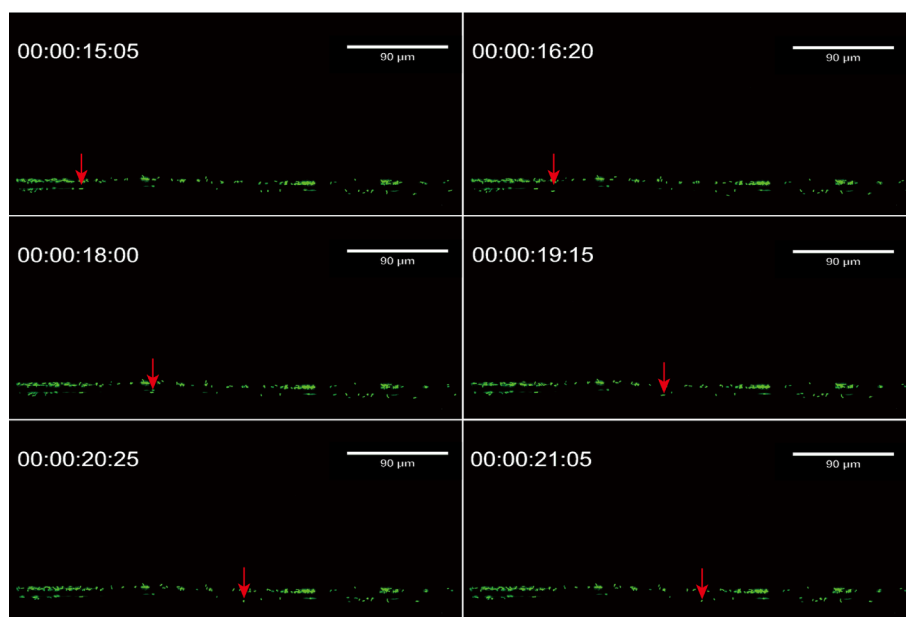


Figure 18. Time-lapse images of *Sinorhizobium meliloti* (expressing green fluorescence) movement along *Rhizophagus irregularis* hyphae.

For cytoplasmic/protoplasmic flow, the “Measure” tool (Schindelin et al., 2012) was used to mark the positions of visible particles within the hyphae in both frames. These particles were visually identified based on their distinct shapes, sizes, or colors (**Figure 19**).

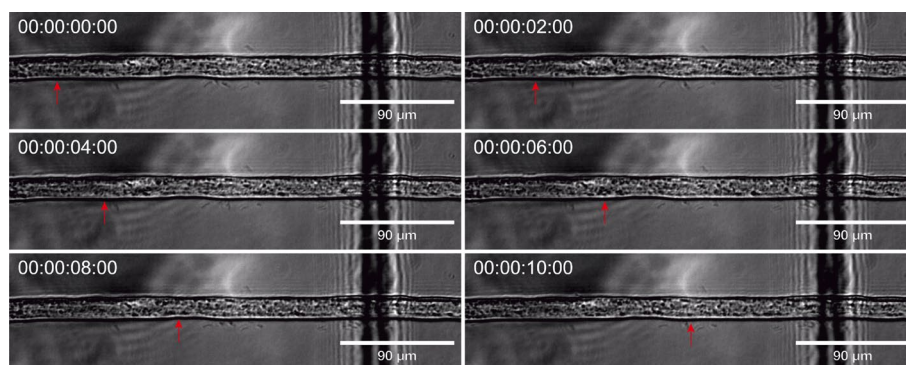


Figure 19. Time-lapse images of cytoplasmic/protoplasmic flow in *Rhizophagus irregular* hyphae.

Calculation of velocity - The linear distance traveled by *S. meliloti* (for migration) or particles (for cytoplasmic flow) between the two frames was measured. Velocity was then calculated using the formula:

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$$Velocity (\mu\text{m} \cdot \text{s}^{-1}) = \frac{\text{Distance Traveled } (\mu\text{m})}{\text{Time Interval } (\text{s})}$$

Bacterial film thickness - For *S. meliloti* migration, the thickness of the bacterial film around the hyphae was also quantified at each time point (**Figure 20**). This was done by measuring the width of the bacterial layer at multiple points along the hyphal surface using the “Measure” tool in Fiji (Schindelin et al., 2012).

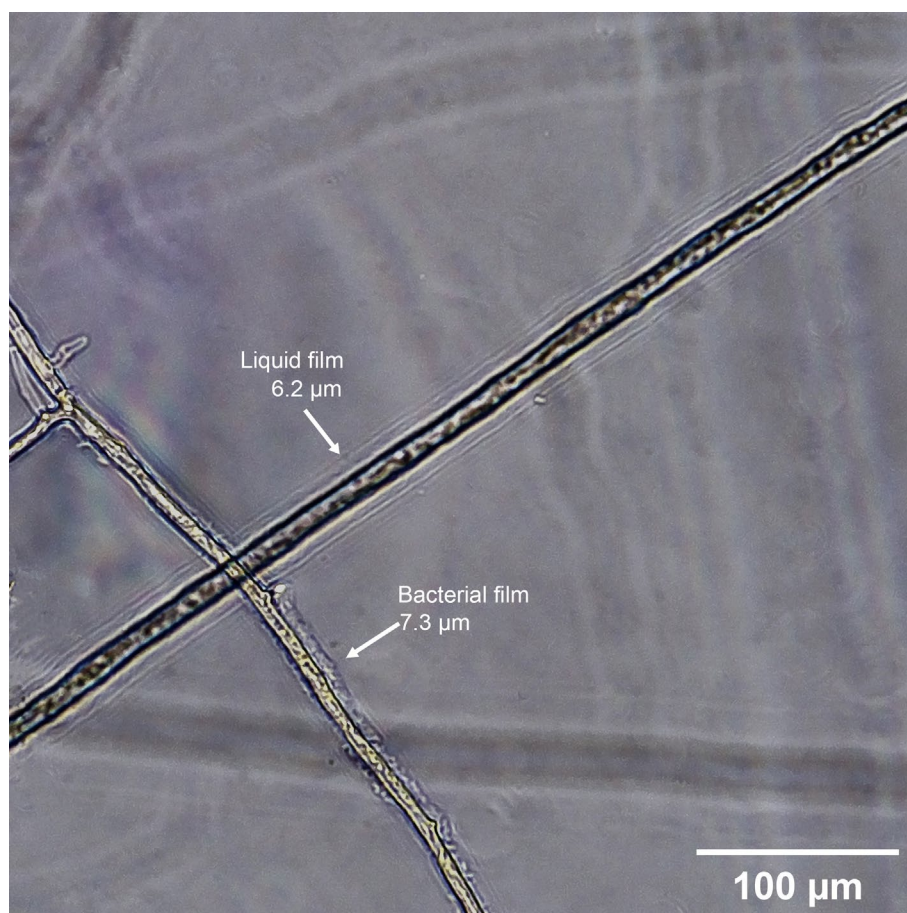


Figure 20. Bacterial film and liquid film around the hyphae of *Rhizophagus irregularis* MUCL 41833.

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Chapter I

Arbuscular mycorrhizal hyphae facilitate rhizobia dispersal and nodulation in legumes

Based on the article published in 2024 in The ISME Journal, volume 18, issue 1 (article number: wrae185), with appropriate modifications.

Impact factor: 10.8

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Preface

In nature, plants form intricate associations with microbial communities, which provide numerous ecological and physiological benefits. Among these, the symbiosis between legumes and arbuscular mycorrhizal (AM) fungi and rhizobia is of particular importance because it promotes plant growth and nitrogen (N₂) fixation. While previous studies have extensively documented the short-distance recruitment of rhizobia via root exudates, the mechanisms enabling long-distance rhizobial migration toward legume roots remain unclear.

In **Chapter I**, we will explore how AM fungal extraradical hyphae facilitate the migration of *Sinorhizobium meliloti* to the roots of *Medicago truncatula*. Using an array of experimental systems, including *in vitro* cultivation setups and greenhouse microcosms, we investigate (i) the ability of AM fungal hyphae to transport rhizobia over long distances, (ii) the role of plant-derived flavonoids in mediating this interaction, and (iii) the preferential migration of rhizobia toward leguminous plants compared to non-leguminous plants.

By using metabolomics approaches to analyze flavonoid profiles and time-lapse microscopy to track rhizobial movement, we demonstrated that AM fungal hyphae act as conduits for rhizobia, transporting chemical signals that enhance rhizobial migration and nodulation gene expression. The results highlighted a previously underexplored, indirect pathway for legume-rhizobia interaction, mediated by fungal networks.

This research provides novel insights into the ecological and molecular mechanisms underlying plant-microbe symbiosis and underlines the potential application of AM fungi and rhizobia in sustainable agriculture.

Abstract

In soil ecosystems, rhizobia occupy the rhizosphere of legume roots to form nodules, a process triggered by microbial recognition of specific root-derived signals (i.e., flavonoids). However, soil conditions can limit bacterial motility, restricting signal perception to the area directly influenced by roots. Legumes, like most plants of agricultural interest, associate with arbuscular mycorrhizal fungi, whose hyphae develop extensively in the soil, potentially providing an effective dispersal network for rhizobia. We hypothesized that mycelial networks of arbuscular mycorrhizal fungi play a role in signal transmission and act as a highway, enabling rhizobia to migrate from distant soil to the roots of leguminous plants. Using *in vitro* and greenhouse microcosm systems, we demonstrated that *Rhizophagus irregularis* helps *Shinorhizobium meliloti* to migrate towards the legume *Medicago truncatula*, triggering nodulation, a mechanism absent without the arbuscular mycorrhizal fungus. Metabolomics analysis revealed eight flavonoids unique to the compartment containing extraradical hyphae of the arbuscular mycorrhizal fungus linked to *M. truncatula* roots, associated with *S. meliloti* growth and *nod* gene expression. Rhizobia plated on the extraradical hyphae connecting two plants (the legume *M. truncatula* and non-legume *Solanum tuberosum*) by a common mycelium network, showed preference for the legume, suggesting the chemoattraction by specific signals transported by the fungus connected to the legume. Simultaneously, *S. meliloti* stimulated the cytoplasmic/protoplasmic flow in the hyphae, likely increasing the release of nutrients and signals. Our results highlight the importance of extraradical hyphae (i.e. the mycorrhizal pathway) of arbuscular mycorrhizal fungi for the migration of rhizobia over long distances to the roots, leading to nodulation.

Introduction

Leguminous crops have the ability to interact symbiotically with arbuscular mycorrhizal (AM) fungi and with nitrogen-fixing soil bacteria collectively known as rhizobia (Saeki, 2011). It has been shown that the contribution of AM fungi to N uptake was 27% in *Vicia faba* colonized by *Rhizophagus irregularis* in a pot experiment conducted on phosphorus-deficient soil (Wahbi et al., 2016), and even 52% in a two-compartment growth system of *Glycine max* L. colonized by *R. intraradices* (Wang et al., 2016b). Similarly the rhizobia association has been shown to mobilize high amounts of N, estimated to be about 65 to 335 kg of N·ha⁻¹·year⁻¹ for alfalfa, red clover, pea, soybean, cowpea, and vetch (Tate, 1995). It is estimated that 70 million tons of N per year are contributed to soil ecosystems by the relationship between legumes and their rhizobial symbionts (Brockwell et

al., 1995). Along with rhizobia, AM fungi are thus considered to be the most crucial terrestrial symbioses that “help feed the world” (Marx, 2004).

Legume–rhizobia interactions are initiated when plant roots release flavonoids into the soil, the perception of which activates the production and secretion of rhizobial Nod factors. However, the perception of these signals is limited to a few millimeters in the rhizosphere (Zhang et al., 2020) and several abiotic factors (e.g., soil organic matter, sorption by minerals) may attenuate flavonoid-based plant-microbe communication (Del Valle et al., 2020). In addition, soil conditions can limit the mobility of rhizobia, subject to water mass flow and other vectors to cross air-filled gaps between soil aggregates (Horiuchi et al., 2005; Vicario et al., 2015). Until recently (Zhang et al., 2020), it was unclear whether and how rhizobia migrate from long distance to the rhizosphere of legumes. These authors demonstrated that mycelial networks of the facultative biotrophic ascomycota *Phomopsis liquidambaris* help with the migration of rhizobia from the bulk soil to the rhizosphere of *Arachis hypogaea*, increasing nodulation in this legume. This study laid the foundation for the hypothesis that legumes might also recruit rhizobia via the ERM of AM fungi. Indeed, the hyphae of AM fungi extends several cm away from plant roots (Awaydul et al., 2019), represents 82 to 111 $\text{m}\cdot\text{cm}^{-3}$ in prairie and 52 to 81 $\text{m}\cdot\text{cm}^{-3}$ in ungrazed pasture (Miller et al., 1995) and mobilize between 4 and 20% of the total carbon synthesized by plants (Shi et al., 2023). In addition, bacterial activities (e.g., the solubilization of organic P), are stimulated by the release of compounds or nutrients from the hyphae, which function as signals (Zhang et al., 2018a). Recently, it has been demonstrated that bacteria could move along the hyphae to nutrient patches in the soil (Jiang et al., 2021). Hence, we hypothesized that rhizobia can also use the extraradical mycelium (ERM) of AM fungi as a highway to reach the roots of legumes from long distances, thus representing an indirect route for legumes to recruit root-nodulating bacteria.

In the present study, we performed a series of *in vitro* and greenhouse experiments to study the migration potential of *Sinorhizobium meliloti* along the ERM of AM fungi connected to the leguminous plant *Medicago truncatula* or the non-leguminous plants *Plantago lanceolata* and *Solanum tuberosum*. We first developed an *in vitro* cultivation system with *M. truncatula* to study whether AM fungal hyphae of *Rhizophagus irregularis* could transport rhizobia from a long distance from the root to the root surface to result in nodulation. Next, we conducted a comparative metabolomics study on the growth media containing AM fungal hyphae connected to *M. truncatula* or *S. tuberosum* in a compartmented system to reveal the presence and potential differences in nodule-inducing flavonoids content. Finally, we carried out an *in vitro* and a pot

microcosm experiment with a leguminous plant connected by a common mycorrhizal network (CMN) of the AM fungus to non-leguminous plants to assess the preferred migration direction of rhizobia.

We show that AM fungal hyphae can serve as a route for *S. meliloti* migration, showing a preference for the legume over the non-legumes. This preference is probably due to the transport of specific signals (i.e., flavonoids) from the legume root to the bacteria via the hyphae, initiating the distant interaction between the legume and *S. meliloti*.

Materials and methods

Biological material

Experiments utilized *Rhizophagus irregularis* MUCL 41833, *Sinorhizobium meliloti* 2011 pHc60-GFP, *Medicago truncatula* L. cv. Jemalong A17, *Plantago lanceolata* L., and *Solanum tuberosum* L. cv. Bintje, sourced and cultured as described in Section 1 of MATERIALS AND METHODS.

Optimization of MSR medium for growth and nodulation of *M. truncatula* *in vitro*

Seven-day-old *M. truncatula* plants were cultured in 90 mm Petri plates under conditions detailed in Section 3 of MATERIALS AND METHODS (**Figure 21a**; **Figure S1**). Two Modified Strullu-Romand (MSR) media variants, MSR^{min0N} (0 mM N) and MSR^{min½N} (1.99 mM N), were used as described in Section 2 of MATERIALS AND METHODS. Roots were inoculated with 150 µL of *S. meliloti* in phosphate-buffered saline (PBS) at 9×10^5 CFU·mL⁻¹. Controls received 150 µL PBS without bacteria. Four treatments, each with 18 plant replicates, were evaluated: MSR^{min0N+S.meliloti}, MSR^{min0N-S.meliloti}, MSR^{min½N+S.meliloti}, and MSR^{min½N-S.meliloti}. For each treatment, 6 replicates were randomly harvested after 1, 2, and 3 months to determine shoot and root dry weights (after 72 hours at 70°C) and number of nodules.

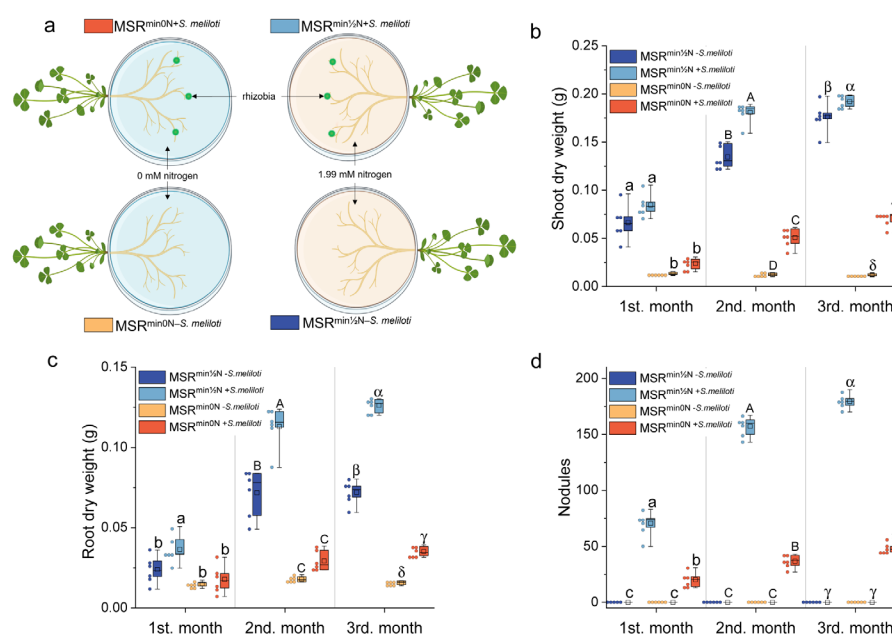


Figure 21. Impact of *S. meliloti* on the *in vitro* growth and nodulation of *M. truncatula* on the MSR^{min} medium containing 0 mM or 1.99 mM nitrogen (N). (a) Schematic representation of the *in vitro* Petri plate system. (b) Shoots dry weight, (c) Roots dry weight, and (d) Number of nodules, after 1, 2, and 3 months of growth of *M. truncatula*. The box plot displays the maximum, upper quartile, median, lower quartile, minimum values. Hollow squares represent mean values, dots represent individual measurements. Data ($n = 6$) were analyzed by a one-way ANOVA followed by a Tukey post-hoc test ($P \leq 0.05$). Different lowercase letters, uppercase letters, and Greek letters, respectively, indicate significant differences between treatments within month 1, 2 or 3, separately.

In vitro experimental design for analyzing flavonoids released by the ERM of *R. irregularis* in the HC

Medicago truncatula plants were pre-mycorrhized using a mycelium donor plant (MDP) system, as detailed in Section 3 of MATERIALS AND METHODS (Figure 15). Pre-mycorrhized plants were transferred to the RC of bi-compartmented Petri plates (90 mm diameter) (Figure 22a; Figure S2). At week 9, a profuse ERM growth was observed in the RC and on the slope in the hyphal compartment (HC) containing MSR^{min/2N} medium. During the subsequent four weeks, 10 mL of MSR^{min/2N} medium cooled to 40°C was added weekly to the RC. Similarly, the HC was supplemented twice a week with liquid MSR^{min/0N} medium to keep the medium volume at 10 mL. Four treatments were considered: *M. truncatula* or *S. tuberosum* grown in the RC in absence of AM fungus in the HC

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(RC^{*M.truncatula*}/HC^{*-R.irregularis*} and RC^{*S.tuberosum*}/HC^{*-R.irregularis*}, respectively) and *M. truncatula* or *S. tuberosum* grown in the RC in presence of the AM fungus in the HC (RC^{*M.truncatula*}/HC^{*+R.irregularis*} and RC^{*S.tuberosum*}/HC^{*+R.irregularis*}, respectively). At week 13, a high number of hyphae was observed in the HC. At that time, the liquid medium in the HC was removed and 10 mL of fresh liquid MSR^{min0N} was added for an extra week. At week 14, all the liquid MSR^{min0N} medium was collected with a VWR Powerpette Plus and transferred to a 15 mL centrifuge tube, quickly frozen in liquid N, and stored at -80°C for flavonoids analysis.

Flavonoid analysis involved extracting 6 mL MSR^{min0N} from each HC with 6 mL methanol for 1 hour at room temperature, followed by evaporation to dryness under vacuum conditions. The dry methanol extracts were resolubilized in 100 µL cyclohexane and 100 µL Milli-Q water, vortexed thoroughly, and then centrifuged at 14,000 rpm. Eighty µL of the aqueous phase was filtered using a 96-well filter plate. Samples were subjected to Ultra Performance Liquid Chromatography High Resolution Mass Spectrometry (UPLC-HRMS) at the VIB Metabolomics Core Ghent (VIB-MCG). Ten µL of the sample was injected on a Waters Acquity UHPLC (Waters) device connected to a Synapt XS high-definition mass spectrometer (Waters). Chromatographic separation was carried out on an ACQUITY UPLC BEH C18 (150 x 2.1 mm; 1.7 µm) column (Waters) with the column temperature maintained at 40 °C. A gradient of two buffers was used for separation, i.e., buffer A (water + 0.1% formic acid, pH 3) and buffer B (acetonitrile + 0.1% formic acid, pH 3). Buffer A was decreased from 99% to 50% in 30 min, decreased to 30% in 30 to 35 min, and further decreased to 0% in 35 to 37 min. The flow rate was set to 0.35 mL·min⁻¹. Electrospray Ionization (ESI) was applied, and the LockSpray ion source was operated in negative ionization mode under the following conditions: capillary voltage, 3 kV; reference capillary voltage, 2.5 kV; source temperature, 120°C; desolvation gas temperature, 550°C; desolvation gas flow, 800 L·h⁻¹; and cone gas flow, 50 L·h⁻¹. The collision energy for full MS scan was set at 4 eV. For data-dependent acquisition–tandem mass spectrometry (DDA-MS/MS), the low mass ramp was ramped between 6-20 eV, and the high mass ramp was ramped between 20-70 eV. The mass range was set from 50 to 1500 Da and scan time was set at 0.3 s. The N (greater than 99.5 %) was employed as desolvation and cone gas. Leucine-enkephalin (100 pg·µL⁻¹ solubilized in water:acetonitrile 1:1 [v/v], with 0.1% formic acid) was used for the lock mass calibration, scanning every 0.75 min at a scan time of 0.3 s. Profile data was recorded through Masslynx (Waters). Data processing was performed with Progenesis QI software version 3.0 (Waters) for chromatogram alignment and compound ion detection. The detection limit was set at medium sensitivity with a minimum peak width of 0.04 min. 20,081 compound ions were detected and aligned to “PooledSample_02n”. The data was normalized to all compound

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ions. The following filters were applied to analyze the data: a minimum ion intensity of 1,000 counts in at least one sample group (resulting in 13,513 features) and an ANOVA $P \leq 0.05$ (resulting in 11,662 features). Statistical analyses were performed on ArcSinh-transformed and pareto scaled ion intensities. Structural annotation of the significant features was attempted using MS-FINDER (*in silico* fragmentation) (Tsugawa et al., 2016). The following parameter settings were applied: formula prediction and structural elucidation by *in silico* fragmenter using all available local databases, cut off score for structural elucidation: 5, MS¹ mass tolerance: 5 ppm, MS² mass tolerance: 10 ppm, relative abundance cut off: 0.1%, LEWIS and SENIOR check: TRUE, element ration check: common range (99.7%), element selection: C, H, N, tree depth: 2.

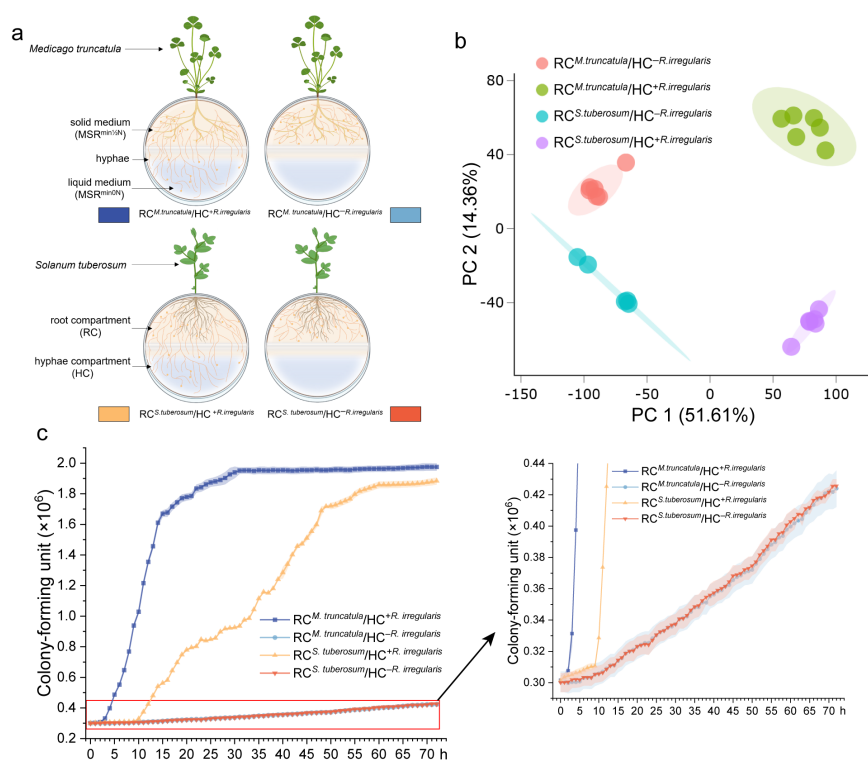


Figure 22. Impact of ERM of *R. irregularis* on transport of flavonoids and on growth in *S. meliloti*. **(a)** Schema of the bi-compartmented Petri plate system. The root compartment (RC) is separated from the hyphal compartment (HC) by a plastic barrier. *M. truncatula* roots were placed in the RC with shoots extending outside through a 2 mm hole. RC contained 20 mL solidified MSR^{min1/2N} medium (1.99 mM N), and HC contained 4 mL solidified MSR^{min0N} medium (0 mM N) in a slope. Once profuse ERM growth was observed in the slope, 10 mL of liquid MSR^{min0N} medium lacking Ca(NO₃)₂·4H₂O was added to the HC. **(b)** Principal

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component analysis (PCA) of compounds in the MSR^{min0N} medium collected from the HC. **(c)** Growth of *S. meliloti* in liquid MSR^{min0N} medium from different treatments: RC^{M.truncatula}/HC^{+R.irregularis}, RC^{M.truncatula}/HC^{-R.irregularis}, RC^{S.tuberosum}/HC^{+R.irregularis}, and RC^{S.tuberosum}/HC^{-R.irregularis}. Data are means $\pm$ SD ($n = 12$, 72 hours, OD = 595). Shaded areas represent error bars.

***In vitro* experimental design for analyzing growth and gene expression of *S. meliloti* in presence of ERM exudates**

Another set of 24 Petri plates initiated simultaneously under identical conditions to above. At week 14, two mL liquid MSR^{min0N} was collected from the HC of each treatment in 2.5 mL centrifuge tubes, frozen in liquid nitrogen, and stored at -80°C for *S. meliloti* growth studies. At week 14, two mL liquid MSR^{min0N} was collected from the HC of each Petri plate per treatment and transferred to a 2.5 mL centrifuge tube, followed by rapid freezing in liquid N and subsequent storage at -80°C . This solution was used to study its effects on the growth of *S. meliloti* (**Figure S6**). One hundred and eighty μL of liquid MSR^{min0N} from each Petri plate was distributed in two wells (90 μL per well). Each treatment (RC^{M.truncatula}/HC^{+R.irregularis}, RC^{M.truncatula}/HC^{-R.irregularis}, RC^{S.tuberosum}/HC^{+R.irregularis} and RC^{S.tuberosum}/HC^{-R.irregularis}) comprised 6 Petri plates, thus 12 wells in total. Twenty μL of a solution of *S. meliloti* at a concentration of $9 \times 10^5 \text{ CFU} \cdot \text{mL}^{-1}$ (OD₆₀₀ = 0.1) was gently mixed to the liquid MSR^{min0N} in the wells. To avoid boundary effects, the wells with MSR^{min0N} and bacteria were located at the center of the microplate, whereas 200 μL of fresh MSR^{min0N} medium without bacteria were added to the surrounding wells (thus corresponding to 48 wells). The microplate was then placed in a Multiskan FC Microplate Photometer (Thermo Fisher Scientific Inc., Waltham, MA) and incubated at 30°C for 72 hours. The absorbance at OD = 595 nm was recorded each hour to evaluate the bacterial growth in each well. To convert OD₅₉₅ to CFU, 0.4 mL of *S. meliloti* in its logarithmic growth phase was introduced into 20 mL of YEB medium. This mixture was then incubated at 28°C during 4 hours to reach a stationary phase. The culture at stationary phase was serially diluted at specified ratios (5, 10, 50, 100, and 200), utilizing PBS as the diluent. Following the calibration of the spectrophotometer with PBS, the absorbance at 595 nm was recorded for varying concentrations of the bacterial solution. Subsequently, 1 mL from each dilution was plated on YEB agar medium ($15 \text{ g} \cdot \text{L}^{-1}$ agar) supplemented with $5 \mu\text{g} \cdot \text{mL}^{-1}$ tetracycline and evenly spread using an L-shaped cell spreader (VWR). The Petri plates were incubated for 24 hours at 28°C in a dark chamber, after which the CFUs of *S. meliloti* were counted visually. The number of bacterial colonies for each dilution was documented, and the average colony count for each dilution level was calculated. A standard curve correlating absorbance values (X-axis) to

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the CFU (Y-axis) was generated using Microsoft Excel. The conversion from OD₅₉₅ to CFU was done using the derived formula:

$$\text{CFU} = \frac{\text{OD value} - 0.0072}{0.1016} \times 10^6$$

For gene expression, two mL of *S. meliloti* at a concentration of 5.4×10^6 CFU·mL⁻¹ was added to the HC in each treatment. Samples were taken at 0, 2, 4, 6, 12, and 24 hours, frozen in liquid N, and stored at -80°C for RNA extraction and gene expression analysis. The complete genome sequence of *S. meliloti* 2011 can be accessed at the National Center for Biotechnology Information (NCBI) under GenBank assembly accession number NC_020528.1. Eight genes (i.e., *nodA*, *nodB*, *nodC*, *nodD1*, *nodD2*, *nodD3*, *nodI*, *nodJ*) from *S. meliloti* were selected. The primers for the selected genes and their subunits were designed using Primer 5 software. Detailed information regarding the primer sequences and selected genes are presented in **Table S1**. Total RNA was extracted from the frozen solution of *S. meliloti* using the RNeasy Mini Kit (Qiagen), following the manufacturer's protocol, and subsequently treated with the TURBO DNAfree Kit (Ambion) to eliminate possible DNA contamination. To generate single-stranded cDNA, reverse transcription was conducted using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche) with 1 µL random primer. The reaction comprised the following steps: incubation at 65°C for 10 min, 55°C for 20 min, and 85°C for 5 min. The final volume of the cDNA was 20 µL and was diluted to 100 µL. The qRT-PCR was conducted using the LightCycler 96 Real-Time PCR System (Roche). The reaction mixture consisted of 2.5 µL cDNA, 5 µL 2 × FastStart Essential DNA Green Master (Roche), and 0.5 µL of each primer (5 µM), in a total reaction volume of 10 µL. The PCR was performed according to the following program: initial denaturation at 95°C for 60 s, followed by 45 cycles at 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. The run ended with a melting curve analysis to avoid nonspecific PCR products. The Ct values and baseline range were determined using the LightCycler 96 software. The relative quantification was evaluated using the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen, 2001), with *SMc00128* serving as the housekeeping gene. The transcript levels were normalized against the expression value in the RC^{*S.tuberosum*}/HC-*R.irregularis* treatment.

***In vitro* experimental design for the analysis of *S. meliloti* migration along AM fungal hyphae and cytoplasmic flow velocity within hyphae**

The experimental setup followed the flavonoid analysis design, with the HC consisting of solid MSR^{min0N} medium (Section 3; **Figure 23a**). At week 13, approximately 80% of the HC surface was covered with an extensive ERM.

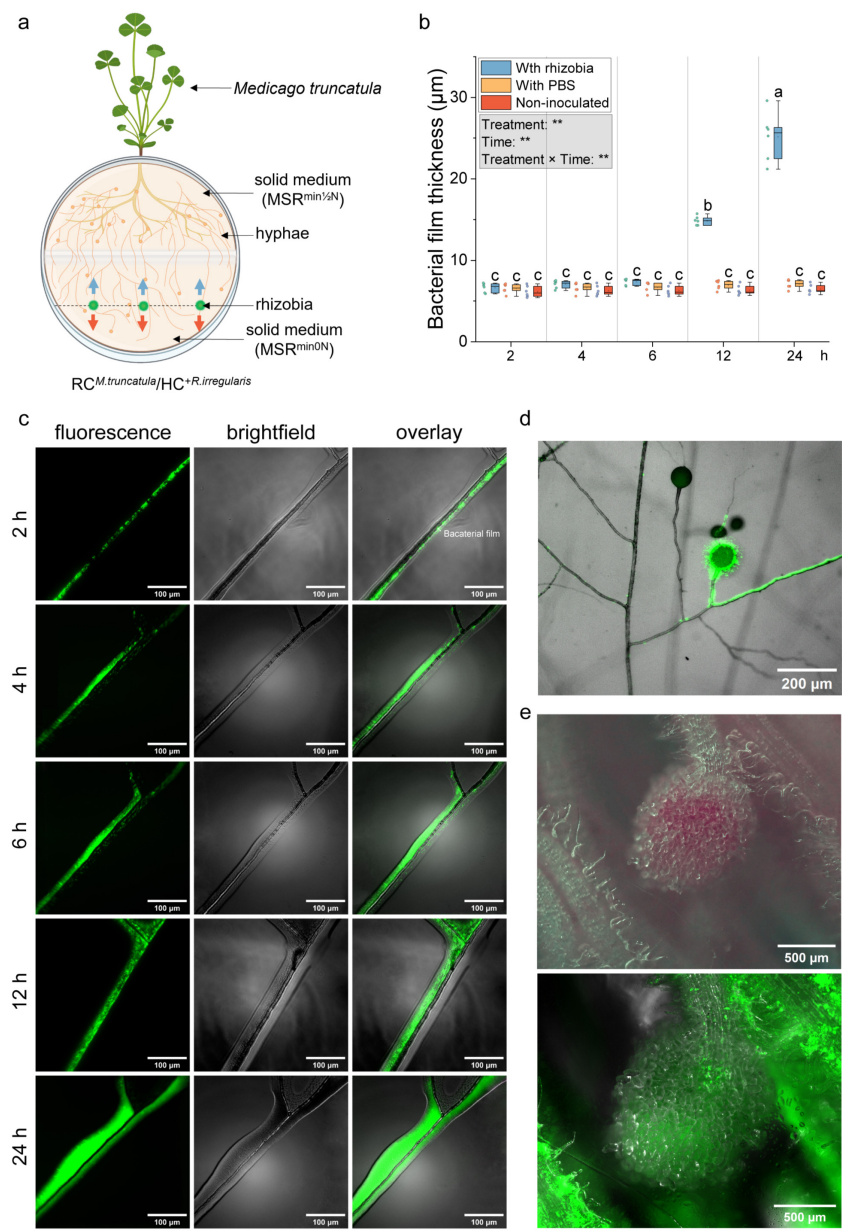


Figure 23. Interactions between *R. irregularis* hyphae and *S. meliloti*. **(a)** Schema of the bi-compartmented Petri plate system used to study the impact of ERM on *S. meliloti* migration. The system includes a root compartment (RC) and a hyphal compartment (HC) separated by a plastic barrier. *M. truncatula* roots were placed in the RC, with shoots extending through a 2 mm hole. RC contained 20 mL MSR^{min¹/₂N} medium (1.99 mM N), and HC contained 20 mL MSR^{min⁰N} medium (0 mM N). Green dots indicate *S. meliloti* inoculation sites; blue and red arrows show the directions of bacterial growth along hyphae towards and away from the RC. **(b)** Bacterial film thickness (μm) of *R. irregularis* inoculated with *S. meliloti* or PBS or non-inoculated, measured at 2, 4, 6, 12, and 24 hours. The box plot displays the maximum, upper quartile, median, lower quartile, minimum values. Hollow squares represent mean values, dots represent individual measurements. Data ($n = 6$) were analyzed by a one-way ANOVA followed by a Tukey post-hoc test ($P \leq 0.05$). Different lowercase letters above the box plots indicate significant differences among each treatment. In the gray box, the results of the two-way ANOVA on “Treatment” and “Time” are presented, where ** represents $P \leq 0.01$. **(c)** *S. meliloti* migration along *R. irregularis* hyphae in the HC of Petri plates with *M. truncatula* in the RC, shown under fluorescence, brightfield, and overlay views at 2, 4, 6, 12, and 24 hours. **(d)** Microscopic picture of *S. meliloti* colonizing the surface of spores. **(e)** Microscopic picture of the nodule formed on the root of *M. truncatula*. The upper picture is the mature nodule under bright-field microscopy, and the lower picture is the nodule under fluorescent microscopy.

***S. meliloti* migration along hyphae** – In each Petri plate, three hyphae, with active cytoplasmic/protoplasmic flow, developing on the surface of the HC and separated from each other by several mm, were individually inoculated with one drop of 1 μL of *S. meliloti* at a concentration of 9×10^5 CFU·mL⁻¹ (OD₆₀₀ = 0.1). Two control treatments were included under strictly identical conditions: (1) hyphae inoculated with 1 μL PBS and (2) non-inoculated hyphae. Six Petri plates (i.e., replicates) were considered per treatment. The speed of colonization of the hyphae surface was quantified by measuring the distance traveled by *S. meliloti* along the hyphae from the point of inoculation divided by the time post inoculation (i.e., after 2, 4, 6, 12 and 24 h).

Velocity of cytoplasmic/protoplasmic flow in hyphae – Another set of 18 Petri plates were used to evaluate cytoplasmic/protoplasmic flow within the hyphae. Three hyphae developing on the surface of the HC and separated from each other by several mm were each inoculated with one drop of 1 μL of *S. meliloti* at a concentration of 9×10^5 CFU·mL⁻¹ (OD₆₀₀ = 0.1) and cytoplasmic/protoplasmic flow within the hyphae measured. Two control treatments were included under

strictly identical conditions: (1) hyphae inoculated with PBS and (2) non-inoculated hyphae. Six Petri plates (i.e., replicates) were considered per treatment.

Migration speed, flow velocity, and bacterial film thickness were measured as per Section 6 of MATERIALS AND METHODS.

In vitro* and greenhouse experimental designs with legumes linked to non-legumes by a CMN for mycelia-based migration assay of *S. meliloti

***In vitro* experimental design** – A modified quadri-compartmented Petri plate (12.4×8.5 cm) was used, comprising three compartments: two RCs containing *Medicago truncatula* in one and *Solanum tuberosum* in the other, both associated with *Rhizophagus irregularis*, separated by a plastic barrier from a central hyphal compartment (HC) (**Figure 24a**; **Figure S3**), as modified from Voets et al. (2006). Each RC received 25 mL of $\text{MSR}^{\text{min}/2\text{N}}$ medium, and the HC 25 mL of $\text{MSR}^{\text{min}0\text{N}}$ medium, both solidified with $3 \text{ g} \cdot \text{L}^{-1}$ Phytagel. Mycorrhizal plants were prepared using the mycelium donor plant (MDP) system (**Figure 15**) described earlier (St-Arnaud et al., 1996). Five mL of $\text{MSR}^{\text{min}/2\text{N}}$ medium cooled to 40°C was added each week in the RCs to keep medium at an adequate level. At week 13, the ERM crossed the partition wall separating the RCs from the HC and developed profusely in the HC. A significant number of hyphae connected in the HC creating a CMN between both plants. Three single hyphae per system developing on the surface of the $\text{MSR}^{\text{min}0\text{N}}$ medium and clearly connecting the plants in both RCs were inoculated with one drop of $1 \mu\text{L}$ of bacterial suspension at a concentration of $9 \times 10^5 \text{ CFU} \cdot \text{mL}^{-1}$ ($\text{OD}_{600} = 0.1$). Inoculation was done on the hyphae in the middle of the HC (**Figure 24a**). The distance moved by *S. meliloti* in the direction of *M. truncatula* or *S. tuberosum* as well as the cytoplasmic flow velocity in the hyphae and thickness of the bacterial film around hyphae were measured as described above. Three hyphae of six independent replicates (i.e., quadri-compartmented Petri plates) were considered.

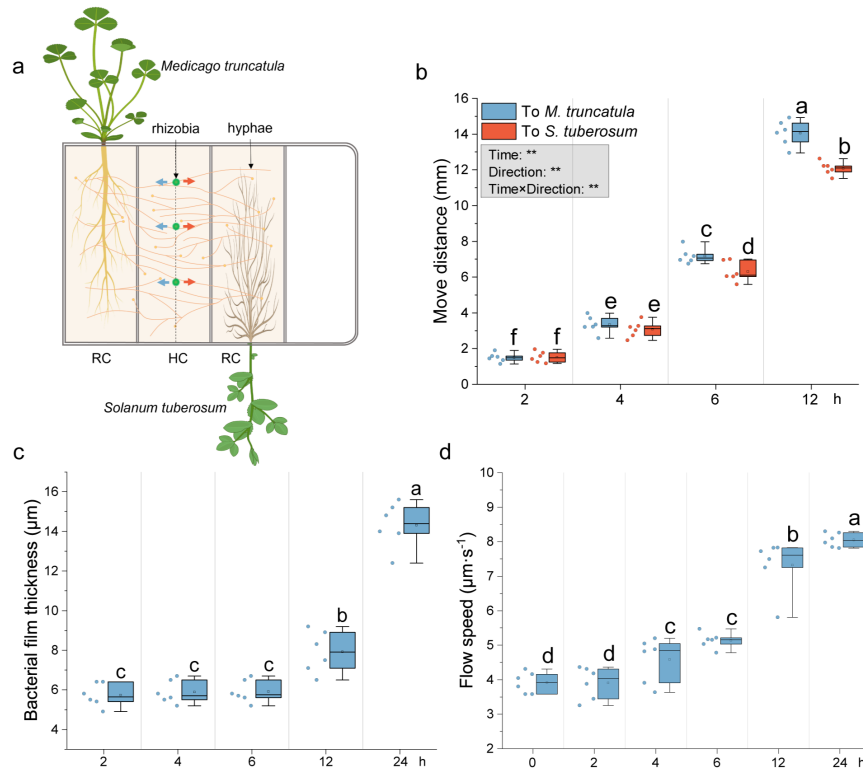


Figure 24. Migration of *S. meliloti* on the surface of hyphae connecting a leguminous plant (*M. truncatula*) to a non-leguminous plant (*S. tuberosum*). **(a)** Schema of the *in vitro* tri-compartmented system. This system uses three compartments of a quadri Petri plate: two root compartments (RCs) containing *M. truncatula* and *S. tuberosum* and a hyphal compartment (HC) where hyphae connect the two plants. After 13 weeks, hyphae from both RCs crossed into the HC, forming numerous anastomoses. *S. meliloti* was then added to the surface of hyphae in the middle of the HC, and its migration was measured in both directions. Green dots indicate *S. meliloti* inoculation points. Blue arrows show bacterial growth towards *M. truncatula*; orange arrows show growth towards *S. tuberosum*. **(b)** Migration distance (mm) of *S. meliloti* along hyphae towards *M. truncatula* and *S. tuberosum*. **(c)** Effects of *S. meliloti* on bacterial film thickness (μm) at 2, 4, 6, and 24 hours. **(d)** Cytoplasmic/protoplasmic flow speed (μm·s⁻¹) in *R. irregularis* hyphae connecting *M. truncatula* and *S. tuberosum*, measured at 0, 2, 4, 6, and 24 hours post-inoculation. The box plot displays the maximum, upper quartile, median, lower quartile, minimum values. Hollow squares represent mean values, dots represent individual measurements. Data ($n = 6$) were analyzed by a one-way ANOVA followed by a Tukey post-hoc test ($P \leq 0.05$). Different lowercase letters above the box plots indicate significant differences among each

treatment. In the gray box, the results of the two-way ANOVA on “Treatment” and “Time” are presented, where ** represents $P \leq 0.01$.

Greenhouse experimental design – A quadri-compartmented pot system was designed comprising a central compartment (CC, 0.3 L) connected to three satellite compartments (0.3 L each) with plastic pipes (PVC pipe, 1.7 cm diameter $\times$ 10 cm length) (Figure 25a; Figure S5). The pipes were perforated in the upper part with six holes (6 mm diameter) separated from each other by 1 cm. The holes were used to collect the substrate (including the hyphae and bacteria). *P. lanceolata* was planted in the CC, and the three satellite compartments with *M. truncatula* associated (*M. truncatula*^{+R.i.}) or not (*M. truncatula*^{-R.i.}) to *R. irregularis* and *P. lanceolata* not associated to *R. irregularis* (*P. lanceolata*^{-R.i.}). The substrate was a 2:1 (v/v) sand-vermiculite mixture, autoclaved twice at 0.11 MPa, 121°C for 15 min. The *M. truncatula*^{+R.i.} satellite compartment received 5 g of *R. irregularis* inoculum and was connected to the CC via a pipe sealed at both extremities with a 41 μ m mesh to allow only hyphae and not the roots to proliferate in the tube. The *M. truncatula*^{-R.i.} and *P. lanceolata*^{-R.i.} satellite compartments were not inoculated by the AM fungus and were connected to the CC with the pipes sealed at both extremities with 5 μ m mesh to prevent the crossing of hyphae and roots coming from the other compartments. *P. lanceolata* was planted in the CC only to check whether the hyphae from the *M. truncatula*^{+R.i.} reached the CC. Once this was evidenced by root colonization assessment (McGonigle et al., 1990), it indicates that the AM fungi hyphae from the *M. truncatula*^{+R.i.} treatment had reached the CC through the pipes. *P. lanceolata* in the CC were then removed, and after another 4 weeks (to allow mycelium regrowth in the CC), *S. meliloti* (5 mL of 9×10^6 CFU $\cdot$ mL⁻¹ - OD₆₀₀ = 1) was inoculated in the CC using a 5 mL NORM-JECT silicone-free Luer Lock Syringe (Henke-Sass Wolf) and substrate samples were collected from the pipes of 5 randomly selected pot systems after 1, 3, 5, 7, and 9 days to evaluate *S. meliloti* migration. Samples were collected using 1 mL pipette tips cut at 3 cm length from the extremity. The tips were inserted into each hole of the pipes and connected to a 1.5 mL centrifuge tube to collect the sample. The pipes were inverted and tapped to allow the substrate samples to fall into the 1.5 mL centrifuge tubes. The substrate samples in the pipes were subsequently weighed. One mL of PBS was further added to each centrifuge tube and the tubes were vortexed on an MS1 shaker (IKA, Germany) at 1000 rpm for 20 s to extract the bacteria. A 20 μ L suspension was retrieved from the aforementioned centrifuge tube and mixed with 180 μ L of PBS to achieve a 10-fold dilution. For the growth of *S. meliloti*, a 20 μ L volume of the diluted solution was added to YEB solid medium (15 g $\cdot$ L⁻¹ agar) supplemented with 5 μ g $\cdot$ mL⁻¹ of tetracycline. The mixture was then spread using an L-Shape Cell Spreader (VWR). Bacteria were cultured for 48 hours in a dark

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chamber at 28 °C. Then, the number of CFUs of *S. meliloti* were counted in each Petri plate using the Echo Revolve RVL2-K microscope (<https://discover-echo.com/revolve/>) and converted into CFUs per g of substrate sample.

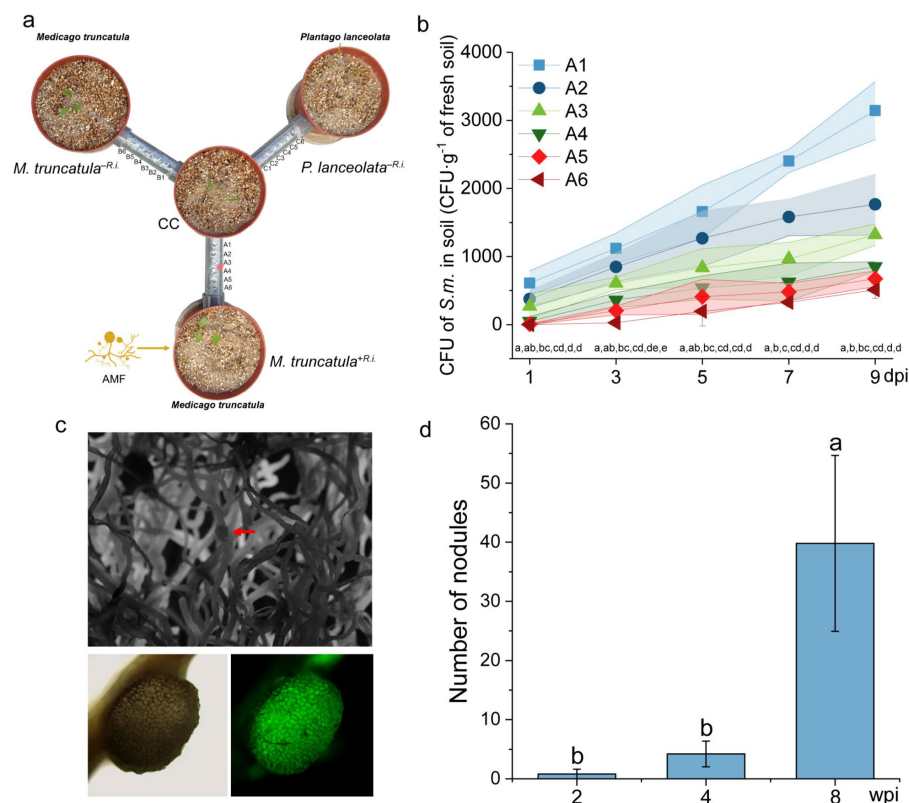


Figure 25. Impact of extraradical mycelium of the AM fungus on the migration of *S. meliloti* in a quadri-compartmented pot device. **(a)** Schema of the quadri-compartmented pot device. *P. lanceolata* was planted in the central compartment (CC), whereas *M. truncatula* (with or without AM fungus, *M. truncatula*^{+R.i.} and *M. truncatula*^{-R.i.}) and *P. lanceolata* (without AM fungus, *P. lanceolata*^{-R.i.}) were planted in three satellite compartments. These were connected to the CC via PVC pipes (1.7 cm diameter, 10 cm long) with six evenly spaced 6 mm holes for substrate sampling (**Figure S5**). The *M. truncatula*^{+R.i.} compartment was connected to the CC with pipes covered by a 41 µm mesh to allow only hyphae passage. The other compartments were connected via pipes covered with 5 µm mesh to block both hyphae and roots. *P. lanceolata* in the CC was used to confirm hyphal growth into the CC; after confirmation, the plants were removed. After one month of ERM regrowth, *S. meliloti* was inoculated in the CC, and substrate

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samples were collected over time to assess rhizobia motility. **(b)** Concentration of *S. meliloti* (CFU·g⁻¹ fresh substrate) in the different holes (A1 to A6) of the pipes connecting the CC to the *M. truncatula*^{+R.i.} pots at 1, 3, 5, 7, and 9 dpi. Data are means ± SD (*n* = 5). Shaded areas represent error bars. **(c)** Microscopic pictures of nascent nodules developing on *M. truncatula*^{+R.i.} roots two weeks after inoculation with *S. meliloti* in the CC. **(d)** Average number of nodules on *M. truncatula*^{+R.i.} roots at 2, 4, and 8 weeks post-inoculation. Data (means ± SD, *n* = 5) were analyzed by one-way ANOVA followed by the Tukey post-hoc test (*P* ≤ 0.05). Different lowercase letters above the bars indicate significant differences among each treatment. No nodules were observed in *M. truncatula*^{-R.i.} and *P. lanceolata*^{-R.i.} treatments throughout the experiment (Data not shown in the Figure).

AM fungal root colonization, hyphal length in pipes, and nodule maturity were assessed at 2, 4, and 8 weeks post-inoculation in five randomly selected systems, using methods detailed in sections 4 and 5 of MATERIALS AND METHODS chapter.

Data analysis

Prior to statistical analysis, the homogeneity of variance and normality of distribution were assessed using the Levene and Shapiro-Wilk tests, respectively. To attain normality and homoscedasticity of data, all the variables were transformed by taking the base 10 logarithm. Analysis of variance was conducted in SAS (8.1) to evaluate the statistical significance of the experimental data. Significance between treatments was determined at 0.05 using the Tukey post-hoc test.

The *in silico* binding studies was made according to the protocol described in Bag et al. (2022). Fourteen flavonoids were considered (see **Table S2**) as well as three proteins from *Sinorhizobium meliloti*: NodD1 (UniProtKB-P03031), NodD2 (UniProtKB-P08719), and NodD3 (UniProtKB-P23190) from the UniProt Knowledgebase (<https://www.uniprot.org/help/uniprotkb>). The 3D structures of the flavonoids were obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov>) as SDF files and subsequently converted into PDB files using PyMOL 3.0 (Schrödinger, LLC, 2015). For flavonoids lacking available 3D structures in PubChem, the Chem3D (version 21.0.0) software was utilized to generate their 3D conformations. The 3D structures of the NodD proteins (NodD1, NodD2, and NodD3) were modeled using the SWISS-MODEL tool (<https://swissmodel.expasy.org>) based on their respective FASTA sequences.

Molecular docking studies were conducted using AutoDock 4.2 to form complexes between each NodD protein and each flavonoid, to determine their binding affinities ($\text{Kcal}\cdot\text{mol}^{-1}$) (Morris et al., 2009). Additionally, LigPlot was used to identify the specific interacting residues within these complexes, providing further insight into the molecular interactions between the flavonoids and the NodD proteins (Laskowski and Swindells, 2011).

Results

***S. meliloti* promotes the growth of *M. truncatula* on the AM fungi-compatible MSR medium containing reduced or no N**

We first tested whether the MSR^{min} medium developed for growing AM fungi on root organs (Declerck et al., 1998) or whole plants (Voets et al., 2009) was adequate for the growth and nodulation of *M. truncatula* in the presence of *S. meliloti*. We compared the growth and nodulation of the plants associated or not to the bacterium on the $\text{MSR}^{\text{min}0\text{N}}$ and $\text{MSR}^{\text{min}1/2\text{N}}$ media (**Figure 21a; Figure S1**). In the $\text{MSR}^{\text{min}0\text{N}+S.meliloti}$ treatment, the growth of *M. truncatula* was strongly limited (**Figure 21b, c**), and no nodule was observed (**Figure 21d**). Conversely, in the presence of *S. meliloti*, shoot and root dry weight increased significantly in the $\text{MSR}^{\text{min}1/2\text{N}+S.meliloti}$ treatment, with increases of 253.6% and 104.9% in month 1, 256.0% and 285.2% in month 2, and 174.2% and 259.1% in month 3, respectively, compared to the $\text{MSR}^{\text{min}0\text{N}+S.meliloti}$ treatment (**Figure 21b, c**). Whatever the time of observation, the number of nodules was three to four times higher in the $\text{MSR}^{\text{min}1/2\text{N}+S.meliloti}$ treatment than in the $\text{MSR}^{\text{min}0\text{N}+S.meliloti}$ treatment (**Figure 21d**). Overall, it appeared that the $\text{MSR}^{\text{min}1/2\text{N}}$ medium is suitable for the growth and nodulation of *M. truncatula* in presence of *S. meliloti* and was therefore selected for all following *in vitro* experiments.

ERM transports flavonoids from roots to medium

We used a bi-compartmented system separating AM fungal-colonized plants (RC) from ERM of *R. irregularis* (HC) (**Figure 22a**) to assess flavonoid transport by hyphae. LC-MS/MS analysis of the HC revealed 20,081 m/z features across four treatments. Principal component analysis (PCA) (**Figure 22b**) showed distinct clustering, i.e., the samples from both plant species, both in the presence or absence of the AM fungus, are clustering separately, indicating the crucial influence of AM fungal hyphae on the transport of plant compounds such as flavonoids.

We retained features with a minimum intensity of 1,000 counts in at least one sample group, resulting in 13,513 features. ANOVA analysis ($P \leq 0.05$) identified 11,662 significant features. MS-FINDER structural annotation of these features recorded MS/MS spectra for 2,056 features, identifying 441 potential metabolites, with 23 annotated as flavonoids (**Supplemental dataset 1**). Eight flavonoids were found exclusively in $RC^{M.truncatula}/HC^{+R.irregularis}$ treatment, suggesting transport from *M. truncatula* roots via AM fungal hyphae. One of these was a luteolin derivative, known to attract *S. meliloti* and induce nodulation genes (Peters et al., 1986). Three compounds were present in both $RC^{M.truncatula}/HC^{+R.irregularis}$ and $RC^{S.tuberosum}/HC^{+R.irregularis}$ treatments, and two features were unique to $RC^{S.tuberosum}/HC^{+R.irregularis}$ treatment.

ERM exudates impact the growth and gene expression in *S. meliloti*

We investigated the influence of exudates from ERM in the HC connected to *M. truncatula* or *S. tuberosum* in the RC on *S. meliloti* growth and nodulation genes expression. The growth of *S. meliloti* was analyzed over a 72-h period (**Figure 22c**). In treatments without hyphae in the HC (control), slow bacterial growth was observed with no significant differences between $RC^{M.truncatula}/HC^{-R.irregularis}$ and $RC^{S.tuberosum}/HC^{-R.irregularis}$ treatments. Conversely, with hyphae in the HC (with ERM exudates), *S. meliloti* growth was significantly higher. The growth pattern was sigmoidal, with a minimal increase during the first 4 and 10 hours for $RC^{M.truncatula}/HC^{+R.irregularis}$ and $RC^{S.tuberosum}/HC^{+R.irregularis}$ treatment, respectively (**Figure 22c**), followed by exponential growth, plateauing at 30 and 60 hours, respectively. From the 3rd hour onward, CFUs in $RC^{M.truncatula}/HC^{+R.irregularis}$ exudates were significantly higher than with $RC^{S.tuberosum}/HC^{+R.irregularis}$ exudates. These results indicate that ERM exudates stimulate bacterial growth, especially when connected to the legume.

We further investigated whether the MSR^{min0N} medium in the HC containing hyphae connected to *M. truncatula* or *S. tuberosum* in the RC could trigger Nod factor expression in *S. meliloti*. The expression of eight *nod* genes (*nodD1*, *nodD2*, *nodD3*, *nodA*, *nodB*, *nodC*, *nodI*, and *nodJ*) was analyzed at 0, 2, 4, 6, 12, and 24 hours after adding the bacterium to the medium (**Figure 26**; **Figure 27**). No significant differences in gene expression were observed between the two control treatments at any time point.

Focusing on the *nodD* genes, which code for the transcription factor NodD, we found that *nodD1* expression was significantly higher in the $RC^{M.truncatula}/HC^{+R.irregularis}$ treatment compared to both controls and the $RC^{S.tuberosum}/HC^{+R.irregularis}$ treatment at 6, 12, and 24 hours. In the

$RC^{S.tuberosum}/HC^{+R.irregularis}$ treatment, *nodD1* expression was significantly higher at 6 hours but lower at other times compared to both controls (**Figure 26a**). For *nodD2*, expression was significantly higher in the $RC^{M.truncatula}/HC^{+R.irregularis}$ treatment at 2, 4, 6, 12, and 24 hours compared to both controls, and except at 0 and 2 hours, it was also higher than in the $RC^{S.tuberosum}/HC^{+R.irregularis}$ treatment. In the $RC^{S.tuberosum}/HC^{+R.irregularis}$ treatment, *nodD2* expression was only significantly higher than both controls at 2 hours (**Figure 26b**). The *nodD3* expression was significantly higher in the $RC^{M.truncatula}/HC^{+R.irregularis}$ treatment at 0, 2, 6, 12, and 24 hours, and in the $RC^{S.tuberosum}/HC^{+R.irregularis}$ treatment at 0, 2, 6, and 24 hours compared to both controls. Additionally, the $RC^{S.tuberosum}/HC^{+R.irregularis}$ treatment showed higher *nodD3* expression compared to the $RC^{M.truncatula}/HC^{+R.irregularis}$ treatment at 0 hours, with the reverse observed at later times (**Figure 26c**).

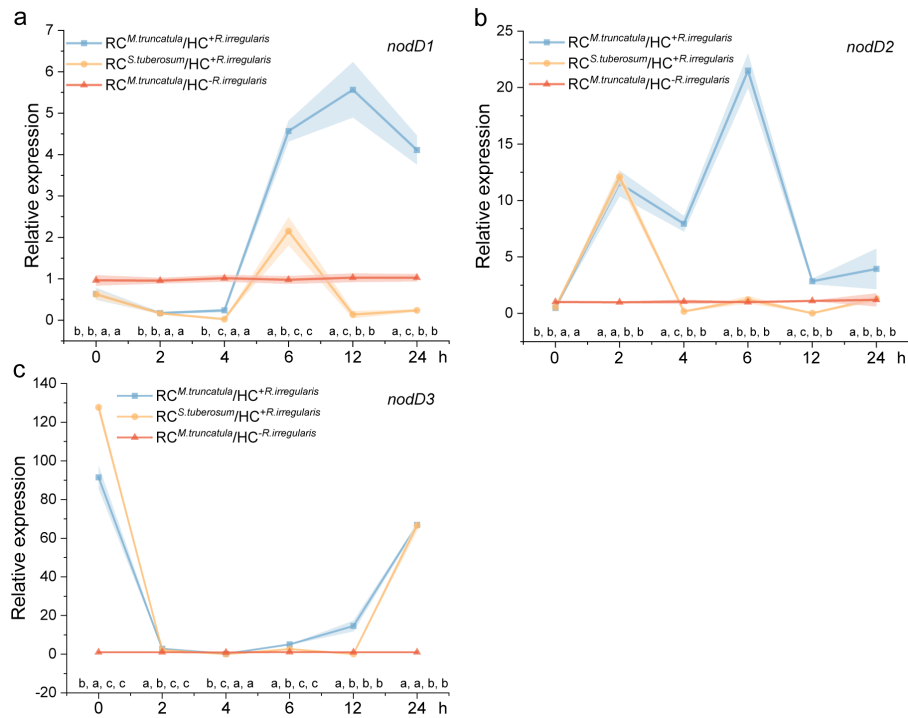


Figure 26. Impact of ERM exudates on the *nod* gene expression of *S. meliloti*. Relative expression of *nodD1* (a), *nodD2* (b), and *nodD3* (c) in *S. meliloti* at 0, 2, 4, 6, 12, and 24 hours after inoculation in the hyphal compartment (HC) of four treatments: $RC^{M.truncatula}/HC^{+R.irregularis}$, $RC^{M.truncatula}/HC^{-R.irregularis}$, $RC^{S.tuberosum}/HC^{+R.irregularis}$, and $RC^{S.tuberosum}/HC^{-R.irregularis}$. Expression values were normalized to the $RC^{S.tuberosum}/HC^{-R.irregularis}$ treatment (not shown). Shaded areas represent error bars. Data ($n = 6$) are means $\pm$ SD and were analyzed by one-way ANOVA with Tukey's post-hoc test ($P \leq 0.05$). Different lowercase letters above

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each time point on the X-axis indicate significant differences between treatments at that time point, with the letters in sequence representing $RC^{M.truncatula}/HC^{+R.irregularis}$, $RC^{M.truncatula}/HC^{-R.irregularis}$, $RC^{S.tuberosum}/HC^{+R.irregularis}$, and $RC^{S.tuberosum}/HC^{-R.irregularis}$.

The structural *nod* genes were further analyzed. The expression of *nodA* was significantly higher in the $RC^{M.truncatula}/HC^{+R.irregularis}$ treatment at 4, 6, 12, and 24 hours compared to both controls and the $RC^{S.tuberosum}/HC^{+R.irregularis}$ treatment (**Figure 27a**). In the $RC^{S.tuberosum}/HC^{+R.irregularis}$ treatment, *nodA* expression was significantly higher at 2, 4, 6, and 12 hours compared to both controls (**Figure 27a**). For *nodB*, the $RC^{M.truncatula}/HC^{+R.irregularis}$ treatment showed significantly higher expression compared to both controls at all time points (**Figure 27b**). In contrast, the $RC^{S.tuberosum}/HC^{+R.irregularis}$ treatment had significantly higher *nodB* expression than the controls at 0, 2, and 6 hours, but significantly lower at 12 hours (**Figure 27b**). When comparing both treatments, *nodB* expression was significantly higher in the $RC^{M.truncatula}/HC^{+R.irregularis}$ treatment at 4, 6, 12, and 24 hours, and significantly lower at 0 and 2 hours, compared to the $RC^{S.tuberosum}/HC^{+R.irregularis}$ treatment (**Figure 27b**). The expression of *nodC* was significantly higher in both $RC^{M.truncatula}/HC^{+R.irregularis}$ and $RC^{S.tuberosum}/HC^{+R.irregularis}$ treatments compared to both controls at 0, 2, and 24 hours (**Figure 27c**). The *nodC* expression was not significantly different between the two treatments, except at 2 hours where it was higher in the $RC^{S.tuberosum}/HC^{+R.irregularis}$ treatment, and at 4, 12, and 24 hours where it was higher in the $RC^{M.truncatula}/HC^{+R.irregularis}$ (**Figure 27c**). For *nodI*, both $RC^{M.truncatula}/HC^{+R.irregularis}$ and $RC^{S.tuberosum}/HC^{+R.irregularis}$ treatments showed significantly higher expression compared to both controls only at 24 hours, with lower expression at all other time points, except 0 and 4 hours (**Figure 27d**). The expression of *nodJ* in the $RC^{M.truncatula}/HC^{+R.irregularis}$ treatment was significantly higher compared to both controls at 2 and 4 hours, and significantly lower at 6 and 12 hour (**Figure 27e**). In the $RC^{S.tuberosum}/HC^{+R.irregularis}$ treatment, *nodJ* expression was significantly higher than both controls only at 2 hours and significantly lower at all other time points, except at 24 hours where there was no difference (**Figure 27e**). Additionally, *nodJ* expression was significantly higher in the $RC^{M.truncatula}/HC^{+R.irregularis}$ treatment at 0, 2, 4, and 6 hours compared to the $RC^{S.tuberosum}/HC^{+R.irregularis}$ treatment (**Figure 27e**). Overall, the expression of most genes (*nodA*, *nodB*, *nodD1*, *nodD2* and *nodD3*) significantly increased after the 6th hour in the $RC^{M.truncatula}/HC^{+R.irregularis}$ treatment suggesting that these genes play a crucial role throughout the regulatory process.

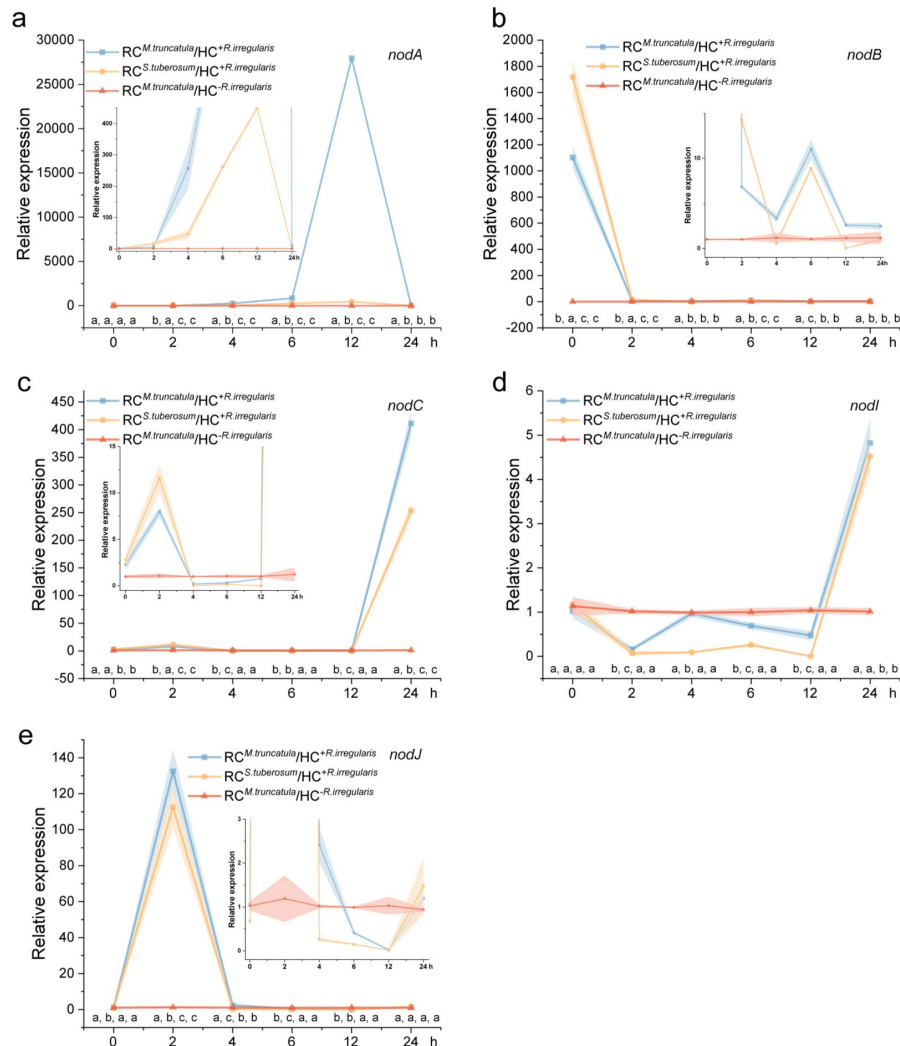


Figure 27. Impact of ERM exudates on the relative expressions of *nodA* (a), *nodB* (b), *nodC* (c), *nodI* (d) and *nodJ* (e) in *S. meliloti* at 0, 2, 4, 6, 12, and 24 hours after inoculation of the bacteria in the HC of $RC^{M.truncatula}/HC^{+R.irregularis}$, $RC^{M.truncatula}/HC^{-R.irregularis}$, $RC^{S.tuberosum}/HC^{+R.irregularis}$ and $RC^{S.tuberosum}/HC^{-R.irregularis}$ treatments. The results were normalized by comparing the transcripts to the relative expression value in $RC^{S.tuberosum}/HC^{-R.irregularis}$ treatment (not shown in the figures). The light-colored background behind the lines on the figure represents the connecting error bars. Values are averages with SD. Data ($n = 6$) were analyzed by a one-way ANOVA followed by the Tukey post-hoc test ($P \leq 0.05$). Different lowercase letters above each time point on the X-axis indicate significant differences between treatments at that time point, with the letters in sequence representing $RC^{M.truncatula}/HC^{+R.irregularis}$, $RC^{M.truncatula}/HC^{-R.irregularis}$,

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RC*S.tuberosum*/HC⁺*R.irregularis* and RC*S.tuberosum*/HC[−]*R.irregularis*.
 RC*M.truncatula*/HC⁺*R.irregularis*: with *M. truncatula* in the RC and hyphae in the HC;
 RC*M.truncatula*/HC[−]*R.irregularis*: with *M. truncatula* in the RC but no hyphae in the HC;
 RC*S.tuberosum*/HC⁺*R.irregularis*: with *S. tuberosum* in the RC and hyphae in the HC;
 RC*S.tuberosum*/HC[−]*R.irregularis*: with *S. tuberosum* in the RC but no hyphae in the HC.

We then conducted a *in silico* binding study between eight flavonoids from RC*M.truncatula*/HC⁺*R.irregularis* treatment and two from RC*S.tuberosum*/HC⁺*R.irregularis* treatment with NodD1, NodD2, and NodD3 (**Figure 28f**; Annex II). Among all the ten flavonoids, sagittain B exhibited the most stable binding, with binding energies of -9.46 , -9.39 , and -9.08 Kcal·mol^{−1} for NodD1, NodD2, and NodD3, respectively. These results suggest that sagittain B, which is particularly present in RC*M.truncatula*/HC⁺*R.irregularis*, has a particularly strong binding affinity for NodD factors, suggesting a potentially dominant role in *S. meliloti* chemotaxis.

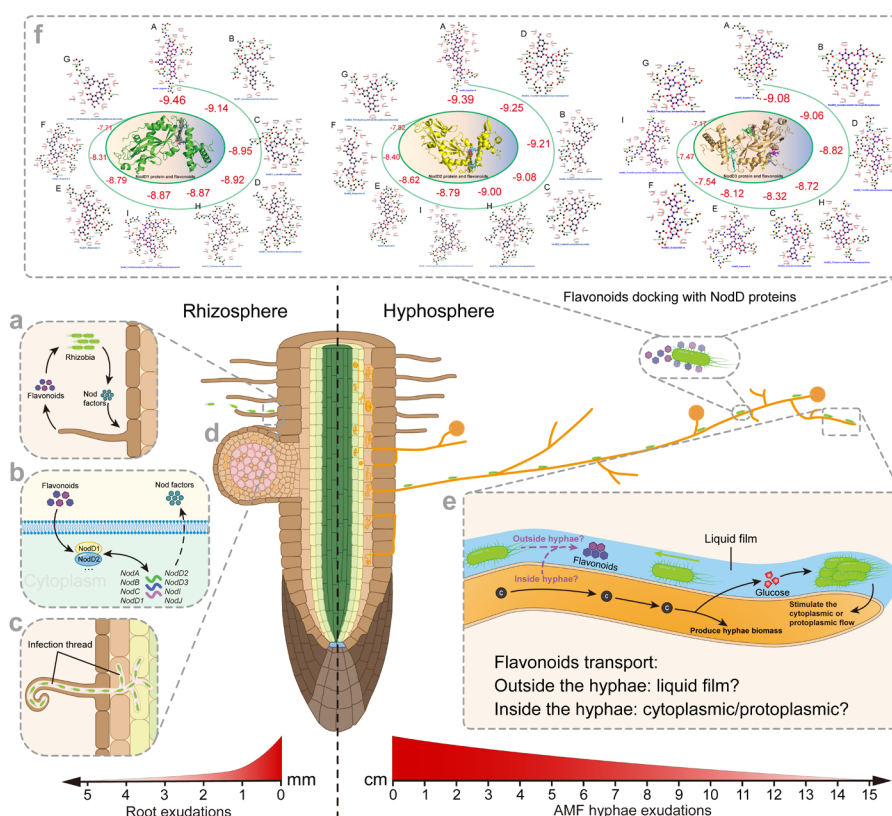


Figure 28. Schematic representation of the two pathways for AM fungi-colonized legumes to recruit Rhizobia. In the direct pathway (left side), root exudates mediate the short-distance recruitment (a few millimeters) of rhizobia via the

release of flavonoids **(a)**, the perception of which activates the production and secretion of rhizobial Nod factors **(b)**, which subsequently stimulate the root hairs to curl around the rhizobia, penetrating the hairs via an infection thread **(c)**, ultimately stimulating the formation of the nodule **(d)**. In the indirect pathway (right side), the extraradical mycelium of the AM fungus connected to the legume release sugars (i.e., glucose), stimulating rhizobial growth, and flavonoids, acting as signal molecules allowing the bacteria to sense the presence of a compatible host at far distances in the soil ($> 10\text{cm}$) **(e)**. In return, the rhizobia stimulate the cytoplasmic/protoplasmic flow increasing the release of exudates (i.e., nutrients and signals). A gradient of exudates/signals is established from roots to hyphal tips, attributable to the combination of (i) a dilution effect of exudates from base to tip of hyphae, (ii) a decrease in number and density of hyphae, (iii) a combination of root hairs and potentially hyphae release of exudates in the rhizosphere. This gradient plays a role as chemoattractant allowing the bacteria to move in the direction of the host plant. At this stage it is unknown whether the transport of flavonoids by hyphae is via a liquid film at the hyphae surface or via cytoplasmic/protoplasmic streaming within the hyphae. In silico binding studies of NodD1 (left), NodD2 (middle), and NodD3 (right) proteins of *S. meliloti* and 10 flavonoids **(f)**. Here NodD–flavonoid complexes were arranged based on their binding affinities (the more negative the values, $(\text{Kcal}\cdot\text{mol}^{-1})$, the stronger the interactions). Each NodD–flavonoid complex form hydrogen (green dotted line between amino acid and flavonoid) and hydrophobic (red half circles) bonds during NodD–flavonoid interaction. The central picture (inside the oval circle) predicts the binding sites of 9 flavonoids in NodD protein of *S. meliloti*. Individual figures are shown below. A: sagittain B1 and sagittain B2; B: isosakuranetin-hexosyl-deoxyhexose; C: luteolin-acetylhexuronide; D: taxifolin-deoxyhexosyl-oxanepentol; E: haploside C; F: sudachiin A; G: tetrahydroxy-tetramethoxyflavone-hexoside; H: trihydroxy-flavanone-hexosyl-pentose; I: trimethoxy-hydroxy-methyl-flavanone-dihexosyl-deoxyhexose. Sagittain B1 and Sagittain B2 were merged into a single flavonoid as their comparison results in the PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) were identical. The H and I are exclusively found in the $\text{RC}^{S.tuberosum}/\text{HC}^{+R.irregularis}$ treatment. Note: The proportions of elements in this schematic, including hyphae, roots, and rhizobia, are exaggerated for illustrative purposes and do not reflect their actual scale.

S. meliloti* efficiently colonizes the surface of *R. irregularis* hyphae and spreads rapidly along the ERM network to form nodules in *M. truncatula

To investigate the interactions between *S. meliloti* and the ERM of *R. irregularis*, we examined the movement of *S. meliloti* along the hyphae connected to *M. truncatula* (**Figure 23a; Figure S2**). Using time-lapse microscopy imaging, we

observed that *S. meliloti* inoculated onto hyphae moved as motile cells and formed thick (6.1 to 22.9 μm) multicellular colonies, resembling a biofilm (**Figure 23b, c**). The cells also colonized spore surfaces, forming multilayers (**Figure 23d**). After 14 d, the bacteria inoculated on the hyphae in the HC had migrated to the RC, forming functional nodules (**Figure 23e**).

We analyzed *S. meliloti* migration along hyphae towards plant roots in the RC. Starting 12 hours post-inoculation, *S. meliloti* migrated 47.1% farther toward RC than away from RC (**Figure 29a; Figure S4; Video S1**). The velocity of cytoplasmic flow in the hyphae significantly increased at 12 and 24 hours after inoculation, with no significant changes observed in the PBS and non-inoculated treatments (**Figure 29b; Video S2**).

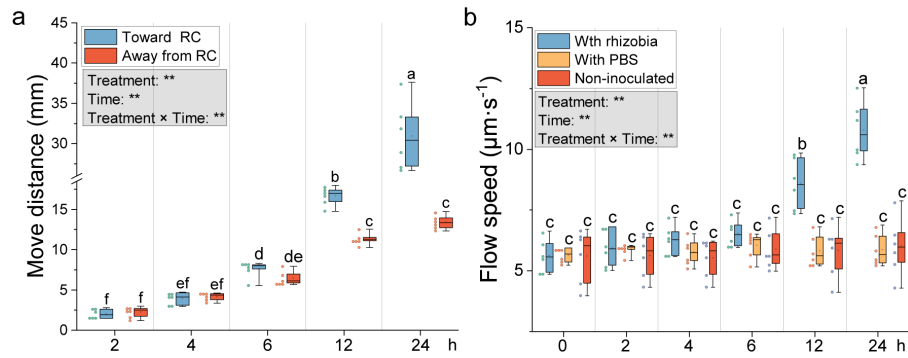


Figure 29. Microbial migration and cytoplasmic flow in mycorrhizal hyphae. **(a)** Migration of *S. meliloti* along *R. irregularis* hyphae in the HC connected to *M. truncatula* in the RC. Migration was measured towards the root (Toward RC) and away from the root (Away from RC) at 2, 4, 6, 12, and 24 hours after bacterial plating on the hyphae surface. **(b)** Cytoplasmic/protoplasmic flow within *R. irregularis* hyphae inoculated with *S. meliloti*, PBS or non-inoculated, measured at 2, 4, 6, 12, and 24 hours. The box plot displays the maximum, upper quartile, median, lower quartile, minimum values. Hollow squares represent mean values, dots represent individual measurements. Data ($n = 6$) were analyzed by a one-way ANOVA followed by a Tukey post-hoc test ($P \leq 0.05$). Different lowercase letters above the box plots indicate significant differences among each treatment. In the gray box, the results of the two-way ANOVA on “Treatment” and “Time” are presented, where ** represents $P \leq 0.01$.

Hyphae connecting *M. truncatula* to *S. tuberosum* favor the migration of *S. meliloti* towards the legume

To further investigate the role of plant-connected AM fungal hyphae in recruiting rhizobia, a tri-compartment setup (**Figure 24a**; **Figure S3**) was used. Microscopic observations indicated that rhizobia preferentially migrate towards *M. truncatula* at 6 and 12 hours (**Figure 24b**). Bacterial multilayer thickness increased significantly at 12 hours and was 1.8 times thicker at 24 hours, indicating rapid proliferation (**Figure 24c**). Cytoplasmic flow velocity in hyphae increased from 4 hours post-inoculation, suggesting high nutrient demand by proliferating rhizobia (**Figure 24d**).

In the quadri-compartment pot device (**Figure 25a**; **Figure S5**), the CMN was well established, with $82.5 \pm 4.4\%$ total colonization and $30.3 \pm 4.3\%$ arbuscule colonization in *P. lanceolata* within the CC (**Table S3**). This provided a solid foundation for studying *S. meliloti*'s ability to migrate along AM fungal hyphae. Bacterial migration along hyphae significantly increased, with $196 \text{ CFU} \cdot \text{g}^{-1}$ in hole A6 by day 5 (**Figure 25b**). Without ERM, CFUs were almost $0 \text{ CFU} \cdot \text{g}^{-1}$ in hole A6 (**Figure 30**). Fourteen days post-inoculation, the first nodule appeared (**Figure 25c**, d). After 4 weeks, *M. truncatula*^{+R.i.} had 4 ± 2 nodules per pot (10.5% mature), and after 8 weeks, 40 ± 15 nodules (63.1% mature) (**Figure 25d**). No nodules were observed in treatments without ERM, indicating that AM fungal hyphae are a primary pathway for the migration of rhizobia over long distances to the roots, leading to nodulation.

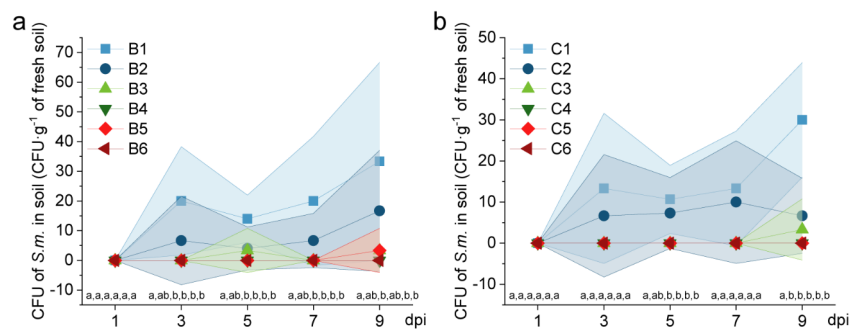


Figure 30. Concentration of *S. meliloti* ($\text{CFU} \cdot \text{g}^{-1}$ of fresh substrate) in the different holes in the pipes connecting the CC to the *M. truncatula*^{-R.i.} (B1 to B6) (**a**) or *P. lanceolata*^{-R.i.} (C1-C6) (**b**) at 1, 3, 5, 7, and 9 d post inoculation (dpi) after *S. meliloti* inoculation in the CC. Data ($n = 5$) are represented as means $\pm$ SD. The light-colored background behind the lines on the figure represents the connecting SD bars.

Discussion

The extraradical hyphae of AM fungi extend from plant roots to the soil, where they interact with microbial communities in the hyphosphere (the narrow zone of the soil influenced by the hyphae). This interface shapes microbial communities (Jin et al., 2024) and aids plants in acquiring nutrients such as P (Zhang et al., 2018a) and N (Hestrin et al., 2021). Studies indicate an increase in legume nodulation when colonized by AM fungi (Andrade et al., 2004; Hao et al., 2019), suggesting these microorganisms enrich the rhizosphere with competent rhizobia. However, the mechanisms by which AM fungal hyphae facilitate the movement of N-fixing rhizobia along their surface towards legume roots remain largely unknown. Here we have developed *in vitro* plate systems to test the migration of *S. meliloti* to leguminous and non-leguminous plants connected to AM fungi, and a greenhouse-based quadri-compartment pot device to test this migration in the presence or absence of AM fungal ERM connections. We demonstrated that, in addition to the short-distance direct pathway via root epidermal cells and root hairs releasing flavonoids (Cesco et al., 2010; Zhang et al., 2020), there is an indirect “mycorrhizal” pathway. In this pathway, rhizobia is able to move over long distances along the hyphae surface to the roots, leading to nodulation (**Figure 25**).

Similar to root exudates in mediating plant–microorganism interactions, fungal hyphae release C-rich compounds like sugars and organic acids into their environment (Hao et al., 2019; Hestrin et al., 2021), feeding the microorganisms developing at their surface. Some exudates also act as signaling molecules, such as fructose from *R. irregularis*, which triggers phytate mineralization by phosphate-solubilizing bacteria (Zhang et al., 2018a). In legume-rhizobia interactions, flavonoids released by plant roots are essential signaling molecules that activate nod gene transcription and Nod factor synthesis in rhizobia, leading to nodulation (Vierheilig et al., 1998b; Perret et al., 2000; Yuhashi et al., 2000; Maj et al., 2010). Our metabolomics approach compared flavonoid compounds transported by hyphae connected to *M. truncatula* and *S. tuberosum*. Flavonoids were detected in the hyphae-containing HC of both plants. However, eight unique flavonoids were found in the HC connected to *M. truncatula*, including three flavonol glycosides, two flavanone glycosides, two flavone glycosides, and one flavone glucuronide. Quercetin-3-O-galactoside and luteolin-7-O-glucoside (flavone glycosides) are known to promote *S. meliloti* growth (Hartwig et al., 1991), which may explain the increased growth observed in the RC^{*M. truncatula*}/HC^{+*R. irregularis*} treatment (**Figure 22c**). In contrast, only two kinds of flavanone glycosides were detected exclusively in the HC connected to *S. meliloti*.

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It remains unclear whether these compounds were transported inside the hyphae or along their surface.

Luteolin, which induces nodulation gene expression in *S. meliloti* (Peters et al., 1986; Hartwig et al., 1989), was detected as luteolin-acetylhexuronide in our study. Our metabolomic analysis showed that flavonol glycosides, which stimulate rhizobia, were exclusive to the RC^{*M.truncatula*}/HC^{*+R.irregularis*} treatment. *NodD1*, *nodD2*, and *syrM2* regulate the flavonoid-induced symbiotic gene expression and nodule growth (Kobayashi et al., 2004). External addition of isoflavones enhances nod-lacZ fusion expression in *Bradyrhizobium japonicum* (Banfalvi et al., 1988). Therefore, we hypothesize that flavonol glycosides may play an important role in regulating nod gene expression in *S. meliloti*. Further investigation, such as gene knockout studies or reporter gene assays, is needed to elucidate their precise mechanisms and effects.

NodD, beyond activating nod genes, indirectly influences chemotaxis by regulating genes involved in exopolysaccharide production, flagellar function, and chemotaxis receptors like McpV, facilitating rhizobial movement toward host plants (Suominen et al., 2003; Barnett and Long, 2015; Compton et al., 2018). NodD1 and NodD2 are the main flavonoid-sensing regulatory factors in *S. meliloti* (Machado et al., 1998; Peck et al., 2006; Barnett and Long, 2015). These highly homologous proteins directly respond to flavonoids secreted by host plant roots, activating the expression of *nod* genes, a crucial step in root nodule formation (Honma and Ausubel, 1987; Machado et al., 1998). In contrast, NodD3 functions through the transcriptional regulator SyrM, exerting its role under specific conditions and activating *nod* gene expression even in the absence of flavonoids (Dusha et al., 1999; Barnett and Long, 2015). This observation highlights the greater importance of NodD1 and NodD2 in flavonoid sensing, as they directly participate in the response to host plant signals. To validate the function of NodD binding to flavonoids, we performed *in silico* binding studies (Annex II). More negative binding affinity indicates stronger interaction (Owoloye et al., 2022). Due to the high homology between NodD1 and NodD2 proteins, our molecular docking analysis revealed that all flavonoids bind to the same region of these proteins (Annex II). Among the ten flavonoids detected in the RC^{*M.truncatula*}/HC^{*+R.irregularis*} and RC^{*S.tuberosum*}/HC^{*-R.irregularis*} treatments, sagittain B (belonging to flavonol glycosides) showed the highest negative binding affinity (i.e., $-9.46 \text{ Kcal} \cdot \text{mol}^{-1}$ with NodD1 and $-9.39 \text{ Kcal} \cdot \text{mol}^{-1}$ with NodD2) (**Figure 28f**). It was only found in the RC^{*M.truncatula*}/HC^{*+R.irregularis*} treatment and exhibited the highest integrated peak area simultaneously (**Supplemental dataset 1**). Meanwhile, NodD3 also plays a vital role in symbiotic signaling, particularly under flavonoid-independent conditions (Barnett and Long, 2015). It regulates the

expression of symbiotic genes through SyrM, thereby influencing the symbiotic process (Dusha et al., 1999; Barnett and Long, 2015). This flavonoid-independent role explains the high expression of *nodD3* at 0 hours (**Figure 26c**) in our study, followed by a rapid decrease due to the subsequent upregulation of *nodD2* (**Figure 26b**). Even though NodD3 does not require exogenous flavonoids for *nod* gene activation, the molecular docking analysis showed a high negative binding affinity for sagittain B (i.e., $-9.08 \text{ Kcal}\cdot\text{mol}^{-1}$) (Annex II). Thus, we could conclude that although flavonoids are less essential for NodD3, they could still act as signaling molecules to bind with NodD3 and modulate its interaction with other NodD proteins when sufficient external flavonoids are present. Moreover, NodD2 primarily induces *nod* gene expression and can compensate for the lack of NodD1 activity (del Cerro et al., 2015). Our results supported this, showing that when *S. meliloti* was plated on the medium containing AM fungal hyphae, NodD1 was not rapidly activated, as evidenced by the lack of early (0, 2, and 4h) *nodD1* gene expression upregulation (**Figure 26a**). Instead, NodD2 quickly responded to external flavonoid signals, activating *nodD2* gene expression to compensate for the delayed NodD1 response, thereby initiating the downstream nodulation process (**Figure 26b**). It has been reported that *S. meliloti* is not attracted to flavonoids found in alfalfa seed exudates (Compton et al., 2020). However, that study was restricted to four exogenous flavonoids (hyperoside, luteolin, luteolin-7-glucoside, and chrysoeriol), which do not overlap with the eight flavonoids detected solely in the RC^{*M.truncatula*}/HC^{+*R.irregularis*} treatment in our study. Therefore, their conclusion could not be extended to our results. Moreover, we performed a molecular docking analysis of the four flavonoids mentioned in previous study (Compton et al., 2020) with NodD1, NodD2, and NodD3 and observed that their most stable binding affinities were -8.36 , -8.37 , and $-8.32 \text{ Kcal}\cdot\text{mol}^{-1}$, respectively (Annex II, **Figure S13**, **Figure S15**, **Figure S17**), which were much lower than the -9.46 and $-9.36 \text{ Kcal}\cdot\text{mol}^{-1}$ observed in our study. Based on these findings, we conclude that the distinct regulatory mechanisms of the NodD proteins underscore the complexity of the symbiotic signaling network in *S. meliloti*, where NodD1 and NodD2 are central to the initial flavonoid-mediated activation of *nod* genes.

Our *in vitro* data support that *S. meliloti* uses ERM exudates for catabolism and growth (Leveau and Preston, 2008), forming biofilm-like multilayers on hyphae surfaces and spores, suggesting that *R. irregularis* provides nutrients for *S. meliloti* reproduction. Without ERM exudates, growth was significantly limited, whereas exudates from hyphae connected to *M. truncatula* or *S. tuberosum* promoted sigmoidal growth, with faster growth observed from *M. truncatula* hyphae exudates. This suggests that hyphae connected to *M. truncatula* release not only nutrients but also signals that may enhance *S. meliloti* growth, extending

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microbial perception beyond the rhizosphere. These observations need to be extended to other AM fungal species but *S. meliloti* seems to behave as a true fungiphile well-adapted to live in the hyphosphere of AM fungi.

In the migration experiment (Fig 4a), *S. meliloti* preferentially migrated to the RC, indicating attraction by signals from hyphae connected to *M. truncatula*. Analysis of exudates from these hyphae suggests some of these signals may be the eight unique flavonoids detected in the RC^{*M.truncatula*}/HC^{+*R.irregularis*} treatment. *S. meliloti* on the hyphae surface also stimulated cytoplasmic flow within the hyphae, suggesting that unknown molecules from *S. meliloti* enhance *R. irregularis* cytoplasmic flow. In the presence of rhizobia, flow speed was 10.78 $\mu\text{m}\cdot\text{s}^{-1}$, compared to 6.01 $\mu\text{m}\cdot\text{s}^{-1}$ without rhizobia, and 5.87 $\mu\text{m}\cdot\text{s}^{-1}$ with PBS. A higher resource allocation is therefore expected in presence of rhizobia, potentially stimulating the bacterial growth.

Mycelial networks, which are hydrophilic, expedite bacterial dispersal by providing continuous bacterial films around their hyphae (Kohlmeier et al., 2005; Banitz et al., 2013; Zhou et al., 2022). The ERM can interconnect plants of the same or different species, forming CMNs (Mikkelsen et al., 2008; Voets et al., 2008; Song et al., 2010). These networks allow plants to receive warning signals and potentially exchange nutrients (Simard et al., 2012; Durant et al., 2023). Our study showed that *S. meliloti* inoculated on hyphae linking *M. truncatula* to *S. tuberosum* preferentially migrate toward *M. truncatula* (**Figure 24b**), likely due to a gradient of chemoattractant signals (e.g., flavonoids) from *M. truncatula* connected hyphae, explaining slower migration towards *S. tuberosum*. This suggests that signal molecule concentration plays a crucial role in attracting *S. meliloti*, affecting its migration within the CMN. We detected eight unique flavonoids in the RC^{*M.truncatula*}/HC^{+*R.irregularis*} treatment. Their effects on nod gene expression and preferential migration towards *M. truncatula* suggest these flavonoids may drive *S. meliloti*'s selective preference. Previous studies support this, demonstrating that chemotaxis response to root exudates initiates rhizobia recruitment and root interactions (Dakora et al., 1993; Feng et al., 2021).

Flavonoids, also well-known as signaling molecules in AM symbiosis (Singla and Garg, 2017), were shown in this study to be transported by AM fungal hyphae, enabling the stimulation of rhizobia at distances far beyond the rhizosphere, thereby suggesting a mechanism for rhizobial migration and potential symbiosis establishment with legumes. Although our *in vitro* experiments provided initial evidence of rhizobial transport via AM fungal hyphae, the quadri-compartment pot experiment in the greenhouse, using a sand-vermiculite substrate, further demonstrated that this transport also occurs under more soil-like conditions,

facilitating long-distance rhizobial dispersal and nodule formation. Hyphae could be a preferential route for long-distance *S. meliloti* migration, as shown in the greenhouse experiment. *S. meliloti* placed in the CC migrated toward *M. truncatula* connected by hyphae (approx. 15 cm). No nodules were observed without hyphae connections. Thus, hyphae likely serve as conduits, with their exudates acting as chemoattractants by providing nutrients and signaling molecules that help rhizobia sense compatible host plants. In addition, hyphae function as conduits, facilitating the rapid migration of rhizobia through soil to the roots, resulting in nodulation. Although ambient rhizobial levels in bulk soil are generally sufficient for nodulation of endemic legumes, their spatial heterogeneity can result in microsites with suboptimal densities, potentially limiting nodulation efficiency (Hirsch, 1996; Andrade et al., 2000; Bever and Simms, 2000; Sindhu et al., 2020; Young and Crawford, 2004; Parr et al., 2017). AM fungal hyphae can enhance rhizobial dispersal towards roots, especially in heterogeneous environments, aiding rapid colonization of new roots (Westhoek et al., 2017; Mendoza-Suárez et al., 2020; D. Wang et al., 2022; Knights et al., 2024). This dispersal mechanism may be particularly beneficial under natural ecosystem conditions where rhizobial distributions are uneven and competition in the rhizosphere is intense (Sprent, 2009; Simon et al., 2017; Zhang et al., 2020; Zhou et al., 2020; Jin et al., 2024). Our results suggest that, without being the only pathway, AM hyphae constitute an additional means of root acquisition of rhizobia, which could confer advantages in specific environmental scenarios. Further research needs to be conducted to validate these findings across different soil conditions and ecosystems.

Conclusion

Root exudates can mediate the short-distance recruitment of rhizobia. For long distances (several cm) water mass flow (Vicario et al., 2015) or other vectors (Zhang et al., 2020) are often mentioned. Our study suggested that the ERM of AM fungi can serve as a route for rhizobia to migrate from long distance to the root, therefore representing an indirect pathway to initiate legume-rhizobia interaction. Given that all legumes are host to AM fungi, that hyphae are hospitable microhabitats for rhizobia and that AM fungal mycelium vastly extend in soil (from 82 to 111 m·cm⁻³ in prairie and 52 to 81 m·cm⁻³ in ungrazed pasture (Miller et al., 1995)), these below-ground fungi can be considered as important contributors in rhizobia dispersal in the soil. In addition to nutrients provision, the hyphae release signaling chemoattractant molecules facilitating the acquisition and movement of rhizobia to the legume. Our study supports the combined application of AM fungi and rhizobia to improve the efficiency of nitrogen fixation in legume crops. It also raises the hypothesis that agricultural practices

that disturb the mycelial networks of AM fungi (e.g. physical disturbances) could indirectly influence rhizobia-legume interactions, particularly in soils where rhizobial density is low and/or in the absence of rhizobia seed coating, a relatively common agricultural practice, although further research is needed to explore these effects in concrete agronomic contexts. (**Figure 28**).

Chapter II

Legume-specific recruitment of rhizobia by hyphae of arbuscular mycorrhizal fungi

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Preface

In agricultural ecosystems, legumes form complex relationships with soil microorganisms, including arbuscular mycorrhizal (AM) fungi and nitrogen (N₂)-fixing rhizobia, which enhance plant nutrition and growth. While AM fungal extraradical mycelia (ERM) have been shown to facilitate rhizobial migration toward legume roots over long distances (see Chapter I), the specificity of rhizobial recruitment in complex plant-microbe networks remains poorly understood. The role of a common mycorrhizal network (CMN) in selectively recruiting host-specific rhizobia in mixed legume systems has yet to be fully elucidated.

In **Chapter II**, we build on the findings of Chapter I to investigate whether rhizobia exhibit a preference for their specific legume hosts when mediated by CMNs. Using *in vitro* cultivation systems and greenhouse microcosms, we explored (i) whether host-specific flavonoid signals are transmitted through CMNs to influence rhizobial behavior, and (ii) whether rhizobia preferentially migrate toward their compatible host plants in mixed legume systems.

By employing two fluorescently labeled rhizobial species (*Sinorhizobium meliloti* and *Bradyrhizobium diazoefficiens*) and metabolomics to analyze fungal secretions, we demonstrate that CMNs function as both information highways, transmitting host-specific chemical signals, and physical networks, facilitating rhizobial migration. Our results revealed that rhizobial migration is guided by host-specific signals transmitted via the CMN, highlighting a remarkable selectivity in plant-microbe interactions.

This research advances our understanding of the mechanisms driving selective rhizobial recruitment and emphasizes the critical role of AM fungal networks in shaping legume-rhizobia symbioses, with implications for improving N₂ fixation in sustainable agriculture.

Abstract

The legume-rhizobia symbiosis possesses great potential for sustainable agriculture because of its ability to fix atmospheric nitrogen, reducing crop dependence on nitrogen fertilizers. Rhizobia recognize the host legume through flavonoids released by the roots. These signals are detected by bacteria typically over a few millimeters. Recent research has shown that arbuscular mycorrhizal fungi extend this recognition beyond 15 cm by transporting flavonoids along their hyphae. In soil, common mycorrhizal networks linking plants are formed by arbuscular mycorrhizal fungi. We hypothesized that such networks linking different legumes can transmit host-specific signals, guiding rhizobia to their appropriate hosts. Using *in vitro* and greenhouse microcosms, we linked *Medicago truncatula* and *Glycine max* via a common mycorrhizal network of *Rhizophagus irregularis* and inoculated GFP-labeled *Sinorhizobium meliloti* and mCherry-labeled *Bradyrhizobium diazoefficiens* on the hyphae. *S. meliloti* preferentially migrated towards *M. truncatula*, whereas *B. diazoefficiens* preferentially migrated towards *G. max* (155 ± 8 and 13 ± 3 nodules, respectively). This was confirmed in the greenhouse with a higher concentration of *S. meliloti* ($2.1\text{--}2.5 \times 10^5$ CFU·g⁻¹) near *M. truncatula* and a higher concentration of *B. diazoefficiens* ($1.5\text{--}1.6 \times 10^5$ CFU·g⁻¹) near *G. max* (71-82 and 15-18 nodules, respectively). Metabolomics revealed host-specific flavonoids in hyphal exudates: *M. truncatula*-connected hyphae released DL-liquiritigenin, naringenin, sakuranetin, and 3,7-dimethylquercetin, whereas *G. max*-connected hyphae released daidzin, 6''-O-malonyldaidzin, irilone, and erylatissin A. These findings establish that common mycorrhizal networks constitute a “navigation system”, using chemical signals to orient rhizobia towards their specific hosts, thereby improving nodulation with potential applications in agriculture.

Introduction

Nitrogen-fixing bacteria (collectively known as rhizobia) can establish beneficial interactions with legumes and fix atmospheric dinitrogen (N₂) by forming a specialized organ, the nodule (Masson-Boivin et al., 2009). However, they can only fix N₂ when they form nodules with roots of compatible leguminous plants (Schultze and Kondorosi, 1998; Chen et al., 2021). For example, *Medicago truncatula* forms indeterminate nodules with *Sinorhizobium meliloti*, whereas *Glycine max* forms determinate nodules with *Bradyrhizobium diazoefficiens* (Gubry-Rangin et al., 2010; Liu et al., 2018).

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Nodulating rhizobia exhibit limited mobility in soil and typically originate from the soil environment rather than from seeds (Zhang et al., 2020). They are attracted by specific compounds (flavonoids) released over a short distance (a few mm) by root epidermal cells and root hairs (Bais et al., 2004). As a result, rhizobia separated from their hosts by a long distance are unable to detect the host. In addition, the presence of air-filled gaps between soil aggregates prevents rhizobia from moving toward the roots using flagella without the aid of flowing water or other vectors (Horiuchi et al., 2005; Vicario et al., 2015).

Previous research demonstrated that *S. meliloti* can migrate to the roots of legumes (i.e., *M. truncatula*) from long distances (several cm) via the surface of the hyphae of root-associated arbuscular mycorrhizal (AM) fungi, leading to nodulation (He et al., 2024). Because the vast majority of legumes are hosts to AM fungi (their hyphae provide a hospitable environment for rhizobia and the mycelium of these obligate root symbionts is extensive and abundant (Soudzilovskaia et al., 2019; Hawkins et al., 2023), spreads widely in the soil – from 82 to 111 m·cm⁻³ in grassland and from 52 to 81 m·cm⁻³ in ungrazed pasture) (Miller et al., 1995), these belowground fungi represent an important indirect route for legumes to recruit root-nodulating bacteria.

In soils, individual plants can be interconnected by common mycorrhizal networks (CMNs) formed by AM fungal hyphae (Giovannetti et al., 2004; Mikkelsen et al., 2008; Voets et al., 2006). These networks have been explored for their potential roles in mediating interactions between connected plants, including the transfer of carbon and nutrients (Barto et al., 2012), and the transmission of signals involved in processes such as defense (Babikova et al., 2013; Wu et al., 2017). However, the extent, net directionality, and ecological significance of these functions, are subjects of ongoing research and critical debate. Recent analyses have urged caution against overinterpretation and highlighted the influence of methodological choices and potential citation biases in some areas of CMN research (Karst et al., 2023; Kuyper and Jansa, 2023). Nonetheless, experimental studies have provided evidence that certain signaling molecules can be transported between plants via CMNs under specific conditions, for instance, in the context of induced plant defense against herbivores (Song et al., 2010; Gilbert and Johnson, 2015; Johnson and Gilbert, 2015). These experimental observations of the movement of signaling molecules between plants via mycelial connections suggest that CMNs could also facilitate the transport of host-specific signals, such as flavonoids, involved in the recruitment of rhizobia.

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It has been demonstrated that *S. meliloti*, plated on AM fungal hyphae linking a legume (*M. truncatula*) to a non-legume (*Solanum tuberosum*) by a CMN, migrated preferentially towards the legume suggesting the chemoattraction by specific signals transported and released by the AM fungus connected to the legume (He et al., 2024). This migration pattern was confirmed by a metabolomics study revealing the presence of eight specific flavonoids released by AM fungal hyphae linked to *M. truncatula* (He et al., 2024). However, it is not known whether signals transmitted by two different legumes connected by a CMN can enable rhizobia to specifically recognize their compatible host and migrate towards it selectively.

In the present study we conducted a series of *in vitro* and greenhouse experiments with two stable fluorescent rhizobia (*S. meliloti* pHC60-GFP and *Bradyrhizobium diazoefficiens* mChe-1) specific to *M. truncatula* and *G. max*, respectively, connected by a CMN of *Rhizophagus irregularis* MUCL 41833, for studying preferential migration. We first confirmed *in vitro* that *S. meliloti* form nodules with *M. truncatula* and not *G. max* and conversely that *B. diazoefficiens* from nodules with *G. max* and not with *M. truncatula*. Next, we conducted a comparative metabolomics study on the growth media containing AM fungal hyphae connected to *M. truncatula* or *G. max* in a compartmented system to reveal the presence and potential differences in flavonoids content. Finally, we carried out an *in vitro* and a pot microcosm experiment with the two leguminous plants connected by a CMN of the AM fungus to explore whether *S. meliloti* pHC60-GFP and *B. diazoefficiens* mChe-1 show selective tropism towards their respective plant hosts.

We show that CMN connecting two different legumes species allow rhizobia to recognize and preferentially migrate towards their specific host and form nodules. This preferential directional migration is probably guided by host-specific flavonoid signals, as shown by comparative metabolomics analysis, which reveals differences between hyphal exudates connected to the two legumes species.

Materials and methods

Biological material

Experiments utilized *Rhizophagus irregularis* MUCL 41833, *Sinorhizobium meliloti* 2011 pHC60-GFP, *Bradyrhizobium diazoefficiens* mChe-1, *Medicago truncatula* L. cv. Jemalong A17, *Glycine max* (L.) Merr., and *Plantago lanceolata*

L., sourced and cultured as described in Section 1 of MATERIALS AND METHODS.

Growth and nodulation of *S. meliloti* and *B. diazoefficiens* on *M. truncatula* and *G. max*.

Seven-days-old *G. max* and *M. truncatula* plants were grown in Petri plates (90 mm diameter) on $\text{MSR}^{\text{min}/2\text{N}}$ medium (MSR medium lacking sucrose and vitamins (MSR^{min})), containing half the normal N concentration (1.99 mM) (He et al., 2024). Six treatments were considered: (1 and 2) *G. max* or *M. truncatula* inoculated with a phosphate buffered saline (PBS) solution ($G.\text{max}^{\text{PBS}}$ and $M.\text{truncatula}^{\text{PBS}}$, respectively), (3 and 4) *G. max* or *M. truncatula* inoculated with *S. meliloti* ($G.\text{max}^{S.\text{meliloti}}$ and $M.\text{truncatula}^{S.\text{meliloti}}$, respectively), and (5 and 6) *G. max* or *M. truncatula* inoculated with *B. diazoefficiens* ($G.\text{max}^{B.\text{diazoefficiens}}$ and $M.\text{truncatula}^{B.\text{diazoefficiens}}$, respectively). The bacterial inoculum consisted of a suspension of 150 μL of the bacteria in PBS solution, at a concentration adjusted to $9 \times 10^5 \text{ CFU} \cdot \text{mL}^{-1}$ ($\text{OD}_{600} = 0.1$). The bacterial suspension was directly pipetted onto the roots of the plants to ensure direct contact between the bacteria and the root surface. Control treatments received an equivalent volume of PBS without bacteria. Six replicates were considered per treatment (**Figure 31A, B**). After 6 weeks, plants were removed from the Petri plates using sterile forceps, rinsed with deionized water, and blotted dry with sterile paper towels. Roots were examined under a stereomicroscope (Olympus SZ61) at $0.67\times$ magnification with a cold light source. Nodules were counted visually and classified based on color and shape. Mature nodules were identified by their pink to red color indicating active N_2 fixation, whereas immature nodules were white, meaning that N_2 fixation had not yet begun (Vishwakarma et al., 2017). Additionally, nodules were categorized as either determinate or indeterminate based on their shape. Determinate nodules are typically spherical, whereas indeterminate nodules exhibit an elongated shape due to continuous meristematic activity (Saeki, 2011).

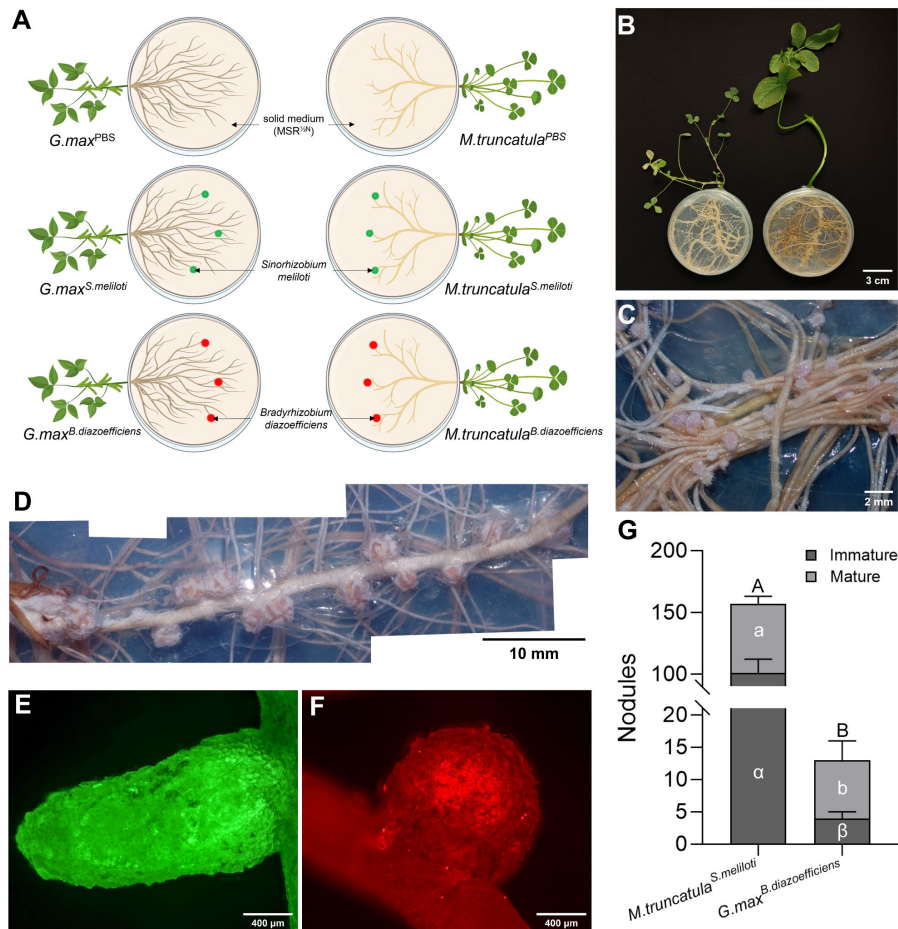


Figure 31. Experimental setup and evaluation of nodulation of *M. truncatula* and *G. max* in vitro. **(A)** Schematic representation of the mono-compartmented Petri plate system, with rhizobial inoculum (green dots: *S. meliloti*; red dots: *B. diazoefficiens*). **(B)** Photograph of the mono-compartmented Petri plate system with *M. truncatula* on the left and *G. max* on the right. **(C)** Nodules formed on *M. truncatula* roots inoculated with *S. meliloti*. **(D)** Nodules formed on *G. max* roots inoculated with *B. diazoefficiens*. **(E)** Epifluorescence microscopy images of an indeterminate immature nodule on a root of *M. truncatula*. **(F)** Epifluorescence microscopy images of a determinate mature nodule formed on a root of *G. max*. **(G)** Number of immature and mature nodules formed on roots of *M. truncatula* inoculated with *S. meliloti* (*M.truncatula*^{*S.meliloti*} treatment) and on roots of *G. max* inoculated with *B. diazoefficiens* (*G.max*^{*B.diazoefficiens*} treatment), respectively, six weeks post-inoculation ($n = 6$ biological replicates). Different Greek letters (α , β), lowercase letters (a, b), and uppercase letters (A, B), respectively, indicate significant differences of immature, mature, and total nodule numbers between

treatments (Student's *t*-test, $P \leq 0.05$). No nodules were observed in the *M. truncatula*^{*B.diazoefficiens*} and *G.max*^{*S.meliloti*} treatments (data not shown).

In vitro* experimental design for analyzing flavonoids released by the ERM of *R. irregularis* connected to *G. max* or *M. truncatula

Following a previous study (He et al., 2024), bi-compartmented Petri plates (90 mm diameter) were used with *G. max* or *M. truncatula* associated with *R. irregularis* in the root compartment (RC) containing 25 mL MSR^{min¹/₂N} medium, and with the AMF fungus extending alone in the hyphal compartment (HC) containing 10 mL liquid MSR^{min⁰N} medium (**Figure 32A, B**). Four treatments were established: RC^{*G.max*}/HC^{+*R.irregularis*}, RC^{*G.max*}/HC^{–*R.irregularis*}, RC^{*M.truncatula*}/HC^{+*R.irregularis*}, and RC^{*M.truncatula*}/HC^{–*R.irregularis*}. At week 13, the HC liquid medium was aspirated with a sterile pipette under a laminar flow hood, and 10 mL fresh liquid MSR^{min⁰N} (prepared fresh, autoclaved, and cooled to 25°C) was added. After one week (week 14), the medium was collected into 15 mL centrifuge tubes (Falcon, Corning, USA), snap-frozen in liquid nitrogen, and stored at –80°C for subsequent flavonoid analysis via UPLC-HRMS at the VIB Metabolomics Core Ghent.

Flavonoid analysis followed the protocol in Chapter I (*In vitro* experimental design for analyzing flavonoids released by the ERM of *R. irregularis* in the HC), with modifications. Six mL of MSR^{min⁰N} medium was extracted with 6 mL methanol for 1 hour, evaporated using a rotary evaporator, and resolubilized in 100 µL cyclohexane and 100 µL Milli-Q water. After centrifugation at 14,000 rpm, 80 µL of the aqueous phase was filtered and analyzed via UPLC-HRMS at the VIB Metabolomics Core Ghent. Data processing with Progenesis QI v3.0 detected 28,122 features, filtered to 11,749 features with ANOVA $P \leq 0.01$. Structural annotation used MS-FINDER with parameters: MS1/MS2 tolerance of 5/10 ppm, relative abundance cut-off of 0.1%, and common element ratio check.

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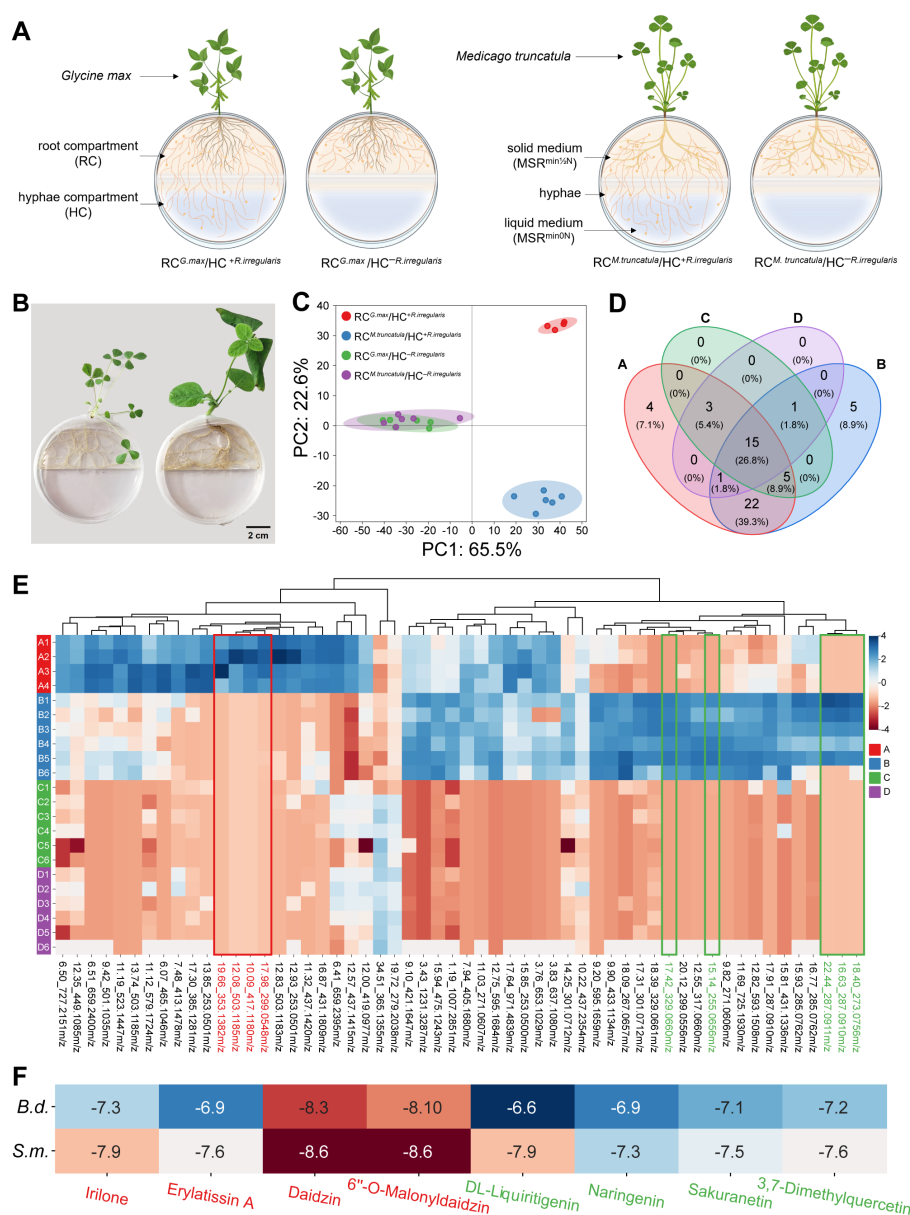


Figure 32. Experimental setup and analysis of extraradical mycelium (ERM) exudates in the *in vitro* bi-compartmented Petri plate system. **(A)** Schematic representation of the bi-compartmented Petri plate system used to analyze the transport and composition of ERM exudates. **(B)** Photograph of the bi-compartmented Petri plate system with *M. truncatula* on the left and *G. max* on the right. **(C)** Principal component analysis (PCA) plot based on compounds detected in the MSR^{min0N} medium collected from the hyphal compartment (HC), showing significant differences (ANOVA, $P \leq 0.01$) between treatments. Ellipses

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represent the 95% confidence interval for each treatment. $RC^{G.max}/HC^{+R.irregularis}$ ($n = 4$ biological replicates); $RC^{G.max}/HC^{-R.irregularis}$ ($n = 6$ biological replicates); $RC^{M.truncatula}/HC^{+R.irregularis}$ ($n = 6$ biological replicates); $RC^{M.truncatula}/HC^{-R.irregularis}$ ($n = 6$ biological replicates). **(D)** Venn diagram summarizing the overlapping flavonoids between the four treatments. Counts of unique flavonoids are shown, with percentages relative to the total unique count in parentheses. **A:** $RC^{G.max}/HC^{+R.irregularis}$ ($n = 4$ biological replicates); **B:** $RC^{M.truncatula}/HC^{+R.irregularis}$ ($n = 6$ biological replicates); **C:** $RC^{G.max}/HC^{-R.irregularis}$ ($n = 6$ biological replicates); **D:** $RC^{M.truncatula}/HC^{-R.irregularis}$ ($n = 6$ biological replicates). **(E)** Heatmap displaying relative abundance profiles of flavonoids (columns) across different samples (rows, A1-D6). Different flavonoids were clustered using hierarchical clustering (complete linkage, Euclidean distance) to group similar profiles. **A:** $RC^{G.max}/HC^{+R.irregularis}$ ($n = 4$ biological replicates); **B:** $RC^{M.truncatula}/HC^{+R.irregularis}$ ($n = 6$ biological replicates); **C:** $RC^{G.max}/HC^{-R.irregularis}$ ($n = 6$ biological replicates); **D:** $RC^{M.truncatula}/HC^{-R.irregularis}$ ($n = 6$ biological replicates). **(F)** *In silico* binding studies between NodD1 protein of *B. diazoefficiens* (*B.d.*) and *S. meliloti* (*S.m.*) with flavonoids detected exclusively in $RC^{G.max}/HC^{+R.irregularis}$ and $RC^{M.truncatula}/HC^{+R.irregularis}$ treatments. Numbers in the panels represent binding affinities ($Kcal \cdot mol^{-1}$), where more negative values indicate stronger interactions. Flavonoids in red font (E, F) and red boxe (E) indicate those unique to $RC^{G.max}/HC^{+R.irregularis}$ are: 17.98_299.0548m/z (irilone), 19.66_353.1382m/z (erylatissin A), 10.09_417.1180m/z (daidzin), and 12.08_503.1185m/z (6''-O-malonyldaidzin). Flavonoids in green font (E, F) and green boxes (E) indicate those unique to $RC^{M.truncatula}/HC^{+R.irregularis}$ are: 15.14_255.0656m/z (DL-liquiritigenin), 18.40_273.0756m/z (naringenin), 16.63_287.0910m/z (sakuranetin), 22.44_287.0911m/z (sakuranetin), and 17.42_329.0660m/z (3,7-dimethylquercetin).

In vitro* and greenhouse experimental designs with legumes linked by a CMN for mycelia-based migration assay of *S. meliloti* and *B. diazoefficiens

The experimental design was adapted from (He et al., 2024), with modifications to accommodate the two plant species and two bacterial strains.

***In vitro* experimental design**

A quadri-compartmented Petri plate (Chapter I, *In vitro* experimental design for the analysis of *S. meliloti* migration along AM fungal hyphae and cytoplasmic flow velocity within hyphae) featured two side root compartments (RCs) with 25 mL $MSR_{min}^{1/2}N_{min}^{1/2}$ medium each, containing *M. truncatula* and *G. max* associated with *R. irregularis*, and a central compartment (CC) with 25 mL

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MSR^{min0N} medium (**Figure 33A, B**). The Petri plates were incubated as above for 13 weeks until a CMN was formed in the CC, confirmed by stereomicroscope observation of hyphal bridges. Three hyphae per plate, visibly connecting the two plants and growing on the CC surface, were inoculated with 1 μ L of a 1:1 mixture of *S. meliloti* and *B. diazoefficiens* (0.5 μ L each, 9×10^5 CFU $\cdot$ mL⁻¹).

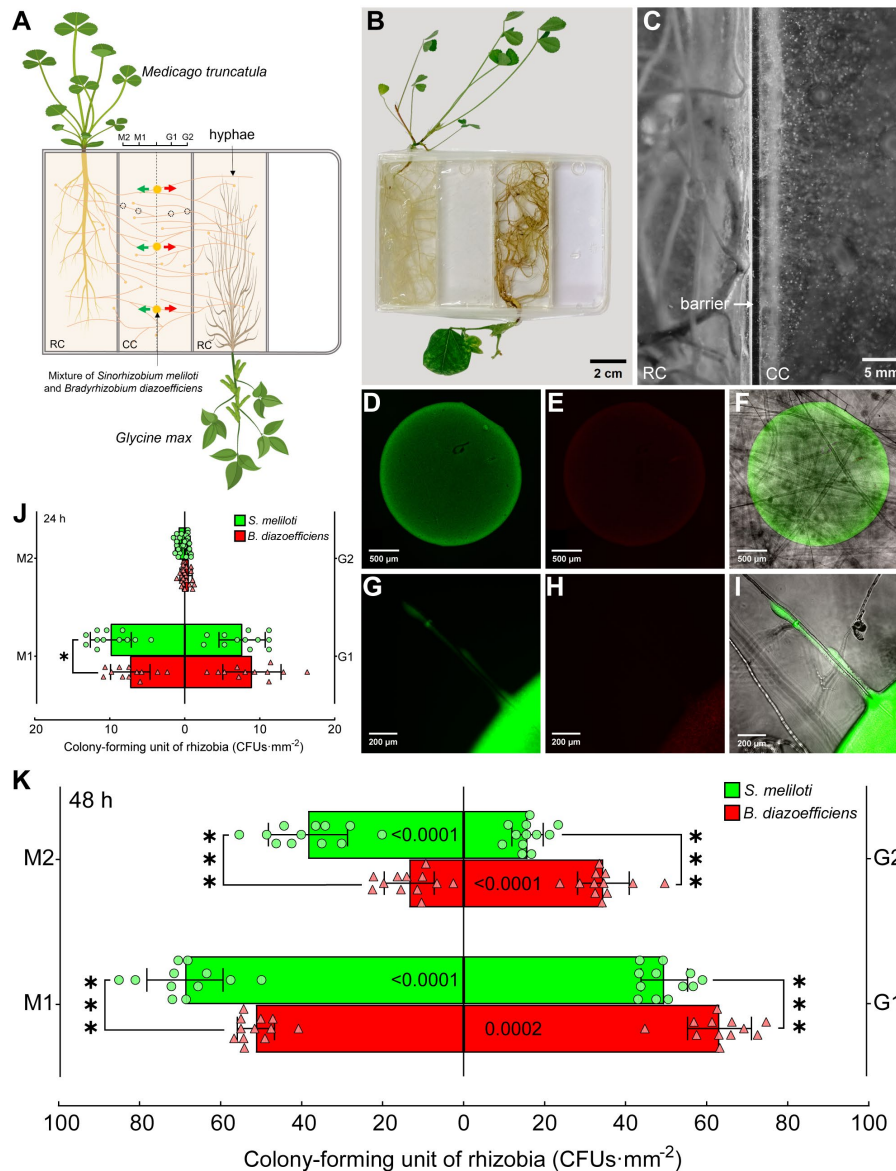


Figure 33. Mycelia-based migration assay of *S. meliloti* and *B. diazoefficiens* in an *in vitro* quadri-compartmented Petri plate system. **(A)** Schematic representation of the quadri-compartmented *in vitro* culture system. Yellow dots

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represent a mixture of *S. meliloti* and *B. diazoefficiens*. Green arrows indicate migration towards *M. truncatula*; Red arrows indicate migration towards *G. max*. RC: root compartment; CC: central compartment. Dashed circles indicate the sampling points. Sampling points M1 and M2 are located 0.7 cm and 1.5 cm from the inoculation site toward *M. truncatula*, respectively; G1 and G2 are located 0.7 cm and 1.5 cm toward *G. max*, respectively. **(B)** Photograph of the quadri-compartmented Petri plate system. **(C)** Hyphae of *R. irregularis* crossing the barrier from the RC to the CC. **(D-F)** Fluorescence micrographs of the inoculation site: (D) *S. meliloti*, (E) *B. diazoefficiens*, and (F) merged image of both. **(G-I)** Fluorescence micrographs of (G) *S. meliloti* and (H) *B. diazoefficiens* migrating along AM fungal hyphae in the CC, and (I) merged image of both. **(J, K)** Colony-forming units (CFU·mm⁻²) of *S. meliloti* and *B. diazoefficiens* at sampling points M1, M2, G1, and G2 at (J) 24 hours and (K) 48 hours post-inoculation. Data are presented as means ± SD ($n = 12$ biological replicates). Asterisks indicate significant differences (* $0.01 \leq P \leq 0.05$; *** $P \leq 0.0001$) determined by Student's *t*-test for each sampling point. Numbers on the bar chart represent *P* value from Student's *t*-test comparing CFU of the same rhizobial species at corresponding sampling points on opposite side of the inoculation site.

Bacterial migration was quantified at 24 and 48 hours post-inoculation by sampling medium with a 2 mm diameter cork borer at 0.7 cm and 1.4 cm from both sides of the inoculation site (**Figure S19**). The medium samples were transferred to 1.5 mL sterile centrifuge tubes containing 0.2 mL of sterile PBS, vortexed for 5 s at 1200 rpm using an MS1 shaker (IKA, Germany), and 20 µL of the suspension was spread onto each selective agar plates using an L-Shape Cell Spreader (VWR). The number of colony forming units (CFUs) of *S. meliloti* was determined on YEB solid medium (15 g·L⁻¹ agar) supplemented with 5 µg·mL⁻¹ tetracycline, while for *B. diazoefficiens*, it was determined on YEB solid medium (15 g·L⁻¹ agar) supplemented with 50 µg·mL⁻¹ spectinomycin. The Petri plates were incubated at 28°C for 48 hours, and CFUs were counted. Three biological replicates were prepared for each sample.

Greenhouse experimental design

Setup: A three-compartment pot system (**Figure 34A**; **Figure S20**) was designed with a central compartment (CC, 0.3 L) connected to two satellite compartments (0.3 L each) via perforated PVC pipes (1.7 cm diameter × 10 cm length, 6 holes of 6 mm diameter spaced 1 cm apart in the upper portion, **Figure S5**). The substrate was a 2:1 (v/v) mixture of sand and vermiculite, autoclaved twice (121°C, 30 min, 0.11 MPa) with a 24 hours interval. The pipes were covered with

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a mesh (5 μm or 41 μm aperture size) at both ends to control hyphal and root passage: the 41 μm mesh allowed only hyphae to proliferate in the pipe, whereas the 5 μm mesh prevented both hyphae and root crossing between compartments.

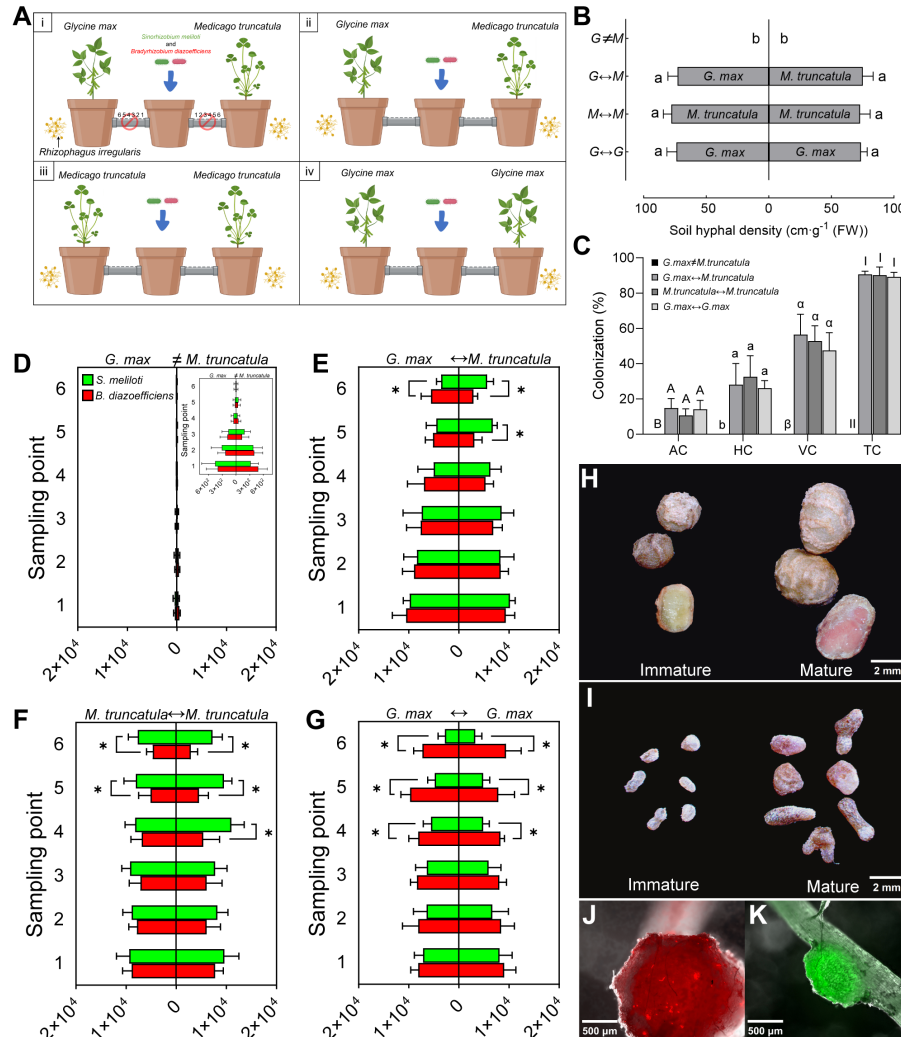


Figure 34. Greenhouse experimental system for studying rhizobial migration and nodulation in *G. max* and *M. truncatula* linked by common mycorrhizal networks. (A) Schematic representation of the greenhouse three-compartment pot system, with numbers (1-6) on the pipes indicating different sampling holes. i: *G. max* and *M. truncatula* in satellite compartments connected to the CC via pipes with 5 μm mesh ($G.\text{max}\neq M.\text{truncatula}$); ii: *G. max* and *M. truncatula* in satellite compartments connected to the CC via pipes with 41 μm mesh ($G.\text{max}\leftrightarrow M.\text{truncatula}$); iii: *M. truncatula* in both satellite compartments and connected to the CC via pipes with 41 μm mesh ($M.\text{truncatula}\leftrightarrow M.\text{truncatula}$); iv: *G. max* in both satellite compartments and connected to the CC via pipes with 41 μm mesh ($G.\text{max}\leftrightarrow G.\text{max}$). Rhizopneumonia is indicated in the first two compartments.

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connected to the CC via pipes with 41 μm mesh (*M.truncatula* $\leftrightarrow$ *M.truncatula*); iv: *G. max* in both satellite compartments and connected to the CC via pipes with 41 μm mesh (*G.max* $\leftrightarrow$ *G.max*). **(B)** Hyphal density in the pipes. Data are presented as means $\pm$ SD ($n = 6$ biological replicates). Different lowercase letters (a, b) indicate significant differences between pipes (one-way ANOVA with Tukey's test, $P \leq 0.05$). **(C)** Mycorrhizal colonization of *P. lanceolata* roots in the CC after 4 months of growth. Data are presented as means $\pm$ SD ($n = 6$ biological replicates). Different uppercase letters (A, B), lowercase letters (a, b), Greek letters (α , β), and Roman numerals (I, II) indicate significant differences between treatments for arbuscular colonization (AC), hyphal colonization (HC), vesicle colonization (VC), and total colonization (TC), respectively (one-way ANOVA with Tukey's test, $P \leq 0.05$). **(D-G)** Colony-forming units (CFU $\cdot\text{g}^{-1}$ fresh substrate) of *S. meliloti* and *B. diazoefficiens* at different sampling points (holes 1-6, with increasing distance from the CC) in the pipes of (D) *G.max* $\neq$ *M.truncatula*, (E) *G.max* $\leftrightarrow$ *M.truncatula*, (F) *M.truncatula* $\leftrightarrow$ *M.truncatula*, and (G) *G.max* $\leftrightarrow$ *G.max* treatments at 9 days post-inoculation (dpi). The inset in Figure 4D displays the same data as the main panel D but uses an adjusted x-axis scale to better visualize variations between sampling points for this treatment. Data are means $\pm$ SD ($n = 3$ biological replicates, with 3 technical replicates per sample). An asterisk (*) indicates $P \leq 0.05$ (Student's *t*-test for each sampling hole). **(H, I)** Mature and immature nodules extracted from roots of (H) *G. max* and (I) *M. truncatula*. **(J, K)** Epifluorescence microscopy images of nodules in the roots of mycorrhizal colonized (J) *G. max* and (K) *M. truncatula*.

Four treatments were established:

- (1) *G.max* $\neq$ *M.truncatula* treatment: *G. max* and *M. truncatula*, both colonized by *R. irregularis* (5 g inoculum per pot), were grown in the two satellite compartments. Pipes to the CC were covered both side with 5 μm mesh, preventing CMN formation.
- (2) *G.max* $\leftrightarrow$ *M.truncatula* treatment: *G. max* and *M. truncatula*, both colonized by *R. irregularis* (5 g inoculum per pot), were grown in the satellite compartments. Pipes to the CC were covered both side with 41 μm mesh, enabling a CMN to link the two species in the CC.
- (3) *M.truncatula* $\leftrightarrow$ *M.truncatula* treatment: Two *M. truncatula* plants, both colonized by *R. irregularis* (5 g inoculum per pot), were grown in the satellite compartments. Pipes to the CC were covered both side with 41 μm mesh, enabling a CMN to link the same species.
- (4) *G.max* $\leftrightarrow$ *G.max* treatment: Two *G. max* plants, both colonized by *R. irregularis* (5 g inoculum per pot), were grown in the satellite compartments.

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Pipes to the CC were covered both side with 41 μm mesh, enabling a CMN to link the same species.

CMN establishment: At the start of the experiment, concurrently with the planting and AM fungi inoculation of *M. truncatula* and *G. max* in the satellite compartments, *P. lanceolata* was initially grown in the CC of all pots for four months in the greenhouse to establish the CMN. After this period, the successful establishment of the CMN was confirmed by assessing AM fungal root colonization in roots of *P. lanceolata* from the CC using standard staining (Walker, 2005) and microscopy (McGonigle et al., 1990) techniques, and by verifying the presence and measuring the density of fungal hyphae within the connecting pipes using established methods (Bethlenfalvay and Ames, 1987). Following this confirmation, all *P. lanceolata* plants were removed from the CC, and the systems were left undisturbed for an additional month to allow the CMN to stabilize before initiating the experimental treatment phase.

Inoculation and sampling: Following the CMN stabilization period, the experimental phase formally began by inoculating the CC with a 5 mL mixture containing *S. meliloti* (2.5 mL, $\text{OD}_{600} = 1$) and *B. diazoefficiens* (2.5 mL, $\text{OD}_{600} = 1$), injected to the center of the substrate surface using a 5 mL NORM-JECT silicone-free Luer Lock Syringe (Henke-Sass Wolf, Germany). To assess rhizobial migration through the CMN, substrate samples were collected from the pipes of three randomly selected systems per treatment at 1, 3, 5, 7, and, 9 days post-inoculation (dpi). Sampling was performed using modified 1 mL pipette tips, cut at 3 cm length from the extremity, inserted into the holes of the pipes; the pipes were then inverted and gently tapped to transfer substrate into 1.5 mL centrifuge tubes (Eppendorf, Germany). For bacterial quantification, samples were mixed with 1 mL PBS, vortexed at 1000 rpm for 20 s, serially diluted 10-fold in PBS, and 20 μL of appropriate dilutions were spread onto selective YEB agar plates (15 $\text{g}\cdot\text{L}^{-1}$ agar). *S. meliloti* was selected using medium supplemented with 5 $\mu\text{g}\cdot\text{mL}^{-1}$ tetracycline, whereas *B. diazoefficiens* was selected with 50 $\mu\text{g}\cdot\text{mL}^{-1}$ spectinomycin, following protocols outlined previously (He et al., 2024). Three biological replicates were considered for each sample. Petri plates were incubated at 28°C for 48 hours, and CFUs were counted using an Echo Revolve RVL2-K microscope at 4 $\times$ magnification, with results converted to CFUs per gram of substrate.

Harvest and assessment: At 6 and 8 weeks post-inoculation (wpi), plants were harvested from the satellite compartments of all treatments for various assessments. AM fungal root colonization was evaluated on harvested roots (the same as previously on *P. lanceolata* roots during CMN establishment) under an

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Olympus BH2-RFCA microscope (Japan) at 10× magnification, scoring 100 intersects per sample.

Soil hyphal density was quantified from fresh substrate samples collected from the pipes. Five grams of fresh substrate was placed in a 50 mL centrifuge tube with 10 mL of 0.1 mol·L⁻¹ PBS (pH 7.8). After gentle shaking, 8 mL of the sample solution was transferred to a 10 mL centrifuge tube, followed by the addition of 2 mL of 2% ink (Parker Blue Ink, United States) in 1% HCl. The tube was mixed gently, incubated at 70°C for 20 min, and cooled to room temperature. Then, 1 mL was extracted for microscopic examination (Olympus BH2-RFCA, 10× magnification) to measure hyphal length, following a modified method outlined previously (Bethlenfalvay and Ames, 1987). Three technical replicates per sample and six biological replicates per treatment were used.

Nodule evaluation was also performed, as described above, on harvested roots from satellite compartments.

Rhizobial quantification in the substrate adjacent to the root systems in the satellite compartments was performed at harvest: 5 g substrate samples were collected, and bacterial concentrations were determined using the same extraction, selective plating (YEB with tetracycline for *S. meliloti*, YEB with spectinomycin for *B. diazoefficiens*), incubation, and CFU counting procedures described for the pipe sampling. Three technical replicates and six biological replicates were considered for each sample.

Data analysis

Prior to statistical analysis, the homogeneity of variance and normality of distribution were assessed using the Levene and Shapiro-Wilk tests, respectively. To attain normality and homoscedasticity of data, all the variables were transformed by taking the base 10 logarithm, except for the root colonization, which were arcsin transformed. Analysis of variance (ANOVA) and student's *t*-tests were conducted in SAS (8.1) to evaluate the statistical significance of the experimental data. Significance between treatments was determined at 0.05 using the Tukey post-hoc test.

In silico binding studies were performed to evaluate the interaction between selected flavonoids and NodD1 proteins. Eight flavonoids were considered (**Table S2**), along with NodD1 from *S. meliloti* (UniProtKB-P03031) and *B. diazoefficiens* (UniProtKB-Q45264). Protein sequences were obtained from the

UniProt Knowledgebase (<https://www.uniprot.org/help/uniprotkb>). Three-dimensional structures (PDB files) for the NodD1 proteins were predicted using AlphaFold (<https://alphafold.ebi.ac.uk>). The 3D structures of the eight flavonoids were retrieved from PubChem (<https://pubchem.ncbi.nlm.nih.gov>). The NodD1 protein structures and flavonoid ligands were prepared for docking using the AutoDockTools (ADT) package within AutoDock 4.2 (Morris et al., 2009). This preparation involved adding polar hydrogen atoms and computing Gasteiger partial charges for both protein and ligand atoms, as well as defining rotatable bonds for the ligands to allow flexibility during docking. Molecular docking was conducted using Autodock Vina (v1.1.2) (Trott and Olson, 2010; Eberhardt et al., 2021) to form complexes between the NodD1 proteins and flavonoids. The binding affinities of the resulting flavonoid-NodD1 complexes were calculated by Autodock Vina and reported in Kcal·mol⁻¹. The docking poses were evaluated based on these predicted affinities, with the most favorable interactions (lowest binding energy) selected for further analysis.

All schematics and composite figures in this study were designed using Adobe Illustrator 2023. Some elements, such as plant models and root system models, were sourced from BioRender. Quantitative results, presented as bar charts, were generated using GraphPad Prism (version 10.1.0). The Venn diagram was created using the Venn Diagram plugin in Origin 2022. Principal Component Analysis (PCA) and heatmap visualizations were performed using the Chiplot online portal (<https://www.chiplot.online/>). No images or figures were created by artificial intelligence (AI) or AI-assisted tools.

Results

Root nodulation of *M. truncatula* and *G. max* by *S. meliloti* and *B. diazoefficiens*

To evaluate the host specificity of rhizobial nodulation, a mono-compartmented Petri plate system was established, with *M. truncatula* or *G. max* inoculated with either *S. meliloti* or *B. diazoefficiens*, or PBS as a control (**Figure 31A, B**). After 6 weeks of growth, nodules were manually counted and categorized as mature or immature based on their color and morphology.

In the *M.truncatula*^{*S.meliloti*} treatment, 155 ± 8 nodules were counted, including 54 ± 6 mature nodules (35% ± 0.04) and 101 ± 11 immature nodules (65% ± 0.04) (**Figure 31C, E, G**). No nodules were detected in the *M.truncatula*^{*B.diazoefficiens*} or *M.truncatula*^{PBS} treatments. For the *G.max*^{*B.diazoefficiens*} treatment, 13 ± 3 nodules

were formed, of which 9 ± 3 were mature ($70\% \pm 0.10$) and 4 ± 1 were immature ($30\% \pm 0.10$) (**Figure 31D**, F, G). No nodules were observed in the *G.max*^{S.meliloti} or *G.max*^{PBS} treatments.

Host-specific flavonoid profiles in AM fungal ERM exudates

To investigate the chemical signals mediating host-specific rhizobial recruitment, exudates from the ERM of *R. irregularis* connected to either *M. truncatula* or *G. max* were analyzed using a bi-compartmented Petri plate system (**Figure 32A**, B). ERM exudates were collected from the HCs of four treatments (**Figure 32A**): $RC^{G.max}/HC^{+R.irregularis}$, $RC^{G.max}/HC^{-R.irregularis}$, $RC^{M.truncatula}/HC^{+R.irregularis}$, and $RC^{M.truncatula}/HC^{-R.irregularis}$. Metabolomic profiling of the four treatments revealed distinct host-specific signatures.

Principal Component Analysis (PCA) showed clear separation of exudates composition based on the plant to which the AM fungus was linked (**Figure 32C**). PC1 (65.5% of variation) separated the $RC^{G.max}/HC^{+R.irregularis}$ treatment from the $RC^{M.truncatula}/HC^{+R.irregularis}$ treatment, indicating distinct metabolomic profiles. The treatments without ERM in the HC clustered together and could also be separated from the two treatments with *R. irregularis*.

Of the 28,122 features identified (minimum intensity ≥ 1000 counts in at least one sample group), the ANOVA ($P \leq 0.01$) revealed 11,749 significant features. Among 1,457 features with MS/MS spectra, 56 were annotated as flavonoids (**Supplemental dataset 2**). The Venn diagram (**Figure 32D**) highlighted host-specific flavonoid profiles: four flavonoids were exclusive to $RC^{G.max}/HC^{+R.irregularis}$: irilone (isoflavone), erylatissin A (isoflavone derivative), daidzin (isoflavone glycoside), and 6''-O-malonyldaidzin (isoflavone glycoside), suggesting transport from *G. max* roots via AM fungal hyphae. Five features (four kinds of flavonoids) were unique to $RC^{M.truncatula}/HC^{+R.irregularis}$: DL-liquiritigenin (flavanone), naringenin (flavanone), sakuranetin (flavonoid derivative), and 3,7-dimethylquercetin (flavonoid derivative). Additionally, 22 flavonoids were found in both treatments with ERM in the HC, indicating common metabolites transported by AM fungal hyphae. The heatmap confirmed distinct clustering of these flavonoids, supporting host-specific chemical signals transported through AM fungal hyphae (**Figure 32E**).

To assess the potential of these host-specific flavonoids to interact with rhizobial NodD1 proteins, *in silico* molecular docking was performed (**Figure 32F**). Flavonoids detected exclusively in $RC^{G.max}/HC^{+R.irregularis}$ and $RC^{M.truncatula}/HC^{+R.irregularis}$ treatments were docked with the NodD1 proteins of *B.*

diazoefficiens and *S. meliloti*, respectively. Daidzin exhibited the strongest binding affinity to *B. diazoefficiens* NodD1 ($-8.3 \text{ Kcal}\cdot\text{mol}^{-1}$), followed by 6''-O-malonyldaidzin ($-8.1 \text{ Kcal}\cdot\text{mol}^{-1}$), irilone ($-7.3 \text{ Kcal}\cdot\text{mol}^{-1}$), and erylatissin A ($-6.9 \text{ Kcal}\cdot\text{mol}^{-1}$). DL-liquiritigenin showed the highest affinity to *S. meliloti* NodD1 ($-7.9 \text{ Kcal}\cdot\text{mol}^{-1}$), followed by 3,7-dimethylquercetin ($-7.6 \text{ Kcal}\cdot\text{mol}^{-1}$), sakuranetin ($-7.5 \text{ Kcal}\cdot\text{mol}^{-1}$), and naringenin ($-7.3 \text{ Kcal}\cdot\text{mol}^{-1}$).

CMN enabled rhizobia to migrate preferentially toward their specific hosts *in vitro*

To investigate whether rhizobia exhibit host-specific preferred migration along the CMN, an *in vitro* quadri-compartmented Petri plate system was used, connecting *M. truncatula* and *G. max* through a CC containing a CMN of *R. irregularis* (**Figure 33A-C**). Following co-inoculation of *S. meliloti* and *B. diazoefficiens* onto AM fungal hyphae in the CC (**Figure 33D-I**), we encountered technical limitations in fluorescence-based quantification due to the stronger signal intensity of GFP-tagged *S. meliloti* (**Figure 33D, G**) compared to mCherry-tagged *B. diazoefficiens* (**Figure 33E, H**), which resulted in complete spectral overlap in the merged images (**Figure 33F, I**). To address this issue, we used selective plating for quantitative comparison of rhizobial migration.

At 24 hours post-inoculation, CFU analysis revealed a significant predominance of *S. meliloti* over *B. diazoefficiens* at the M1 sampling point, whereas no significant differences were observed at other sampling points (**Figure 33J**). At 48 hours, both rhizobia species displayed distinct host-oriented migration patterns via the CMN (**Figure 33K**). *S. meliloti* exhibited significantly higher CFUs than *B. diazoefficiens* at both M1 and M2 sampling points. Conversely, *B. diazoefficiens* preferentially migrated toward *G. max*, showing significantly higher CFUs than *S. meliloti* at the G1 and G2 sampling points (**Figure 33K**). At their respective sampling distances (0.7 cm for M1 and G1, 1.5 cm for M2 and G2), *S. meliloti* and *B. diazoefficiens* demonstrated significantly higher CFU counts than those migrating toward incompatible hosts (**Figure 33K**).

Greenhouse validation of CMN-mediated host-specific rhizobial recruitment

To validate the role of CMN in the migration and in increasing the concentrations of host-specific rhizobia in the substrate close to the plant host roots, a three-compartment greenhouse pot system was used (**Figure 34A; Figure S20**). This setup linked legumes through pipes with either 41 μm or 5 μm mesh barriers, permitting or restricting fungal hyphal growth, respectively. Four treatments were established: *G.max*≠*M.truncatula* (**Figure 34A-i**), *G.max*↔*M.truncatula* (**Figure**

34A-ii), *M.truncatula*↔*M.truncatula* (Figure 34A-iii), and *G.max*↔*G.max* (Figure 34A-iv). Hyphal density, rhizobial migration, and nodulation patterns were analyzed to examine CMN-mediated host specificity.

Fungal colonization and CMN formation

Fungal colonization was assessed by quantifying hyphal density in the pipes (Figure 34B) and by evaluating the AM fungal root colonization of *P. lanceolata* in the CC (Figure 34C). In the *G.max*≠*M.truncatula* treatment, no hyphae in the pipes and no root colonization of *P. lanceolata* were detected. By contrast, in the other three treatments (*G.max*↔*M.truncatula*, *M.truncatula*↔*M.truncatula*, and *G.max*↔*G.max*), hyphae were observed in the pipes and root colonization detected in *P. lanceolata*. Across these three treatments, no significant differences were observed in hyphal density in the pipes (Figure 34B) or in any of the measured parameters (i.e., AC, HC, VC, TC) of AM fungal root colonization of *P. lanceolata* (Figure 34C).

Rhizobial migration along CMN

Rhizobial migration was quantified at six sampling points (i.e., holes 1-6) along the pipes at 1, 3, 5, 7 (Figure 35), and 9 (Figure 34D-G) dpi. Independent of the dpi, a significantly lower concentration of *S. meliloti* and *B. diazoefficiens* was observed in the *G.max*≠*M.truncatula* treatment at all sampling points, compared to the other three treatments with AM fungal hyphae present in the pipes. In this treatment, the concentration of rhizobia progressively decreased with distance from the CC, and by day 9, rhizobia were almost undetectable at the sixth sampling point. There were no significant differences between *S. meliloti* and *B. diazoefficiens* at any sampling point in this treatment.

In the *G.max*↔*M.truncatula* treatment, both *S. meliloti* and *B. diazoefficiens* concentrations decreased with increasing distance from the CC at 1, 3, 5, 7, and 9 dpi. During the first 7 day, no significant differences were observed between the concentrations of the two rhizobia at any sampling point. On day 9, at the fifth and sixth sampling points the concentrations of *S. meliloti* were significantly higher than *B. diazoefficiens* in the pipes connected to *M. truncatula*. Conversely, the concentration of *B. diazoefficiens* was significantly higher than *S. meliloti* at the sixth sampling point in the pipes connected to *G. max*.

In the *M.truncatula*↔*M.truncatula* treatment, at 1 and 3 dpi, *B. diazoefficiens* and *S. meliloti* concentrations decreased with distance from the CC. During these early time points, no significant differences were found between the two rhizobia at any

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sampling point. Starting at 5 dpi, *S. meliloti* concentrations became significantly higher than *B. diazoefficiens* at one side of the sixth sampling point. At 7 dpi, this trend extended to both sides of the sixth sampling point and persisted through 9 dpi, where *S. meliloti* concentrations were significantly higher than *B. diazoefficiens* at the fifth and sixth sampling points. In addition, a similar trend was observed at the fourth sampling point in one of the pipes at 9 dpi.

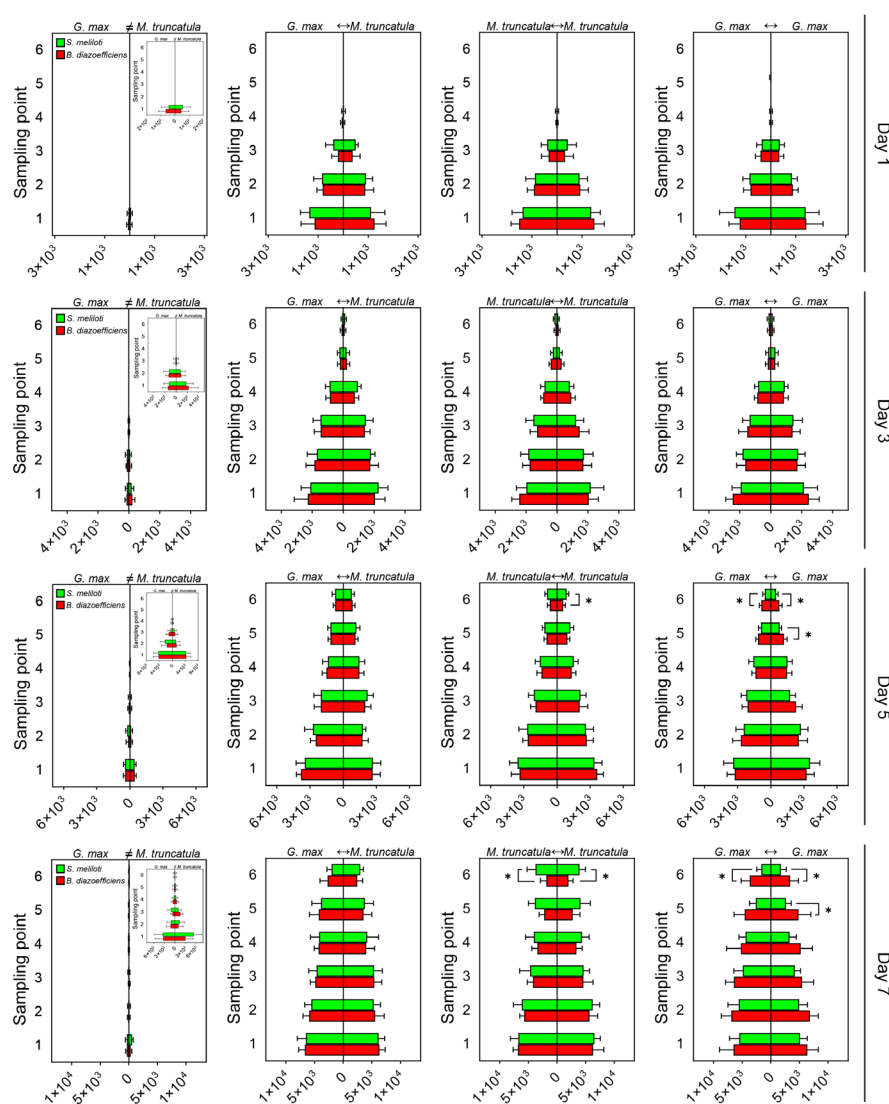


Figure 35. Colony-forming units (CFU·g⁻¹ fresh substrate) of *S. meliloti* and *B. diazoefficiens* at different sampling points (holes 1-6, with increasing distance from the CC) in the pipes of *G.max*≠*M.truncatula*, *G.max*↔*M.truncatula*, *M.truncatula*↔*M.truncatula*, and *G.max*↔*G.max*.

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M.truncatula↔*M.truncatula*, and *G.max*↔*G.max* treatments at 1, 3, 5, and, 7 days post-inoculation (dpi). Data are means ± SD ($n = 3$ biological replicates, with 3 technical replicates per sample). An asterisk (*) indicates $P \leq 0.05$ (Student's *t*-test for each sampling hole). Where insets are shown for the *G.max*≠*M.truncatula* treatment (top-right corner of relevant panels), they display the same data as the main panel but with an adjusted x-axis scale to better visualize variations between sampling points for that specific condition.

In the *G.max*↔*G.max* treatment, *B. diazoefficiens* and *S. meliloti* concentrations decreased with distance from the CC at 1 and 3 dpi. During these early time points, no significant differences were observed between the two rhizobia at any sampling point. At 5 and 7 dpi, *B. diazoefficiens* concentrations became significantly higher than *S. meliloti* at one side of the fifth sampling point and both sides of the sixth sampling point. At 9 dpi, this difference extended to both sides of the sixth sampling point, where *B. diazoefficiens* concentrations were significantly higher than *S. meliloti* at the fourth, fifth, and sixth sampling points. In addition, at 9 dpi, *S. meliloti* concentrations exhibited a pronounced decline with distance, particularly at the sixth sampling point, whereas *B. diazoefficiens* showed no such trend.

Rhizobial establishment in the substrate close to the roots

Rhizobial concentrations in the substrate close to the roots were measured 6 (Figure 36A) and 8 (Figure 37A) wpi. At 8 wpi, we found that in the *G.max*≠*M.truncatula* treatment, rhizobial concentrations in the substrate close to the roots of both *M. truncatula* (222 ± 120 CFU·g⁻¹ for *S. meliloti* and 244 ± 124 CFU·g⁻¹ for *B. diazoefficiens*) and *G. max* (233 ± 150 CFU·g⁻¹ for *S. meliloti* and 200 ± 132 CFU·g⁻¹ for *B. diazoefficiens*) were extremely low. In the *G.max*↔*M.truncatula* treatment, *S. meliloti* concentration ($2.1 \pm 0.3 \times 10^5$ CFU·g⁻¹) was significantly higher than *B. diazoefficiens* ($0.24 \pm 0.03 \times 10^5$ CFU·g⁻¹) in the rhizosphere of *M. truncatula*. Conversely, in the rhizosphere of *G. max*, *B. diazoefficiens* concentration ($1.6 \pm 0.2 \times 10^5$ CFU·g⁻¹) was significantly higher than *S. meliloti* ($0.22 \pm 0.04 \times 10^5$ CFU·g⁻¹). In the *M.truncatula*↔*M.truncatula* treatment, both *M. truncatula* substrate close to the roots were dominated by *S. meliloti* ($2.5 \pm 0.2 \times 10^5$ CFU·g⁻¹) and $2.4 \pm 0.2 \times 10^5$ CFU·g⁻¹, respectively), with significantly higher concentrations than *B. diazoefficiens* ($0.22 \pm 0.03 \times 10^5$ CFU·g⁻¹ and $0.22 \pm 0.04 \times 10^5$ CFU·g⁻¹, respectively). Similarly, in the *G.max*↔*G.max* treatment, both *G. max* substrate close to the root were dominated by *B. diazoefficiens* ($1.6 \pm 0.2 \times 10^5$ CFU·g⁻¹ and $1.5 \pm 0.2 \times 10^5$ CFU·g⁻¹, respectively), with significantly higher

concentrations than *S. meliloti* ($0.16 \pm 0.02 \times 10^5$ CFU·g⁻¹ and $0.15 \pm 0.02 \times 10^5$ CFU·g⁻¹, respectively). These statistically significant patterns were consistently observed at 6 wpi across all treatments (Figure 36A).

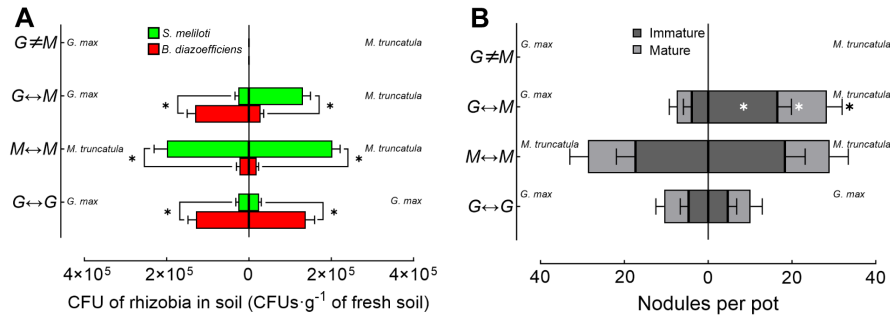


Figure 36. Rhizobial concentrations and nodulation in satellite compartments at 6 weeks post-inoculation (wpi) in greenhouse experiment. **(A)** Colony-forming units (CFU·g⁻¹ fresh substrate) of *S. meliloti* and *B. diazoefficiens* in the satellite compartments in the four treatments at 6 wpi with rhizobia in the CC. Data are means $\pm$ SD ($n = 6$ biological replicates, with 3 technical replicates per sample). An asterisk (*) indicates significant difference ($P \leq 0.05$, Student's t -test) within each satellite compartment. **(B)** Numbers of immature and mature nodules on roots of *G. max* and *M. truncatula* in the satellite compartments of the four treatments at 6 wpi with rhizobia in the CC. Data are means $\pm$ SD ($n = 6$ biological replicates, with 3 technical replicates per sample). White asterisks (*) on the bars for immature nodules (dark gray) and mature nodules (light gray) indicate significant differences between the two satellite compartments within each treatment ($P \leq 0.05$, Student's t -test). Black asterisks (*) adjacent to the bars denote significant differences in total nodule numbers between the two satellite compartments for each treatment ($P \leq 0.05$, Student's t -test). G≠M (*G. max*≠*M. truncatula*): *G. max* and *M. truncatula* in satellite compartments connected to the CC via pipes with 5 μ m mesh; G↔M (*G. max*↔*M. truncatula*): *G. max* and *M. truncatula* in satellite compartments connected to the CC via pipes with 41 μ m mesh; M↔M (*M. truncatula*↔*M. truncatula*): Both satellite compartments with *M. truncatula* and connected to the CC via pipes with 41 μ m mesh; G↔G (*G. max*↔*G. max*): Both satellite compartments with *G. max* and connected to the CC via pipes with 41 μ m mesh.

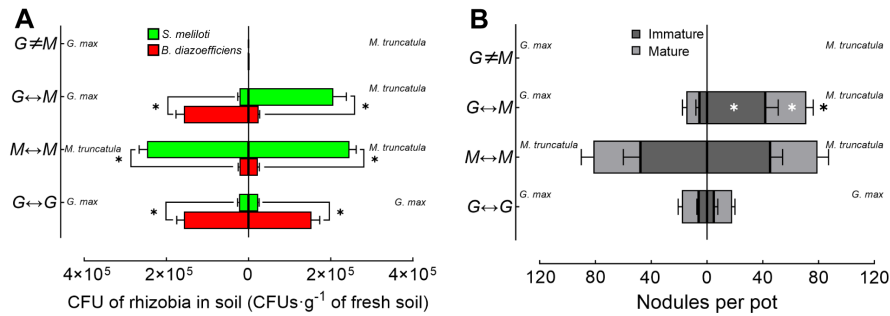


Figure 37. Rhizobial concentrations and nodulation in satellite compartments at 8 weeks post-inoculation (wpi) in greenhouse experiment. **(A)** Colony-forming units (CFU·g⁻¹ fresh substrate) of *S. meliloti* and *B. diazoefficiens* in the satellite compartments in the four treatments at 8 weeks post-inoculation with rhizobia in the CC. Data are means $\pm$ SD ($n = 6$ biological replicates, with 3 technical replicates per sample). An asterisk (*) indicates significant difference ($P \leq 0.05$, Student's *t*-test) within each satellite compartment. **(B)** Numbers of immature and mature nodules on roots of *G. max* and *M. truncatula* in the satellite compartments of the four treatments at 8 wpi with rhizobia in the CC. Data are means $\pm$ SD ($n = 6$ biological replicates, with 3 technical replicates per sample). White asterisks (*) on the bars for immature nodules (dark gray) and mature nodules (light gray) indicate significant differences between the two satellite compartments within each treatment ($P \leq 0.05$, Student's *t*-test). Black asterisks (*) adjacent to the bars denote significant differences in total nodule numbers between the two satellite compartments for each treatment ($P \leq 0.05$, Student's *t*-test). $G \neq M$ (*G. max* $\neq$ *M. truncatula*): *G. max* and *M. truncatula* in satellite compartments connected to the CC via pipes with 5 μ m mesh; $G \leftrightarrow M$ (*G. max* $\leftrightarrow$ *M. truncatula*): *G. max* and *M. truncatula* in satellite compartments connected to the CC via pipes with 41 μ m mesh; $M \leftrightarrow M$ (*M. truncatula* $\leftrightarrow$ *M. truncatula*): Both satellite compartments with *M. truncatula* and connected to the CC via pipes with 41 μ m mesh; $G \leftrightarrow G$ (*G. max* $\leftrightarrow$ *G. max*): Both satellite compartments with *G. max* and connected to the CC via pipes with 41 μ m mesh.

Host-specific nodulation

Nodulation was analyzed 6 (**Figure 36B**) and 8 (**Figure 34H-K**; **Figure 37B**) wpi. At 8 wpi, no nodules were observed on *G. max* or *M. truncatula* roots in the *G. max* $\neq$ *M. truncatula* treatment. In the *G. max* $\leftrightarrow$ *M. truncatula* treatment, 15 ± 4 nodules (10 ± 3 mature and 5 ± 3 immature) were formed on the roots of *G. max* and 71 ± 10 (29 ± 5 mature and 42 ± 9 immature) on the roots of *M. truncatula*.

In the *G.max*↔*G.max* treatment, both plants formed 18 (± 3 and ± 4 , respectively) nodules each, with one plant producing 12 ± 3 mature and 6 ± 1 immature nodules, and the other producing 13 ± 2 mature and 5 ± 3 immature nodules. In the *M.truncatula*↔*M.truncatula* treatment, the two *M. truncatula* plants formed 82 ± 17 and 79 ± 10 nodules, respectively. The nodules included 34 ± 9 and 34 ± 8 mature nodules, and 48 ± 12 and 45 ± 9 immature nodules. Similar trends were observed at 6 wpi, with comparable patterns in nodule formation and distribution across all treatments, and these differences were statistically significant (**Figure 36B**).

Discussion

This study examined the central role of the CMN of the AM fungus *R. irregularis* in mediating the migration of *S. meliloti* and *B. diazoefficiens*, and subsequent nodulation in their respective legume hosts, *M. truncatula* and *G. max*. By combining *in vitro* and greenhouse experiments with metabolomic analyses, we have revealed the dual function of this CMN: it acts both as a fungal highway facilitating the dispersal of rhizobia and as a carrier of chemical signals (i.e., flavonoids) guiding the migration of rhizobia towards their specific host. This selective tropism transforms the CMN into a sophisticated “navigation system”, which orient the rhizobia through the complex soil matrix with great precision.

In our study, the high specificity of rhizobia-legume interaction was confirmed by the exclusive nodulation of *S. meliloti* with the roots of *M. truncatula* and of *B. diazoefficiens* with the roots of *G. max*. This host-specific nodulation reflects the tightly regulated relationships between rhizobia and their legume partners, where recognition signals are fine-tuned to ensure compatibility (Oldroyd, 2013; Ferguson et al., 2019). Such specificity is likely mediated by the exchange of signal molecules, including flavonoids from the plant to the rhizobia and Nod factors from the rhizobia to the plant, a process well-documented as critical to initiate symbiosis (Masson-Boivin and Sachs, 2018). Our results further indicated that this specific recognition mechanism may also be mediated by the hyphae of AM fungi. These belowground conduits transport and release compounds, such as flavonoids, specific to the legume to which they are linked, acting as chemoattractants for rhizobia towards their compatible host.

The notably high number of nodules observed in our *in vitro* experiments, particularly in the *M.truncatula*^{*S.meliloti*} treatment, compared to the *G.max*^{*S.meliloti*} treatment, merits further explanation (**Figure 31C-G**). Several factors likely contribute to this elevated nodulation. Firstly, the *in vitro* Petri dish environment,

using $\text{MSR}^{\text{min}/2\text{N}}$ medium with reduced N, imposes minimal physical constraints on root growth, promoting extended and branched root systems (Gage, 2004). In our experiments, *M. truncatula* developed notably long, fibrous, and highly branched roots, likely increasing the surface area and number of rhizobial infection sites compared to *G. max*, which exhibited thicker, less branched roots *in vitro*. Secondly, the nitrogen-limited medium and controlled conditions (e.g., stable pH, high humidity) create an optimal environment for nodulation, enhancing rhizobial colonization and infection thread formation compared to soil-based systems, where environmental variability can suppress nodule development (Oldroyd and Downie, 2008). The direct application of rhizobial suspensions onto roots ensures high rhizobial density and uniform contact, further increasing infection efficiency. Thirdly, the high compatibility between *S. meliloti* and *M. truncatula* drives efficient nod factor signaling, leading to rapid and extensive nodule initiation, as evidenced by the high proportion of immature nodules (Jones et al., 2007). In contrast, the lower nodule count in *G. max* may result from slower nodulation kinetics of *B. diazoefficiens* or the formation of determinate nodules, which are typically fewer but mature faster, as observed in our results. Additionally, the sterile *in vitro* environment eliminates competing soil microorganisms, allowing unimpeded rhizobial colonization and potentially inflating nodule numbers compared to natural conditions. The six-week experimental duration may also contribute, as *M. truncatula*'s indeterminate nodules continue to form due to persistent meristematic activity, unlike the synchronized formation of determinate nodules in *G. max* (Saeki, 2011). These factors collectively suggest that the *in vitro* setup, combined with host-specific rhizobial interactions and root architecture, promotes exceptionally high nodulation, particularly in the *M. truncatula*^{*S. meliloti*} treatment.

Central to our study was the demonstration that the CMN formed between two different legume species by *R. irregularis* served as a physical conduit, or “fungal highway”, for the migration of rhizobia towards their specific host. This concept of “fungal highway”, as presented in previous studies (Kohlmeier et al., 2005; Simon et al., 2015), indicates that hyphal surfaces, coated with water films, create microenvironments favorable to bacterial motility, particularly in soils where diffusion alone can limit microbial propagation. Using an *in vitro* compartmented system, we observed that *S. meliloti* and *B. diazoefficiens* migrated along the AM fungal hyphae with a higher presence towards *M. truncatula* for the first and towards *G. max* for the second. Interpreting the CFU data from our *in vitro* system (**Figure 33**) requires consideration of whether differences arise from differential migration or potential differential growth at the sampling sites. Although some localized bacterial growth within the central compartment medium over 24-48 hours cannot be entirely discounted, several factors suggest that active, host-

preference-driven migration along AM fungal hyphae is the predominant mechanism. These include the nutrient-limited nature of the hyphal support medium, the clear host-specific directional accumulation of rhizobia, and the visual evidence of bacterial association with fungal hyphae. Thus, we interpret the observed CFU differences as primarily indicative of differential bacterial migration efficiency and/or preference. This specific migration suggests that the CMN not only provides a facilitated pathway for rhizobial dispersal to new niches but also helps the bacteria to move preferentially towards their compatible hosts, at a reduced energetic cost. This fungal path was suggested by recent studies of improved migration efficiency due to facilitated flow velocity and chemical guidance along fungal hyphae, further contextualized within the framework of optimized symbiotic interactions and resource allocation (de Novais et al., 2020; D. Wang et al., 2022; Anckaert et al., 2024; He et al., 2024). The greenhouse experiment using a compartmented pot system further corroborated this mechanism under more natural conditions (**Figure 34**). In the treatment with CMN connecting the two legumes species, rhizobial concentrations decreased progressively with distance from the inoculation point towards the compatible host, indicating a hyphal-mediated transport. In contrast, in the treatment without CMN connecting plants, only a very limited migration was observed, underlining the key role of CMNs in bridging spatial gaps between rhizobia and their hosts. The analysis of CFU of both rhizobia revealed dynamic patterns: in the treatment linking the two different legumes by the CMN, we observed no significant differences in the CFUs of *S. meliloti* and *B. diazoefficiens* during the first 7 dpi at any sampling point in the pipes (**Figure 35**). This suggests that, initially, the migration of both rhizobia along the hyphae may be non-directional, with no clear preference for either host. Conversely 9 dpi, a significant difference was observed (**Figure 34E**). Close to *M. truncatula*, the concentration *S. meliloti* was significantly higher than that of *B. diazoefficiens*, whereas the reverse was observed near *G. max*. This difference indicates that migration becomes increasingly oriented as rhizobia approach their specific hosts, likely due to the increasing concentration of specific flavonoids.

In addition to physical transport, our study revealed an important role for the hyphae of CMN in transporting and releasing signal molecules from the host to which they are connected, thereby orienting the migration of rhizobia towards their specific hosts. The metabolomic profiling of ERM exudates (in particular the flavonoids) collected in the hyphal compartment of the bi-compartmented *in vitro* culture system differed between plants to which the hyphae are connected (**Figure 32A, B**). The perception of these flavonoids by rhizobia is mediated by NodD proteins, transcriptional regulators of the LysR family that undergo a conformational change upon binding specific flavonoids, enabling them to

activate nod genes by binding to conserved DNA sequences known as nod boxes (Peck et al., 2006; Hawkins and Oresnik, 2022). This specificity, driven by unique flavonoid cocktails from each legume and varying NodD isoforms in rhizobia, is a key determinant of symbiotic host range (Liu and Murray, 2016).

For the exudates of ERM connected to *G. max*, isoflavones including daidzin, 6''-O-malonyldaidzin, irilone and erylatissin A were detected (**Figure 32F**), with binding affinities to *B. diazoefficiens* NodD1 of $-8.3 \text{ Kcal}\cdot\text{mol}^{-1}$, $-8.1 \text{ Kcal}\cdot\text{mol}^{-1}$, $-7.3 \text{ Kcal}\cdot\text{mol}^{-1}$ and $-6.9 \text{ Kcal}\cdot\text{mol}^{-1}$, respectively. Daidzin, a glycosylated form of daidzein, stands out as a powerful inducer of nod genes, as previously indicated (Bag et al., 2022; Hawkins and Oresnik, 2022). Its high affinity for NodD1 underlines its central role in the initiation of symbiosis by triggering transcriptional activation of nodulation genes. Similarly, 6''-O-malonyldaidzin not only contributes to *nod* gene induction but also appears to modulate the secretion of other isoflavones (Loh and Stacey, 2003), suggesting a regulatory function that fine-tunes the symbiotic dialogue between *G. max* and *B. diazoefficiens*. Irilone, although more commonly associated with red clover (Lee et al., 2018), shows moderate NodD1 affinity in our study, suggesting a possible secondary role in soybean, perhaps as a chemoattractant attracting *B. diazoefficiens* to the host or as a modulator of wider microbial interactions in the rhizosphere. Erylatissin A, had the lowest affinity among the four compounds above. This compound lacks extensive studies in soybean symbiosis. Its presence in ERM exudates suggests that it might contribute to a broader chemical signature, potentially enhancing *B. diazoefficiens* recognition or subtly influencing rhizosphere ecology.

For the exudates of ERM connected to *M. truncatula*, the most abundant compounds were flavanones like DL-liquiritigenin and naringenin, and alongside flavonoid derivatives like sakuranetin and 3,7-dimethylquercetin, with binding affinities to *S. meliloti* NodD1 of $-7.9 \text{ Kcal}\cdot\text{mol}^{-1}$, $-7.3 \text{ Kcal}\cdot\text{mol}^{-1}$, $-7.5 \text{ Kcal}\cdot\text{mol}^{-1}$ and $-7.6 \text{ Kcal}\cdot\text{mol}^{-1}$, respectively (**Figure 32F**). DL-liquiritigenin appears to be a key player, not only because of its high NodD1 binding affinity, but also because of its demonstrated ability to rescue nodulation in flavonoid-deficient *M. truncatula* mutants (Wasson et al., 2006). This ability indicates that it can either induce nodulation genes or act synergistically with other signals to establish symbiosis. Naringenin, a precursor in flavonoid biosynthesis, can stimulate the binding of *S. meliloti* NodD1 to *nod* gene promoters, although it does not induce gene expression (Compton et al., 2020). It has also been shown to act as a competitive inhibitor of known *nod* gene inducers like luteolin (Peck et al., 2006), which suggests a regulatory role in fine-tuning *S. meliloti* responses. Sakuranetin, although less studied in this specific symbiosis, is known as a

phytoalexin in other plant species (Jiang et al., 2024). It could integrate symbiotic signaling with defense responses, with AM fungal hyphae potentially transporting it to amplify *M. truncatula*'s chemical signature and aid *S. meliloti* in distinguishing its host from competitors (*B. diazoefficiens* in this study) or pathogens (Hasegawa et al., 2014). Similarly, for 3,7-dimethylquercetin, there is a lack of studies on this compound, but its methylated structure, a modification highlighted in *M. truncatula* flavonoids (Jasiński et al., 2009), could reinforce host specificity, possibly by stabilizing NodD1 interactions or modulating rhizosphere microbial interactions. These compounds were absent from the exudates of the hyphal-free controls, highlighting the active participation of AM fungi in the transport and release of signals.

While our study primarily focused on the role of flavonoids in mediating the specificity of rhizobia-legume interactions via the CMN, our metabolomic analysis also revealed a diverse array of non-flavonoid compounds in the ERM exudates from the *G. max* and *M. truncatula* (**Table S5**). For the exudates of ERM connected to *G. max*, 30 unique compounds across 15 chemical categories, dominated by terpenoids (11 compounds, primarily triterpene saponins and terpene glycosides) and glycosides (2 compounds, including hydroxycinnamic acid glycosides and cyanogenic glycosides), alongside amino acid derivatives (e.g., glutamic acid derivatives), aromatic compounds (e.g., anthraquinones, coumestans), carboxylic acids, phenols, nucleosides, ketones, and heterocyclic compounds were identified. This high chemical diversity and compound abundance suggest a complex secondary metabolism in *G. max*, likely tailored to intricate plant-microbe interactions. In contrast, the exudates of ERM connected to *M. truncatula* contained only 6 unique compounds across 6 categories, including terpenoids (1 triterpene saponin), glycosides (1 steroidal glycoside), aromatic compounds (e.g., 1-benzopyrans), saccharides (oligosaccharides), and steroids, with no dominant category and simpler chemical profiles, possibly reflecting specific metabolic pathways in *M. truncatula*. Both treatments shared terpenoids (triterpene saponins) and glycosides, indicating partial overlap in secondary metabolic pathways, likely driven by common plant-AM fungal symbiotic responses. However, the exudates of ERM connected to *G. max* exhibited far greater chemical diversity and quantity compared to the exudates of ERM connected to *M. truncatula*, with unique compounds like anthraquinones, phenols, and nucleosides, whereas the exudates of ERM connected to *M. truncatula* contained exclusive saccharides, coumarins, and steroids, suggesting more specialized metabolic outputs in *M. truncatula*. These non-flavonoid compounds likely play complementary roles in the symbiotic process. Terpenoids, such as triterpene saponins abundant in the exudates of ERM connected to *G. max*, are known for their antimicrobial properties and may shape the rhizosphere

microbiome by selectively inhibiting non-compatible microorganisms, thereby facilitating colonization by compatible symbionts like *B. diazoefficiens* (Badri and Vivanco, 2009; Dunn, 2015). Similarly, glycosides, including cyanogenic glycosides in the exudates of ERM connected to *G. max* and steroidal glycosides in the exudates of ERM connected to *M. truncatula*, may contribute to plant defense mechanisms, indirectly supporting symbiosis by reducing pathogen pressure (Gleadow and Woodrow, 2002; Zaynab et al., 2021). Amino acid derivatives, such as glutamic acid derivatives and N-acyl-alpha amino acids in the exudates of ERM connected to *G. max*, may support metabolic exchanges critical for nitrogen fixation, as rhizobial amino acid metabolism is closely linked to plant metabolism and essential for effective symbiosis (Dunn, 2015). The production and release of these compounds are likely influenced by AM fungal symbiosis, as AM fungi can reprogram plant secondary metabolism, enhancing the biosynthesis of such metabolites (Zhao et al., 2022). By transporting and releasing these non-flavonoid compounds, the CMN facilitates a more complex chemical dialogue than previously appreciated, potentially enhancing the precision of rhizobial navigation and symbiotic efficiency (Gorzelak et al., 2015). Future studies, combining *in vitro* assays and targeted metabolomics, are needed to elucidate the specific functions of these compounds and their interactions with rhizobia in the context of legume-AMF-rhizobia tripartite symbiosis (Badri & Vivanco, 2009; Dunn, 2015).

Regarding the molecular docking results presented in **Figure 32F**, we observed an intriguing phenomenon, while we anticipated *B. diazoefficiens* NodD1 would primarily recognize and bind to *G. max*-specific flavonoids, and *S. meliloti* NodD1 to *M. truncatula*-specific flavonoids, the docking results generally indicated that *S. meliloti* NodD1 exhibited lower binding energies (i.e., higher binding stability) with all tested flavonoids compared to *B. diazoefficiens* NodD1. For instance, the binding energies of *S. meliloti* NodD1 with irilone, erylatissin A, daidzin, and 6''-O-malonyldaidzin were all lower than those of *B. diazoefficiens* NodD1, which somewhat deviates from our expectation that *B. diazoefficiens* would demonstrate more stable binding to *G. max*-specific flavonoids. This discrepancy may reflect the inherent limitations of molecular docking simulations, which are often conducted under simplified models and may not fully capture the complex *in vivo* biological environment, including the presence of other molecules, ionic strength, and the dynamic conformational changes that NodD proteins may undergo upon flavonoid binding. Crucially, the biological “specificity” of NodD proteins is not solely determined by their binding affinity to flavonoids, but also by their ability to effectively induce the correct conformational changes that lead to the activation of nod gene expression. For example, as discussed, naringenin can stimulate *S. meliloti* NodD1 binding to *nod*

gene promoters without inducing gene expression, illustrating that binding stability does not always equate to biological activation effectiveness. Therefore, even if *S. meliloti* NodD1 shows energetically more stable binding to certain flavonoids, this does not necessarily mean these interactions are biologically productive *in vivo*. Conversely, the binding of *B. diazoefficiens* NodD1 to *G. max*-specific flavonoids (such as daidzin), while perhaps not showing the lowest binding energies compared to *S. meliloti* NodD1 in some instances, may be more efficient at triggering the activation of its nodulation pathway. Simultaneously, rhizobia in the CMN encounter a mixture of flavonoids and a concentration gradient. The localized high concentration of host-specific flavonoids near their compatible host is likely the driving force behind selective chemotaxis and eventual nodulation, overriding any differences in molecular-level binding energies. The observed host-specific rhizobial accumulation and nodulation patterns in our *in vivo* experiments strongly support a host-specific recognition mechanism driven by biological activation and concentration gradients.

By transporting and releasing these host-specific signals along a probable root-to-hyphal tip concentration gradient, AM fungi play a crucial role in guiding the rhizobia towards their appropriate host. We hypothesize that in the central region of the CMN (i.e., at equal distance of the two legumes), the signals released by the hyphae linking *M. truncatula* and *G. max* could not be differentiated, resulting in the absence of a clear gradient towards either host (Parniske, 2008; Smith, 2010). In this central zone, rhizobia can move randomly in both directions, with no clear preference for one host or the other. As they migrated closer to one legume or another, they probably perceived greater gradients of host-specific flavonoids, triggering chemotaxis towards their compatible host. This hypothesis is supported by previous studies (Besserer et al., 2006; Zhang et al., 2020), who demonstrated that bacteria can detect and respond to chemical gradients over time, resulting in chemotaxis towards specific attractants. This phenomenon can be amplified by the coenocytic nature of AM fungal hyphae facilitating signal diffusion across the inoculation point, with flavonoids from one plant potentially present on the opposite side, complicating initial directional cues until a significant dominant gradient emerges near the host (Schmieder et al., 2019; Han et al., 2023).

The ability of CMNs to transport and release specific signals from the plant to whom they are connected resembles a sophisticated “navigation system” within the soil, transmitting host-specific cues that serve as “signposts”. These cues guide *S. meliloti* and *B. diazoefficiens* through the complex “labyrinth” of the soil matrix, directing them precisely to their respective host plants, *M. truncatula* and *G. max*.

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This resulted in a relatively higher concentration of *S. meliloti* close to *M. truncatula* roots versus *B. diazoefficiens* close to *G. max*

The high specificity of flavonoid release suggests a co-evolutionary adaptation between AM fungi, legumes, and rhizobia. By transporting and releasing exudates to recruit compatible rhizobia, AM fungi optimize symbiotic outcomes, aligning with a resource exchange framework where they act as intermediaries maximizing nitrogen fixation and nutrient uptake (Duan et al., 2025). This role parallels their transport of phosphate-solubilizing bacteria to nutrient-rich patches, highlighting their mediation in plant-microbe interactions (Jiang et al., 2021).

Conclusion

Legume-rhizobia symbiosis typically relies on short-distance root signals, with long-distance dispersal attributed to external vectors. Our *in vitro* and greenhouse experiments show that *R. irregularis* hyphae, via CMN, fill this gap, directing *S. meliloti* and *B. diazoefficiens* to *M. truncatula* and *G. max*, respectively. These networks integrate physical transport with host-specific flavonoid signals, acting as a sophisticated navigation system that ensures precise rhizobial delivery and selective nodulation. Beyond nutrient exchange, CMN spatially organize rhizobia, enhancing symbiotic efficiency and demonstrating an ecological role for AM fungi as regulators of rhizobia distribution in soil. Given their omnipresence in soils, this mechanism improves rhizobial access to compatible hosts, supporting the co-application of AM fungi and rhizobia for sustainable nitrogen fixation. Future studies under fields across diverse legume systems are essential to translate these observations into agricultural practice. This research repositions AM fungi as key mediators of belowground microbial interactions, paving the way for eco-friendly farming strategies (**Figure 38**).

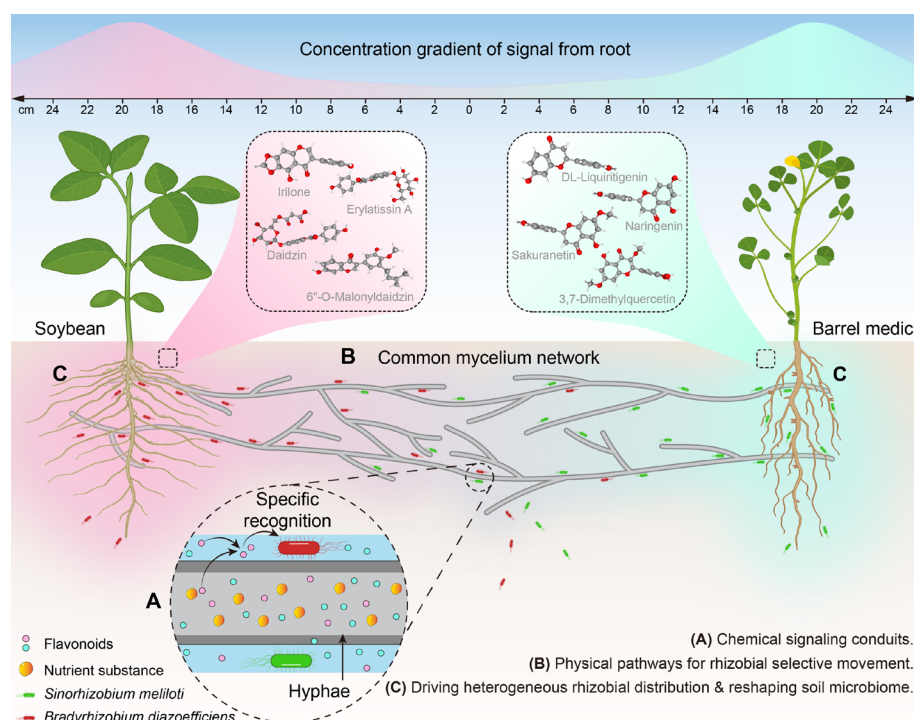


Figure 38. Schematic representation of how the common mycorrhizal network (CMN) of *R. irregularis* guides the selective recruitment of rhizobia to their compatible legume hosts. The CMN connecting soybean (*Glycine max*, left) and barrel medic (*Medicago truncatula*, right) serves as both a fungal highway and a chemical signaling conduit. Host-specific flavonoids – depicted in pink for soybean (including daidzin, 6''-O-malonyldaidzin, irilone, and erylatissin A) and cyan for barrel medic (including DL-liquiritigenin, naringenin, sakuranetin, and 3,7-dimethylquercetin) – produced by the roots of legumes are transported and released by the fungal hyphae. These flavonoids establish signal gradients that act as molecular navigation cues for rhizobia. The magnified inset (A) reveals the detailed mechanism of rhizobial migration, showing how *B. diazoefficiens* (red) and *S. meliloti* (green) move along hyphal surfaces in response to these flavonoid signals, which are transported within the hyphal cytoplasm and/or through the external water film. This chemical signaling drives the preferential movement of rhizobia toward their compatible hosts: *B. diazoefficiens* navigates toward soybean while *S. meliloti* is guided to barrel medic, with sharpening gradients of host-specific flavonoids refining their navigation precision as they approach their targets, enhancing nodulation efficiency and nitrogen fixation potential. This sophisticated biological navigation system illustrates how the CMN spatially organizes rhizobial communities in soil, creating heterogeneous microbial

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distributions that optimize symbiotic partnerships and potentially reshape the broader soil microbiome. The precision of this recruitment mechanism reflects the co-evolutionary adaptations among legumes, rhizobia, and arbuscular mycorrhizal fungi, providing an ecological advantage in nutrient acquisition and highlighting the remarkable complexity of belowground plant-microbe interactions that support sustainable nitrogen cycling in terrestrial ecosystems. Note: The proportions of elements in this schematic, including hyphae, roots, and rhizobia, are exaggerated for illustrative purposes and do not reflect their actual scale.

GENERAL DISCUSSION

Legumes are essential components of agricultural ecosystems, not least because of their ability to form symbiotic relationships with nitrogen (N₂)-fixing bacteria, known collectively as rhizobia (Saeki, 2011; Parniske, 2018; Poole, 2024), thereby reducing crop dependence on N fertilizers. This symbiosis, resulting in the formation of root nodules, allows legumes to thrive in nitrogen-limited environments and contributes significantly to nitrogen (N) accumulation in agricultural soils, with an estimate of 70 million tons of N₂ fixed annually (Brockwell et al., 1995; Pawlowski and Demchenko, 2012; Palmero et al., 2022). However, the efficiency of this symbiosis is highly dependent on the successful encounter and interaction between rhizobia and the roots of compatible legume hosts (Ferguson et al., 2019). While the short-distance recruitment of rhizobia via root exudates, particularly flavonoids, is relatively well-understood (Peters et al., 1986; Bais et al., 2006), the mechanisms governing long-distance dispersal and host-specific recruitment, particularly given the often-limited mobility of rhizobia and the presence of air-filled gaps in soil aggregates making migration difficult (Horiuchi et al., 2005; Vicario et al., 2015), remain less clear.

Arbuscular mycorrhizal (AM) fungi, which are ubiquitous soil microorganisms form symbiotic associations with the roots of about 72% of terrestrial plants, including legumes (Smith and Read, 2008; Brundrett and Tedersoo, 2018; Vigneron et al., 2018). They are known to play an important role in the acquisition of nutrients by plant, in particular phosphorus and nitrogen (Wahbi et al., 2016; Wang et al., 2016a; Zhang et al., 2018a). Their extraradical mycelium (ERM) extends far beyond the rhizosphere, creating an extensive network of hyphae exploring a larger volume of soil than roots alone (Awaydul et al., 2019), with densities reaching 82 to 111 m·cm⁻³ in prairie and 52 to 81 m·cm⁻³ in ungrazed pasture (Miller et al., 1995). This network has been shown to facilitate the transport of nutrients (Jiang et al., 2017; Wipf et al., 2019; Yan et al., 2022; Magkourilou et al., 2024) and even signaling molecules between plants (Song et al., 2010; Babikova et al., 2013; Wu et al., 2017). Recent studies have also demonstrate the role of AM fungi in influencing the composition and activity of microbial communities in the hyphosphere, the zone of soil immediately surrounding the hyphae (Toljander et al., 2007; Zhang et al., 2018a), and even facilitating bacterial movement to nutrient patches inaccessible to roots (Jiang et al., 2021).

In this thesis, we investigated the role of AM fungi, in particular the model strain *Rhizophagus irregularis* MUCL 41833, in mediating the long-distance migration

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of rhizobia and their subsequent specific recruitment by legume roots, thereby influencing the efficiency and specificity of the legume-rhizobia symbiosis. We used a combination of *in vitro* and greenhouse experiments, coupled with molecular and metabolomic analyses, to elucidate the mechanisms underlying this tripartite interaction, building upon the foundational hypothesis that legumes recruit rhizobia via the ERM of AM fungi, similar to how mycelial networks of other fungi have been shown to aid rhizobial migration (Zhang et al., 2020). This work has revealed a completely new indirect pathway, whereby AM fungi play a key role both as physical conduits for rhizobia migration and as transporters and delivery pipes for signals (e.g., flavonoids) originating from the legume and acting as chemoattractants, significantly increasing rhizobia recruitment beyond the limits of the rhizosphere.

In the first chapter, we investigated how the ERM of *R. irregularis* served as a pathway for *Sinorhizobium meliloti* to travel long distances to *Medicago truncatula* roots, through the perception of a gradient of plant-derived flavonoids, increasing nodulation. **In the second chapter**, we studied the specificity of this recruitment in mixed legume systems, demonstrating how a common mycorrhizal network (CMN) directed *S. meliloti* and *Bradyrhizobium diazoefficiens* towards their respective hosts, *M. truncatula* and *Glycine max*, respectively, following the perception of specific chemoattractant chemical signals.

Below, we discuss the most critical results of the doctoral research, around four major integrated thematic.

Physical connectivity: fungal hyphae as dispersal highways

A fundamental challenge in establishing effective legume-rhizobia symbioses is to overcome the spatial limitations imposed by the heterogeneous distribution of rhizobia in the soil and their inherent limited mobility (Horiuchi et al., 2005; Vicario et al., 2015). While root exudates can attract rhizobia over short distances (millimeters), this mechanism is ineffective for recruitment from more distant locations (Zhang et al., 2020). Our research, directly addressed this challenge by demonstrating that the ERM of AM fungi provides a physical network, a “fungal highway”, which considerably improves rhizobial dispersal.

The *in vitro* experiments using *S. meliloti* and those using both *S. meliloti* and *B. diazoefficiens* with their respective legume hosts (*M. truncatula* and *G. max*), consistently showed that rhizobia could migrate along the hyphae of *R. irregularis* connected to the plants. This migration was not a simple non-directional diffusion.

GENERAL DISCUSSION

Time-lapse imaging revealed that *S. meliloti* was traveling at a speed up to $10.78 \mu\text{m}\cdot\text{s}^{-1}$ along hyphae compared to $6.01 \mu\text{m}\cdot\text{s}^{-1}$ without hyphae, suggesting that the hyphal surface is a suitable conduit facilitating bacterial motility. The observed migration was significantly faster and more directional than in absence of hyphae, a pattern consistently observed in both single- and mixed-legume systems. These findings suggest that hyphae serve as preferential pathways for rhizobia migration, probably due to a combination of factors: (i) a continuous water film along the hyphal surface, (ii) a reduced tortuosity relative to the soil matrix, and (iii) the potential supply of nutrients (i.e., carbon sources) and chemoattractant signals by the fungus connected to the appropriate host plant (Kohlmeier et al., 2005; Vieira et al., 2025).

The greenhouse experiments provided compelling evidence for the ecological relevance of this hyphal transport. The quadri-compartmented pot system demonstrated that *S. meliloti* could migrate approximately 15 cm along the hyphae to form nodules on the root of *M. truncatula*. A similar three-compartmented system with two legume species connected by a CMN, revealed that both *S. meliloti* and *B. diazoefficiens* could migrate along the hyphae, albeit with a discernible preference for their respective hosts. This was for instance materialized by the significantly higher density of *S. meliloti* (i.e., $5.7 \times 10^3 \text{ CFU}\cdot\text{g}^{-1}$) near *M. truncatula* compared to *B. diazoefficiens* ($2.9 \times 10^3 \text{ CFU}\cdot\text{g}^{-1}$) for which *M. truncatula* is not the host, highlighting the preferred migration. The lack of substantial nodulation in control treatments (without CMN connecting plants) underlined the crucial role of the fungal network in facilitating long-distance rhizobial dispersion.

Taken together, our findings provide convincing evidence that the ERM of AM fungi acts as a dispersal highway for rhizobia, expanding the zone of rhizobial recruitment far beyond the immediate rhizosphere. This physical connectivity between soil and plant is a prerequisite for the establishment of the symbiosis, under conditions where rhizobia have to overcome spatial barriers (e.g. air gaps), reaching their legume hosts from distances that would otherwise be insurmountable. This “fungal highway” not only bridges gaps in the soil matrix but also represent a novel mechanism that complements traditional vectors like water flow (Vicario et al., 2015; Vieira et al., 2025). This builds upon the concept of “fungal highways” for other soil bacteria (Kohlmeier et al., 2005; Banitz et al., 2013; Christensen and Reck-Peterson, 2022) and establishes it firmly within the context of the legume-rhizobia symbiosis.

Chemical guidance: host-specific signaling via flavonoid gradients

While the physical connection of ERM to the legume species facilitates rhizobial dispersal, it does not alone explain the remarkable specificity observed in legume-rhizobia symbioses. Legumes and rhizobia engage in a highly specific molecular dialogue, with flavonoids released by the plant acting as key signals (Peters et al., 1986; Oldroyd, 2013). Our results revealed that AM fungi play a crucial role in the transport and release of these host-specific signals, guiding rhizobia to their appropriate hosts.

This clear host-specificity preference was demonstrated in the *in vitro* experiment with *M. truncatula* and *G. max* connected by a CMN, favoring the migration of *S. meliloti* towards *M. truncatula* and *B. diazoefficiens* towards *G. max*. This result combined with the findings of the directed migration of *S. meliloti* towards *M. truncatula* rather than to *Solanum tuberosum* demonstrate that the CMN transport and release specific chemical signals from the plant to whom they are connected. This selective tropism transforms the CMN into a sophisticated “navigation system”, directing rhizobia through the complex soil matrix with high precision.

The metabolomic analyses were key to understand this phenomenon. Interestingly, the metabolomic analyses of hyphal exudates revealed distinct flavonoid profiles in our different experiments, with no overlap between the flavonoids identified. In the first experiment comparing *M. truncatula* and non-legume plants, we identified eight unique flavonoids including sagittain B and luteolin derivatives, while in the second experiment comparing *M. truncatula* and *G. max*, we detected different compounds such as DL-liquiritigenin and daidzin. This complete difference in flavonoid profiles warrants explanation.

The fundamental difference lies in the experimental designs and their corresponding research questions. When examining *M. truncatula* in contrast to non-leguminous plants, we were essentially comparing plants with and without nodulation capacity. In this context, the identified flavonoids (sagittain B, isosakuranetin-hexosyl-deoxyhexose, luteolin-acetylhexuronide, taxifolin-deoxyhexosyl-oxanepentol, haploside C, sudachiin A, and tetrahydroxy-tetramethoxyflavone-hexoside) likely represent “general nodulation signals” that primarily function to induce nod gene expression in rhizobia. These compounds exhibited particularly strong binding affinities with *S. meliloti* NodD1 (ranging from -7.7 to -9.4 Kcal·mol⁻¹), suggesting their primary role in activating the nodulation pathway. For instance, sagittain B demonstrated exceptional binding

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affinities ($-9.46 \text{ Kcal}\cdot\text{mol}^{-1}$ with NodD1 and $-9.39 \text{ Kcal}\cdot\text{mol}^{-1}$ with NodD2), indicating its potency as a nodulation trigger.

Conversely, when comparing two leguminous plants (*M. truncatula* and *G. max*), we focused on identifying signals responsible for host-specific rhizobial recruitment. The flavonoids detected in this context (DL-liquiritigenin, naringenin, sakuranetin, and 3,7-dimethylquercetin from *M. truncatula*-connected hyphae; daidzin, 6''-O-malonyldaidzin, irilone, and erylatissin A from *G. max*-connected hyphae) displayed more moderate binding affinities to their respective rhizobial NodD proteins (-7.3 to $-7.9 \text{ Kcal}\cdot\text{mol}^{-1}$ for *M. truncatula* flavonoids to *S. meliloti* NodD1; -6.9 to $-8.3 \text{ Kcal}\cdot\text{mol}^{-1}$ for *G. max* flavonoids to *B. diazoefficiens* NodD1). These lower affinities, compared to the “general nodulation signals”, suggest that these compounds might serve more as “host identity recognition signals” guiding specific rhizobia to their compatible hosts, rather than as primary nodulation inducers. Although both experiments utilized *M. truncatula* connected to *R. irregularis*, the lack of overlap does not indicate entirely dissimilar raw exudate profiles. In untargeted metabolomics, significant metabolites are identified relative to the comparison group. Thus, the legume versus non-legume setup highlighted “general nodulation signals”, while the legume vs. legume setup emphasized host-specific signals.

The statistical methodology further amplified this distinction. In the legume versus non-legume experiment, we employed a threshold of $P \leq 0.05$ to identify significantly different metabolites, a relatively permissive criterion that captured flavonoids with moderate differences in abundance, i.e. those “general signals” distinguishing legumes from non-legumes. In contrast, the legume versus legume comparison used a stricter $P \leq 0.01$ threshold, retaining only the most significantly divergent compounds and likely excluding many “general nodulation signals” common to both species. For example, compounds like luteolin-acetylhexuronide may be produced by both *M. truncatula* and *G. max* at relatively similar levels (Peters et al., 1986; Zhang and Smith, 1996; Pan and Smith, 2000; Adiji et al., 2021), yielding P -values above 0.01 and thus being filtered out. The nature of ANOVA testing reinforced this pattern by highlighting substantial differences in flavonoid production between different plant types (legume vs. non-legume), while in comparisons to closely related legume species, only the most divergent compounds stood out against a backdrop of baseline similarity. This explains why the “general nodulation signals” from the first experiment did not appear as significantly different in the second. They may be present in both legumes' exudates but lack sufficient divergence to meet the stricter threshold.

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From a biological perspective, this pattern aligns with plant signaling in symbiotic relationships. Legumes produce a baseline set of flavonoids that generally promote nodulation (those identified in our first experiment) and species-specific signals that guide their preferred rhizobial partners (those identified in our second experiment). When establishing symbiosis, legumes likely first release “host-specific recruitment signals” to attract compatible rhizobia to the root vicinity; once the rhizobia arrive, the “general nodulation signals” (present at higher concentrations near the root) trigger *nod* gene expression to initiate nodule formation. In our experimental design, each setup was optimized to detect one signal type, resulting in non-overlapping flavonoid sets. This dual mechanism reflects a sophisticated strategy: specific recruitment followed by robust nodulation induction, with the AM fungal network facilitating both processes. This indicates that the AM fungus plays a crucial role in the transport and release of plant-derived signals. The fungal hyphae act as pipelines, transporting and releasing flavonoids produced by the plant. Although it remains unknown if this transport is passive or active.

The quantity of flavonoids released by the hyphae is probably greater close to the root, creating a gradient along the hyphae from root to tip. This gradient guides the rhizobia towards the compatible host plant. Thus, the plant, by sending specific signals via the AM fungus, directs the recruitment of its preferred rhizobial partner, which is similar to the “cry for help” strategy in which plants attacked by pathogens or herbivores modify their root exudation chemistry to attract beneficial microorganisms and assemble protective rhizobia (Rolfe et al., 2019; Liu et al., 2024). The greenhouse experiments showed a progressive decrease in rhizobial concentration with distance from the inoculation point towards the compatible host, further supporting the existence of this gradient. However, this pattern may also be influenced by the gradient of carbon sources and other nutrients for the bacteria along the hyphae, as these compounds likely follow similar concentration patterns decreasing with distance from the root. The results showing high binding affinities between the identified flavonoids and NodD proteins of *S. meliloti*, provide a mechanistic basis for this chemoattraction (Machado et al., 1998; Peck et al., 2006; Barnett and Long, 2015), though multiple factors may contribute to the overall rhizobial migration pattern observed.

This chemical guidance mechanism, combined with the physical connectivity, ensures that the symbiosis is both efficient and specific. The AM fungus facilitates the delivery of plant-derived signals by transporting host-specific flavonoids along its hyphae, thereby guiding rhizobia with remarkable precision via the established chemical gradient. Additionally, the release of carbon compounds by the hyphae, potentially in higher concentrations closer to the roots, may also

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contribute to rhizobial guidance or support. This dual role, functioning as both a physical conduit and mediator of chemical cues, represents a remarkable progress in our understanding of the tripartite symbiosis, highlighting AM fungi as essential mediators in the interaction between plant and rhizobia.

Ecological and evolutionary implications

The results of this thesis improve our understanding of belowground ecological interactions and the evolution of plant-microbe symbioses. The “fungal highway” and “chemical signaling” mechanisms, which work in concert, probably give legumes a competitive advantage, particularly in N-limited soils, by improving the efficiency and specificity of N₂ fixation.

From an ecological perspective, the extension of the rhizobial recruitment zone facilitated by the AM fungal network gives legumes access to a wider pool of potential symbionts (Sprent, 2009). This is particularly important in soils where rhizobial populations are unevenly distributed. Host specificity, maintained through plant-derived signals transported via the AM fungal hyphae, may help sustain the diversity of legumes and rhizobial populations by ensuring that legumes primarily interact with their most compatible partners, enhancing overall N₂ fixation efficiency. For instance, the dominance of *S. meliloti* (66% at the closest sampling point near *M. truncatula*) over *B. diazoefficiens* in our study illustrates how the AM fungal network, by acting as a conduit for plant-specific signals, influences the spatial distribution of rhizobia, optimizing symbiotic outcomes.

From an evolutionary perspective, the fairly high specificity of flavonoids transported and released via the fungal hyphae, depending on which plant the AM fungus is linked, observed in our study, strongly suggests a co-evolutionary relationship between AM fungi, legumes, and rhizobia. Selective pressures have likely favored AM fungi that can effectively serve as extensions of the root system, passively conveying plant signals to attract beneficial rhizobial partners. This represents a form of reciprocal cooperation, where each partner in the tripartite symbiosis benefits: the AM fungus receives C from the plant. Part of this C is released at the hyphae surface to feed microbes, among which rhizobia. At the same time specific signals are also released by hyphae coming from the plant, attracting specific rhizobia to migrate towards their specific host plant. They form nodules, representing a secured habitat where C and N are exchanged. This increase N accumulation in plants result in higher biomass production, deriving thereby more C to the AM fungus. Thus, by transporting these signals, the AM

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fungus enhances the fitness of both the plant and the rhizobia, acting as a critical facilitator in this interaction, similar to its role in delivering phosphate-solubilizing bacteria (Jiang et al., 2021).

Agricultural applications and limitations

Our results reveal a specific mechanism where AM fungal networks facilitate long-distance rhizobial recruitment. This could have major implications in agriculture. On one side, by increasing the volume of soil from which compatible rhizobia are accessible, by analogy with the two phosphorus uptake pathways (direct root uptake and mycorrhizal uptake pathway) (Smith et al., 2011), AM fungi could increase the likelihood and importance of nodulation, particularly when rhizobia are scarce. This opens the door to legumes being less dependent on synthetic N fertilizers, the environmental and economic costs of which are considerable. On the other side, we do not have evidence that in situations where both roots and AM fungi are exposed to rhizobia (in our study the roots were physically separated from the rhizobia and only the hyphae were in contact with the bacteria), the number of nodules is increased. In addition, plants regulate nodule numbers through mechanisms like autoregulation of nodulation (AON) due to the C cost of the symbiosis (Li et al., 2022). In plants heavily colonized by rhizobia, the role of AM fungi in continuing rhizobia recruitment may diminish, as signaling to attract additional rhizobia may decrease once a sufficient density of nodules has been reached. Further research in more realistic soil conditions is thereby essential to validate this hypothesis and to assess the exact agricultural potential of AM fungal-mediated rhizobial recruitment.

Our findings support the potential application of AM fungal inoculants in legume cropping systems to enhance nodulation and N₂ fixation, particularly in agricultural systems that minimize the use of chemical fertilizers inputs. For instance, in organic or regenerative agriculture systems where chemical fertilizers are reduced or not used, the natural recruitment of beneficial rhizobia can become critical for maintaining legume yield. In these specific conditions, applying AM fungal strains that facilitate the transport of host-specific flavonoids may be a judicious strategy. For example, exploiting *R. irregularis* strains that facilitate the transport of flavonoids such as DL-liquiritigenin or daidzin could attract specific rhizobia, increasing nodulation and N₂ fixation efficiency in *M. truncatula* or *G. max*, respectively. This approach offers a sustainable alternative to chemical inputs, supports robust microbial symbioses, and contributes to improved soil structure and nutrient cycling, enhancing soil health and crop productivity.

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Another potential application is the adoption of agricultural practices that promote the establishment and maintenance of AM fungal networks. Minimum tillage, cover cropping, and crop rotations that include AM fungal host plants can all contribute to increase the AM fungal root colonization and network formation (Rosner et al., 2018; Hontoria et al., 2019). These practices can potentially improve legume-rhizobia interactions by providing a more extensive “fungal highway” for rhizobial dispersal, as demonstrated in our thesis.

However, there are also limitations to consider. Disrupting the AM fungal networks, by tillage or by practices affecting the AM fungal viability (e.g., the use of fungicides or herbicides), might affect this indirect pathway, particularly in soils reliant on AM fungi for rhizobial recruitment. Moreover, the effectiveness of AM fungal inoculants can vary depending on soil type, environmental conditions, and the presence of indigenous AM fungal populations (Bakhshandeh et al., 2017; Hydbom et al., 2017; Cloutier et al., 2020). Boosting local AM fungal populations through agricultural practices that are not detrimental to AM fungi (e.g., minimum tillage or cover cropping), can reduce the need for introducing exogenous AM fungal strains, thus reducing competition with local populations. The findings of our study, while demonstrating host specificity, also highlight the complexity of these interactions in mixed-legume systems.

Further research is needed to optimize the use of AM fungi in agricultural systems. This includes identifying the most effective AM fungal strains for different legume species and soil types, developing effective inoculation methods, possibly by co-formulation of AM fungi and rhizobia, and understanding the long-term effects of AM fungal manipulation on soil health and ecosystem functioning. Field trials under a variety of conditions are essential to validate the findings of controlled experiments and to assess the practical benefits of AM fungal-mediated rhizobial recruitment in real agricultural settings.

CONCLUSIONS AND PERSPECTIVES

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The main objective of this Ph.D. thesis was to investigate the role of arbuscular mycorrhizal (AM) fungi in mediating the long-distance migration of rhizobia and subsequent host-specific recruitment by legume roots. This involved exploring both the physical and chemical mechanisms by which AM fungi influence the legume-rhizobia symbiosis, addressing gaps in our understanding of how these interactions occur beyond the immediate rhizosphere.

The thesis was structured to address four key objectives:

1. Investigate if and how AM fungal hyphae facilitate the long-distance migration of rhizobia and promote nodulation in legumes.
2. Determine whether rhizobia recruitment by AM fungi is host-specific, favoring leguminous over non-leguminous plants.
3. Explore the selective tropism of different rhizobia species within a common mycorrhizal network (CMN) toward their respective legume hosts.
4. Identify and characterize the biochemical signals, in particular flavonoids, that mediate rhizobia recruitment and host-specificity in the tripartite interaction between a legume, an AM fungus and a rhizobium.

These objectives were addressed through a combination of *in vitro* and greenhouse experiments, coupled with metabolomic and molecular analyses. In **chapter I**, we focused on the interaction between the extraradical hyphae of *Rhizophagus irregularis* and *Sinorhizobium meliloti*, demonstrating the role of AM fungi as “fungal highway” for rhizobium migration and in the transport and release of flavonoids directing the rhizobium towards its host. In **chapter II**, we extended the study by exploring host-specific rhizobia recruitment via the CMN of *R. irregularis*, using *Medicago truncatula* and *Glycine max* as model legumes and *S. meliloti* and *Bradyrhizobium diazoefficiens* as model rhizobia.

Therefore, four specific research questions were addressed and answers summarized below:

- 1. Do AM fungal ERM facilitate the long-distance migration of rhizobia and enhance nodulation in legumes and what are the potential mechanism involved?**

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Yes, the AM fungal ERM facilitate the long-distance migration of rhizobia and enhance nodulation in legumes by acting as a physical conduit for rhizobial migration and as a transporter and delivery system for plant-derived flavonoids, which serve as chemoattractants to guide rhizobia towards legume roots. This was evidenced by a number of observations:

- The *in vitro* experiments conducted in **chapter I** demonstrated that *S. meliloti* can migrate along the ERM of *R. irregularis* towards *M. truncatula* roots. This migration was significantly faster and more directed than movement in the agar medium alone.
- The greenhouse experiments in **chapter I**, using a quadri-compartment pot culture system, confirmed that *S. meliloti* can migrate approximately 15 cm along the ERM connecting *M. truncatula* to a non-legume (i.e., *Solanum tuberosum*), leading to nodulation of *M. truncatula* roots. No nodulation was observed without the ERM connection.
- The metabolomic analyses of hyphal exudates in **chapter I** allowed to identify eight unique flavonoids, including luteolin derivatives, known to stimulate rhizobial growth and nod gene expression.
- The *in silico* molecular docking conducted in **chapter I** showed high binding affinities between the identified flavonoids and NodD proteins of *S. meliloti*, reinforcing their role in activating symbiosis-related gene expression.

Perspectives:

From these results a number of perspectives can be addressed:

- **Investigate the mechanisms involved in flavonoid transport and release:** The central question arising from this thesis is how AM fungi transport and release plant-derived flavonoids to guide rhizobia. Is this a passive process driven by simple diffusion, or an active process involving specific fungal mechanisms (e.g., vesicle trafficking) ? To resolve this question, advanced imaging techniques (e.g., confocal microscopy with fluorescently labeled flavonoids) for vesicle movement, combined with transcriptomic analyses (to identify potential vesicle-related transporter proteins) can be used. The roles of membrane transporters at the peri-arbuscular membrane (PAM) or vesicle-mediated pathways can also be approached using gene knockouts strategies. Finally, investigate the concentration gradients of signals along the hyphae using micro-sampling or confocal microscopy to map their distribution can also be considered.
- **Explore the role of other hyphal exudates:** Although the untargeted metabolomics approach specifically analyzed flavonoids, it also generated a

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broader set of detectable metabolites. Future studies could leverage this full set of secondary metabolites to identify other potential signaling molecules, beyond flavonoids, that might contribute to rhizobial migration and nodulation. This could involve re-analyzing the existing data or conducting new untargeted metabolomics studies with a wider analytical lens.

- **Examine the dynamics of rhizobial movement on the hyphae:** Time-lapse microscopy with higher resolution could provide more detailed insights into the mechanisms of rhizobial movement along the hyphae (e.g., active vs. passive transport, role of hyphal surface properties, potential involvement of bacterial motility structures, release of a gradient of carbon sources), complementing the chemical findings with physical interaction data.
- **Test the “fungal highway” hypothesis with different AM fungal and rhizobial species:** Expanding the research to include other AM fungal and rhizobial species would help generalizing this phenomenon and identify potential species-specific interactions. This could involve using different *Rhizopagus* species or even AM fungi from other genera with other life history strategies.
- **Investigate the concentration gradients of signals along the hyphae:** Understanding how the concentration of plant-derived signals, such as flavonoids, varies along the hyphae could elucidate how these gradients guide rhizobia towards the roots. Techniques like micro-sampling or confocal microscopy could map these gradients. Specifically, confocal microscopy can utilize the natural fluorescence of certain flavonoids or targeted fluorescent probes to image their distribution along the hyphae with high precision. The optical sectioning capability of confocal microscopy ensures that the fluorescence signal is specifically from the hyphal plane, allowing for accurate mapping of concentration gradients, enhancing our understanding of the chemical guidance mechanism.

2. Does rhizobial recruitment mediated by AM fungal networks exhibit host preference, favoring legumes over non-leguminous plants?

Yes, rhizobial recruitment mediated by AM fungal networks exhibits host preference for legumes by directing rhizobial migration towards legume roots over non-legumes roots through the transport of host-specific signals along the hyphae. This was evidenced by the following observation:

- The *in vitro* and greenhouse experiments conducted in **chapter I**, demonstrated that *S. meliloti* migrated preferentially along the ERM of *R. irregularis* towards the legume *M. truncatula* compared to the non-legumes

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S. tuberosum and *P. lanceolata*. This suggests a degree of selectivity in the interaction, favoring the legume host over the non-legume.

Perspectives:

From this result a number of perspectives can be addressed:

- **Investigate the mechanisms underlying the preferential migration towards legumes:** While our study confirmed flavonoids as key signaling molecules facilitating rhizobial recruitment, the mechanisms by which AM fungi mediate this legume-specific process remain unclear. Future research can focus on comparative transcriptomic analyses of rhizobia exposed to exudates from hyphae connected to legumes versus non-legumes, to determine whether genes related to motility, chemotaxis, or symbiosis are differentially expressed, thus elucidating the molecular basis of preferential migration. In addition, mapping the distribution of flavonoids when a single legume species is colonized by different AM fungal species could reveal whether the transport and release of these signals are AM fungus-specific, enhancing our understanding of the tripartite interaction.
- **Test a wider range of legume and non-legume species:** Expanding the study to include a more diverse set of plant species would help to confirm the generality of the legume preference and identify potential exceptions.

3. Do CMNs connecting different legume species mediate selective recruitment of host-specific rhizobia?

Yes, the CMN connecting different legume species mediate the selective recruitment of rhizobia by transporting and releasing plant-derived, host-specific flavonoids that act as chemoattractants, guiding *S. meliloti* towards *M. truncatula* and *B. diazoefficiens* towards *G. max*. This was evidenced by a number of observations:

- The *in vitro* experiments conducted in **chapter II**, using a CMN connecting *M. truncatula* to *G. max*, showed that *S. meliloti* preferentially migrated towards *M. truncatula*, while *B. diazoefficiens* preferentially migrated towards *G. max*.
- The Greenhouse experiments conducted in **chapter II**, using a three-compartmented pot system, corroborated these findings, showing hyphal-mediated and host-directed transport of rhizobia towards their appropriate host, even in a more complex soil-like environment.

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Perspectives:

From these results a number of perspectives can be addressed:

- **Explore the role of signaling molecules other than flavonoids:** While flavonoids are key, other plant-derived signals (e.g., strigolactones, other secondary metabolites) might also contribute to host specificity. Broader metabolomic analyses of hyphal exudates in mixed-legume systems, using both targeted and untargeted approaches, could help identifying additional or even novel signaling molecules.
- **Test the host-specificity hypothesis with a wider range of legume and rhizobial species:** Expanding the research to include more diverse legume-rhizobia combinations would help determine the generality of the host-specific recruitment mechanism and identify potential co-evolutionary patterns or exceptions.

4. What are the biochemical signals that mediate rhizobial recruitment and host specificity in legume-AM fungi-rhizobia interactions?

Flavonoids are key biochemical signals, with their composition being host-specific. This was evidenced by a number of observations:

- Metabolomic analyses in **chapter I** identified eight unique flavonoids in the hyphosphere of *R. irregularis* connected to *M. truncatula*, including luteolin derivatives known to induce nodulation genes in *S. meliloti*.
- Metabolomic analyses in **chapter II** revealed that *R. irregularis* released distinct flavonoids depending on the connected legume: flavanones (DL-liquiritigenin and naringenin), and flavonoid derivatives (sakuranetin and 3,7-dimethylquercetin) when connected to *M. truncatula*, and isoflavones (daidzin, 6''-O-malonyldaidzin, irilone, and erylatissin A) when connected to *G. max*. These compounds are known chemoattractants for the rhizobial species studied.
- The absence of these specific flavonoids in the controls without hyphae connected to the plant in both chapters emphasize the role of AM fungal hyphae in transporting and releasing plant-derived, host-specific signals.

Perspectives:

From these results the following perspective can be addressed:

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- **Develop bioassays to test the activity of individual flavonoids and flavonoid mixtures:** To complement our understanding of AMF-mediated flavonoid transport and release, we could develop bioassays that test how flavonoids delivered by *R. irregularis* hyphae influence rhizobial behavior. These assays could expose *S. meliloti* and *B. diazoefficiens* to hyphal exudates containing flavonoids (e.g., luteolin derivatives from *M. truncatula*-connected hyphae, genistein from *G. max*-connected hyphae), as identified in our studies, and measure chemotactic responses and nod gene expression levels and their binding affinities to NodD proteins.

Other perspectives on the results of the PhD thesis

Beyond the perspectives related to each research question above, several other perspectives could be addressed:

- **Energetic costs and benefits of the tripartite legume-AM fungi-rhizobia symbiosis:** Transporting and releasing flavonoids, maintaining hyphal networks, and supporting rhizobial populations require metabolic resources from the legume, AM fungi, and rhizobia. Future studies could investigate the energetic costs and benefits of these interactions across the entire tripartite system by quantifying the metabolic resources, particularly carbon, allocated from the legume to *R. irregularis* and rhizobia (*S. meliloti* or *B. diazoefficiens*) and assessing the resulting benefits in terms of enhanced nodulation and N₂ fixation. This could involve using isotopic labeling (e.g., ¹³C) to track the flow of plant-derived carbon not only to the fungus for hyphal growth and flavonoids transport but also to the rhizobia, which utilize carbon to fuel N₂ fixation, subsequently increasing plant N content and carbon production that may indirectly support AM fungal growth. Complementary measurements could include plant growth parameters (e.g., biomass, N content), fungal performance indicators (e.g., hyphal density, spore production), and rhizobial metrics (e.g., nodule number, nitrogenase activity) under varying environmental conditions, such as N availability or water stress. While similar carbon allocation studies exist for plant-fungal interactions, extending this approach to the tripartite level, considering the dynamic interplay where rhizobia enhance N₂ fixation and AM fungi facilitate rhizobial recruitment, opens new research perspectives.
- **Interactions within complex soil microbiomes:** While our thesis elucidated AMF-mediated recruitment of rhizobia, future research should investigate the robustness and specificity of this flavonoid-based mechanism within complex soil microbiomes, reflecting realistic field conditions where numerous microorganisms co-exist and interact. Does the targeted

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recruitment of rhizobia observed in our simplified system also persist under more complex microbiomes? Furthermore, extending beyond symbiotic rhizobia, research could explore whether AM fungi also recruit other bacteria involved in N₂ fixation, particularly free-living nitrogen-fixing bacteria such as *Azospirillum* spp., potentially using transported other signaling molecules delivered via the hyphal network. This was shown for phosphate solubilizing bacteria (i.e., *Rahnella aquatilis* (Zhang et al., 2018a)) opening the door to free-living N-fixation bacteria. Addressing these questions, using experiments with defined microbial consortia or analyzing natural soil ecosystems via metagenomics, metatranscriptomics, and targeted functional assays, is crucial for understanding the full ecological role of AM fungi in structuring beneficial plant-microbe interactions in the soil.

- **Agricultural applications:** Beyond individual strains, exploring consortia of compatible AM fungi and rhizobia offer promising avenues for improving crop yield. These consortia, consisting of mixed microbial communities that naturally coexist and interact, could leverage synergistic effects to boost symbiotic efficiency, potentially increasing nitrogen fixation and crop yields more effectively than single-strain applications. For instance, AM fungi can enhance rhizobial colonization and survival, while rhizobia contribute fixed nitrogen that indirectly supports fungal growth.

In addition, developing effective inoculation methods is critical. Seed coatings or soil amendments incorporating these microbial consortia could be designed to ensure robust delivery and establishment of both AM fungi and rhizobia in the field. Additionally, understanding the long-term impacts of introducing such consortia on soil health and ecosystem functioning remains vital for sustainable management of agricultural systems. This includes assessing changes in soil microbial diversity, nutrient cycling, and ecosystem resilience over time. Field trials across different cropping systems like monocultures versus intercropping, soil types including sandy versus clay-rich, and climates ranging from temperate to tropical, are necessary to validate findings from controlled experiments and evaluate the practical benefits of AM fungal-mediated rhizobial recruitment. Such trials will provide evidence to refine application strategies, ensuring that these microbial partnerships can reliably improve legume performance and reduce reliance on synthetic fertilizers in real-world agricultural settings.

- **Signal Specificity and CMN Dynamics:** A critical question is how signal specificity is maintained within the CMN, particularly when multiple legume species are connected. Does the AM fungus actively regulate signal transport (e.g., through selective transport mechanisms), or is it primarily driven by concentration gradients? To address this question, we propose integrating advanced imaging with mathematical modeling. Imaging

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techniques, such as confocal microscopy with fluorescently labeled flavonoids (e.g., fluorescein-tagged luteolin), can track signal movement in real-time. Mathematical modeling can then simulate transport dynamics to distinguish mechanisms:

- a) A diffusion model (based on Fick's laws) could simulate passive spread, predicting gradual dispersion from source plants.
- b) An active transport model (using Michaelis-Menten kinetics) could test selective uptake and targeted delivery by fungal transporters.
- c) A network model could represent the CMN as a graph, exploring how hyphal connections maintain specificity across species and predict behavior in complex networks.

By calibrating these models with imaging data and comparative predictions (e.g., uniform gradients vs. localized hotspots), we can infer the dominant mechanism, guiding further experimental design and future studies of CMN dynamics.

• **The “cry for help” mechanism:** Building on the concept that plants under stress release signals to attract beneficial microorganisms, future studies could investigate how nitrogen deficiency influences the production and transport of flavonoids through the AM fungal network to recruit rhizobia, similar to a “cry for help” strategy enhancing symbiosis under nutrient limitation.

In conclusion, this Ph.D. thesis has demonstrated that AM fungi play a crucial, multifaceted role in facilitating the legume-rhizobia symbiosis. By acting as both physical conduits for rhizobial migration (the “fungal highway”) and as mediators transporting plant-derived chemical signals, guiding host-specific recruitment, AM fungi significantly enhance the efficiency and specificity of this vital ecological interaction. These findings open up new avenues for research in belowground ecology, evolutionary biology, and sustainable agriculture, with the potential to improve legume productivity in specific contexts, such as nitrogen-poor soils with scarce rhizobial populations, contributing to the reduced reliance of legume crops on synthetic nitrogen fertilizers.

ANNEXES

Annex I



Figure S1. Picture of the mono-compartment autotrophic *in vitro* culture system with *Medicago truncatula*.



Figure S2. Picture of the bi-compartment autotrophic *in vitro* culture system with *M. truncatula*.



Figure S3. Picture of the quadri-compartmented autotrophic *in vitro* culture system.

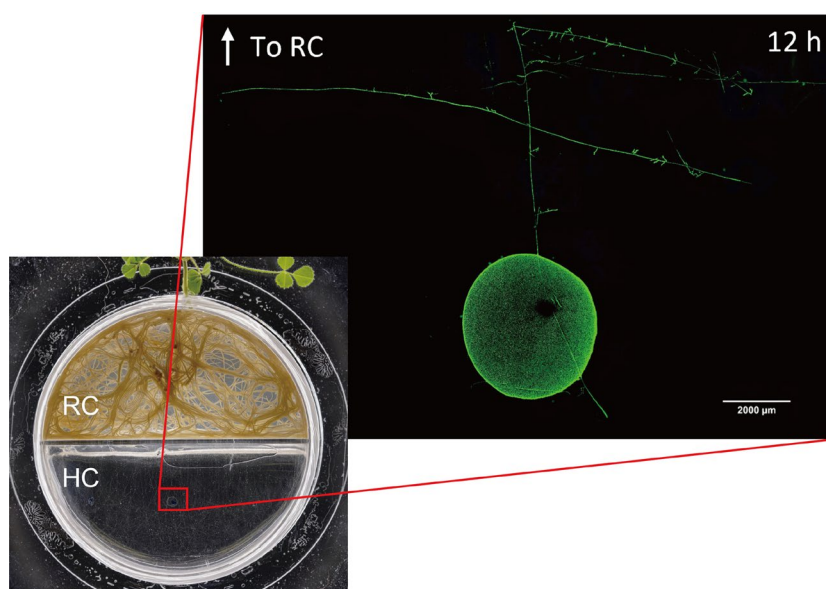


Figure S4. The movement track for *S. meliloti* on the surface of ERM in the hyphal compartment (HC) with *M. truncatula* in the root compartment (RC) of bi-compartmental Petri plates after 12 hours.

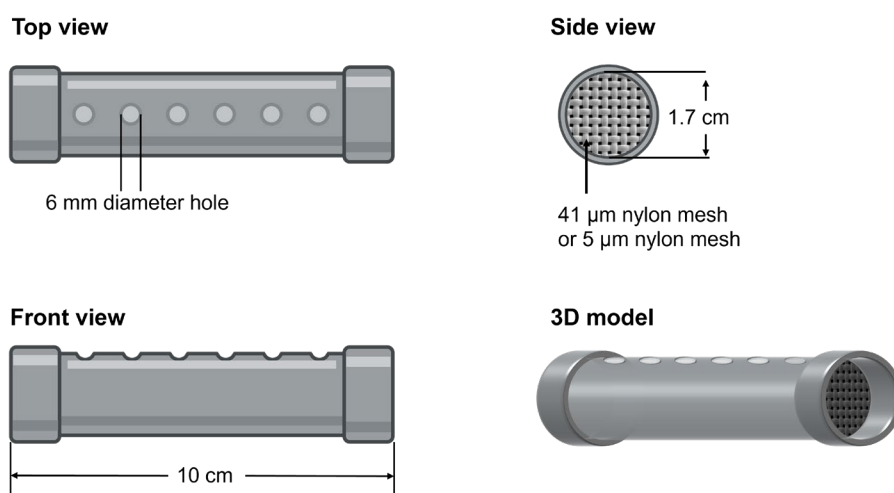


Figure S5. Schematic diagrams of the pipe from different perspectives.

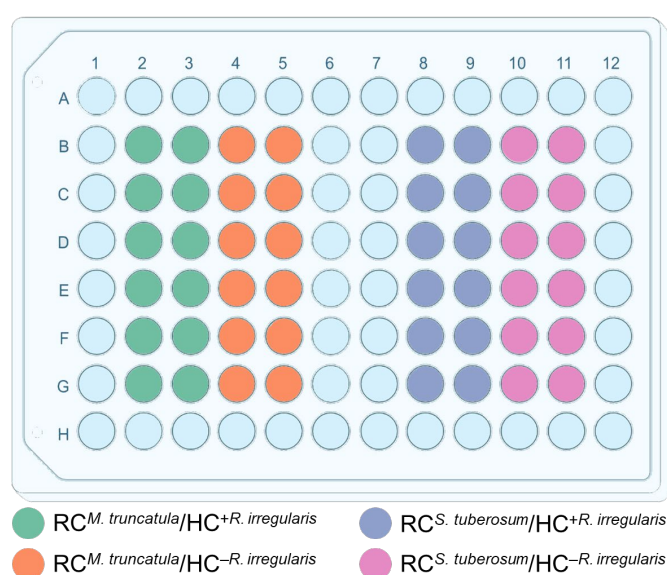


Figure S6. Layout of 96-wells plate culture of *S. meliloti*. Blue wells: 200 µL of fresh MSR^{min0N} medium without bacteria to avoid boundary effects. $RC^{M. truncatula}/HC^{+R. irregularis}$ (green wells): with *Medicago truncatula* in the root compartment and hyphae in the hyphae compartment; $RC^{M. truncatula}/HC^{-R. irregularis}$ (orange wells): with *M. truncatula* in the root compartment but no hyphae in the hyphae compartment; $RC^{S. tuberosum}/HC^{+R. irregularis}$ (dark blue wells): with potato in the root compartment and hyphae in the hyphae compartment; $RC^{S. tuberosum}/HC^{-R. irregularis}$ (pink wells): with potato in the root compartment but no hyphae in the hyphae compartment.

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Table S1. Gene-specific primer sequences used in this study.

Gene	Abbreviations in this study	GenBank accession number	Gene (bp)	length	Primer sequence (5'-3')	Product length (bp)
Multispecies: nodulation N-acyltransferase NodA	<i>nodA</i>	WP_010967455	591		F: GGTCGGGATAGCAAGCCACATG R: CCAGTTCAGCCACAAGGAGATCAG	80
NodB chitooligosaccharide deacetylase (plasmid)	<i>nodB</i>	AGG70157	654		F: CGGAAGGTCACGAAGTGGCTAAC R: CTGAGGACAAGCGGCGATAATGG	122
NodC N-acetylglucosaminyltransferase (plasmid)	<i>nodC</i>	AGG70156	1281		F: TCCAGAGGTCGGTGCGGTTATG R: TTCGTTACAGGCAAGCCAATACTCC	103
NodD1 nod-box dependent transcriptional activator (plasmid)	<i>nodD1</i>	AGG70159	927		F: CCACGCCTATTGTCGGGTACTAAC R: CGGCAGCGGAGATGTGACTATG	105
NodD2 nod box-dependent transcription activator (plasmid)	<i>nodD2</i>	AGG70091	933		F: GCCGCACACCAATCGTATAGCC R: TGGACAGCCTCAGTGAACCAGAG	120
NodD3 nod box-dependent transcriptional activator (plasmid)	<i>nodD3</i>	AGG70144	942		F: ACTCGATGCACTGATGACCAAACG R: ATAGGAGCGTAGGCGAGCGATG	103
NodI ABC transporter, ATP-binding protein (plasmid)	<i>nodI</i>	AGG70155	1008		F: GCGGCAAGACGATTCTCCTGAC R: CTATCACCTGGCAACCGATCTTCTC	147
NodJ ABC transporter, permease (plasmid)	<i>nodJ</i>	AGG70154	789		F: GGTGGAAGTGGATTGCCGTGTG R: CGCCCGACCATCATTCCCTAAGC	139
Hypothetical protein, signal peptide	<i>SMc00128</i>	AGG73587	546		F: TTGCGATCTTCGACAGCGG R: GCAGTTCGACGAGCTGGATC	111

Table S2. The information of flavonoids *in silico* NodD1,2,3–flavonoid binding studies

No.	Flavonoids	PubChem CID	m/z
1	Luteolin-acetylhexuronide	44258127	503.0830
2	Sudachiin A	73829938	521.1300
3	Tetrahydroxy-tetramethoxyflavone-hexoside	44260070	567.1355
4	Isosakuranetin-hexosyl-deoxyhexose	16760075	593.1875
5	Taxifolin-deoxyhexosyl-oxanepentol	163060888	597.1460
6	Haploside C	44260015	695.1832
7	Sagittain B1	44258973	751.2116
8	Sagittain B2	44258973	751.2117
9	Trimethoxy-hydroxy-methyl-flavanone-dihexosyl-deoxyhexose	72828014	813.2828
10	Trihydroxy-flavanone-hexosyl-pentose	42607913	565.1563
11	Luteolin-7-glucoside	5280637	447.0938
12	Hyperoside	5281643	303.0502
13	Luteolin	5280445	287.0543
14	Chrysoeriol	5280666	299.0553

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Table S3. Root mycorrhizal colonization and soil mycelium density in the *in vivo* experiment.

Sampling date (Y-M-D)	Sampling place	Arbuscules (%)	Vesicles/spores (%)	Total (%)	Soil mycelium density (cm ³ ·g ⁻¹ of fresh soil)
2023-07-06	CC	30.3 ± 4.3	19.7 ± 4.5	82.5 ± 4.4	--
2023-08-01	<i>M. truncatula</i> ^{+R.i.}	38.5 ± 2.8	18.8 ± 2.5	79.3 ± 1.3	69.1 ± 10.7
2023-08-01	<i>M. truncatula</i> ^{-R.i.}	0 ± 0	0 ± 0	0 ± 0	0 ± 0
2023-08-01	<i>P. lanceolata</i> ^{-R.i.}	0 ± 0	0 ± 0	0 ± 0	0 ± 0
2023-08-01	CC	--	--	--	51.5 ± 6.5
2023-08-01	<i>M. truncatula</i> ^{+R.i.} tube	--	--	--	73.3 ± 9.6
2023-08-01	<i>M. truncatula</i> ^{-R.i.} tube	--	--	--	0 ± 0
2023-08-01	<i>P. lanceolata</i> ^{-R.i.} tube	--	--	--	0 ± 0

Note: CC: central chamber; *M. truncatula*^{+R.i.}: the chamber with *M. truncatula* and *R. irregularis*; *M. truncatula*^{-R.i.}: the chamber with *M. truncatula* but not *R. irregularis*; *P. lanceolata*^{-R.i.}: the chamber with *P. lanceolata* but not *R. irregularis*; *M. truncatula*^{+R.i.} tube: the tube connects *M. truncatula*^{+R.i.} with the CC; *M. truncatula*^{-R.i.} tube: the tube connects *M. truncatula*^{-R.i.} with the CC; *P. lanceolata*^{-R.i.} tube: the tube connects *P. lanceolata*^{-R.i.} with the CC. Data ($n = 6$) are represented as means ± SD.



Scan the QR code or visit this link (<https://doi.org/10.1093/ismejo/wrae185>) to access the **Video S1**, **Video S2**, and **Supplemental dataset 1**.

Video S1. Movement of *S. meliloti* on the surface of ERM in the hyphal compartment in the direction of the root compartment containing *M. truncatula* at 12 hours following inoculation of the bacteria on the hyphae.

Video S2. Velocity of cytoplasmic/protoplasmic flow in the ERM at 24 hours following *S. meliloti* inoculation on the hyphae, under the bright field of view. (Scan the QR code for the video)

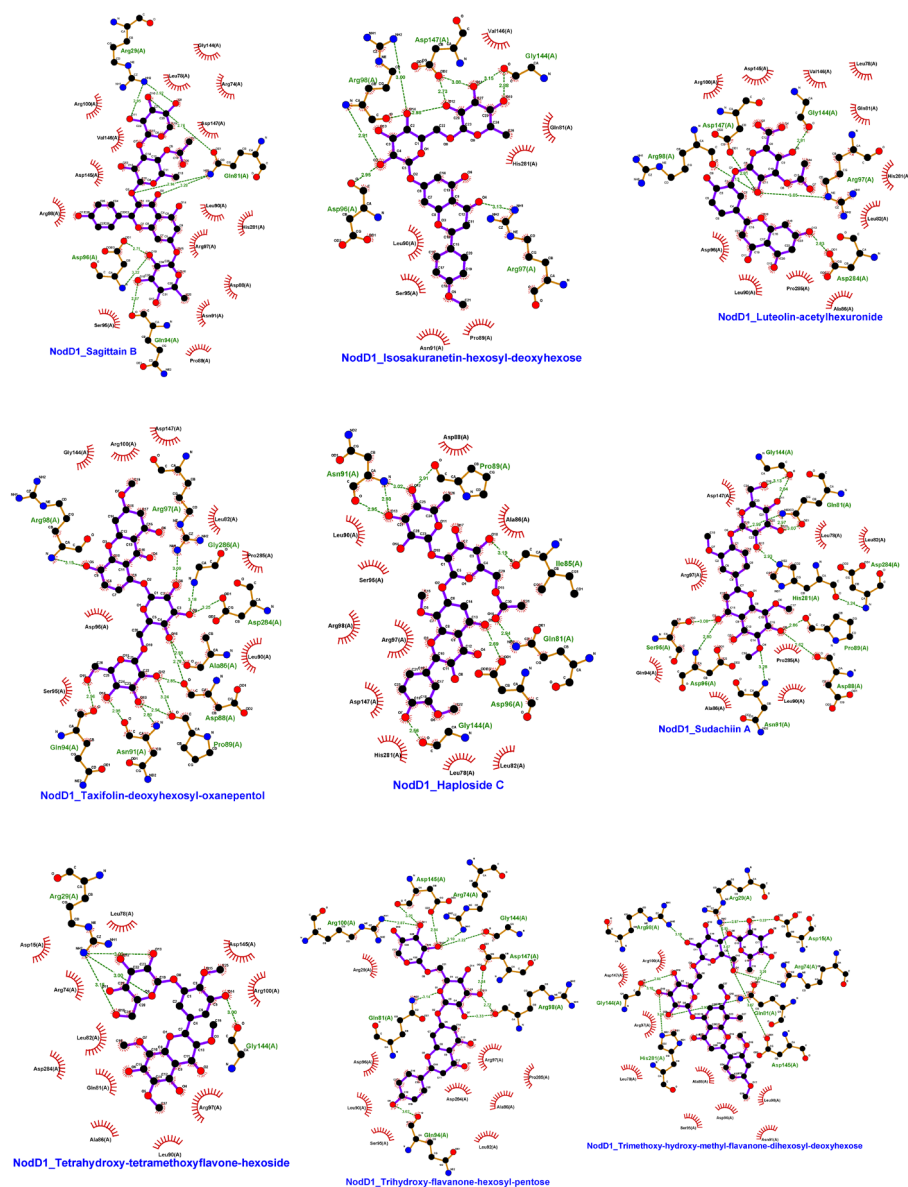


Figure S8. *In silico* binding studies of NodD1 protein of *S. meliloti* and 10 flavonoids. Each NodD1-flavonoid complex form hydrogen (green dotted line between amino acid and flavonoid, with distances) and hydrophobic (red half circles) bond during NodD1-flavonoid interaction. Sagittain B1 and Sagittain B2 were merged into a single flavonoid since their comparison results in the PubChem (<https://pubchem.ncbi.nlm.nih.gov>) were identical.

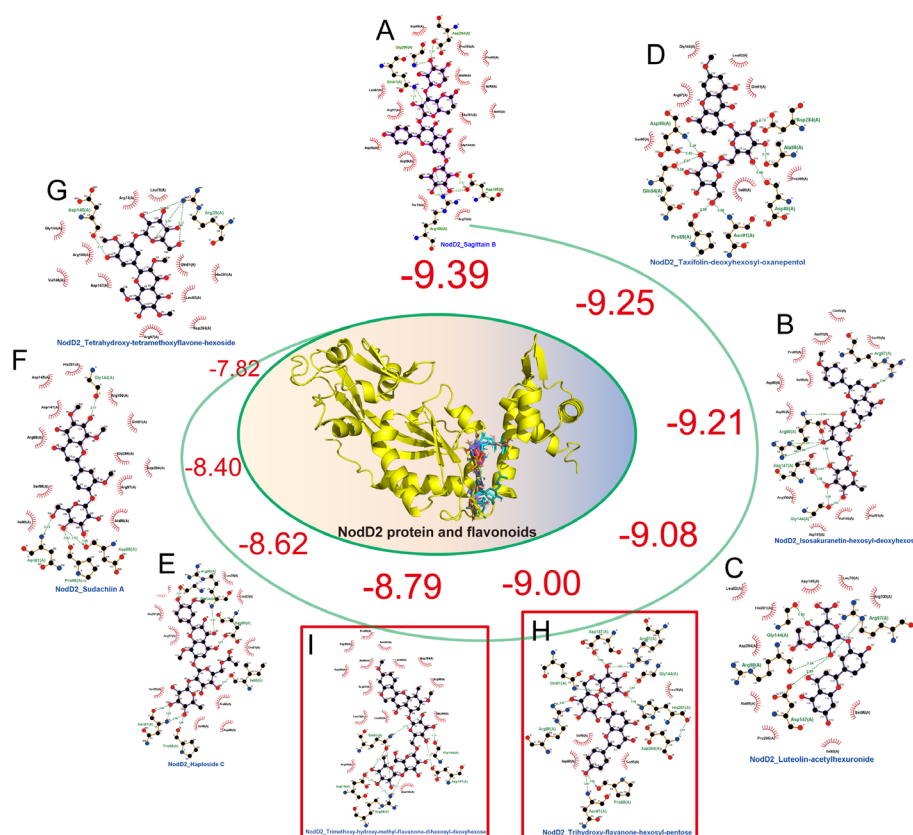


Figure S9. *In silico* binding studies between NodD2 protein of *S. meliloti* and 10 flavonoids. Here NodD2–flavonoid complexes were arranged based on their binding affinities (the more negative the values, (Kcal·mol⁻¹), the stronger the interactions). Each NodD2–flavonoid complex form hydrogen (green dotted line between amino acid and flavonoid) and hydrophobic (red half circles) bonds during NodD2–flavonoid interaction. The red-boxed NodD2–flavonoid indicates the flavonoid found exclusively in the RC*S.tuberosum*/HC+R*.irregularis* treatment. The central picture (inside the oval circle) predicts the binding sites of 9 flavonoids in NodD2 protein of *S. meliloti*. Individual figures are shown below. A: sagittain B1 and sagittain B2; B: isosakuranetin-hexosyl-deoxyhexose; C: luteolin-acetylhexuronide; D: taxifolin-deoxyhexosyl-oxanepentol; E: haploside C; F: sudachiin A; G: tetrahydroxy-tetramethoxyflavone-hexoside; H: trihydroxy-flavanone-hexosyl-pentose; I: trimethoxy-hydroxy-methyl-flavanone-dihexosyl-deoxyhexose. Sagittain B1 and Sagittain B2 were merged into a single flavonoid since their comparison results in the PubChem (<https://pubchem.ncbi.nlm.nih.gov>) were identical. The figure was designed according to Bag et al. (2022).

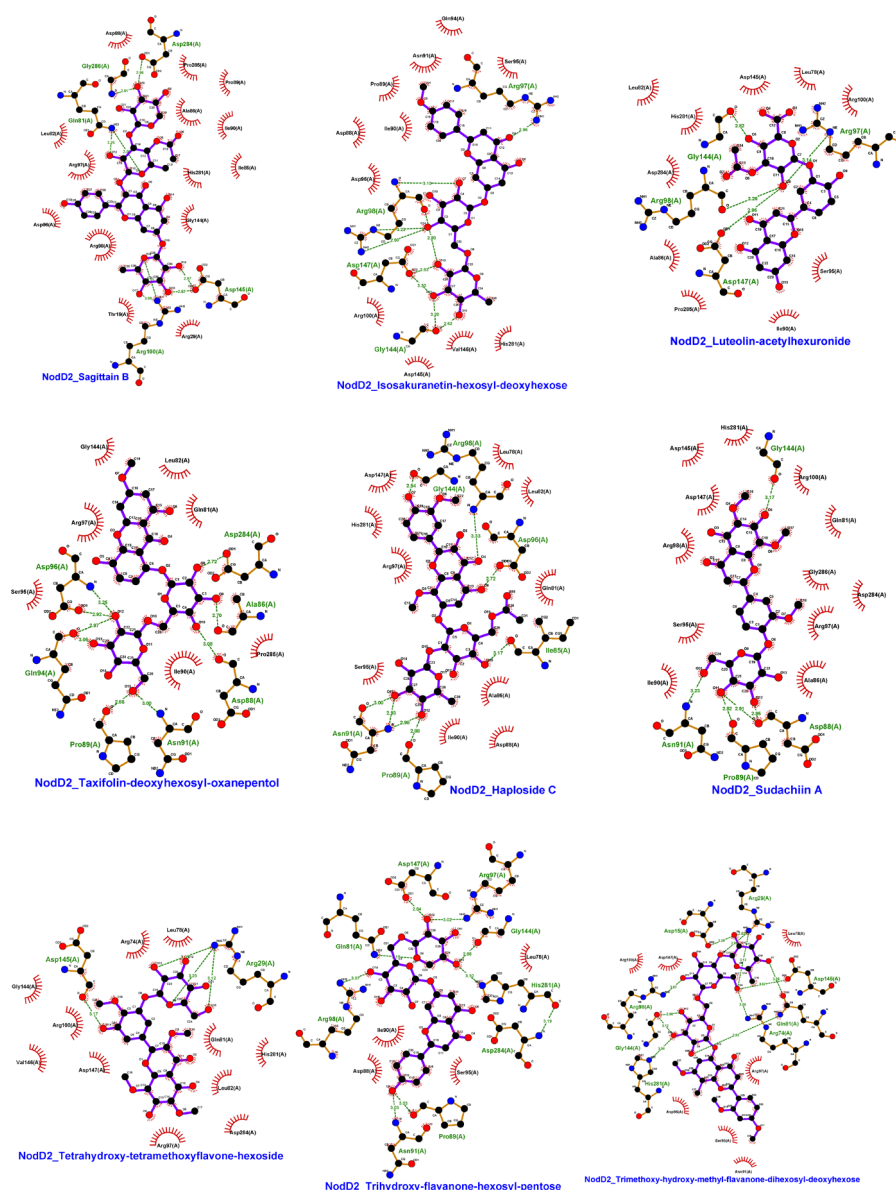


Figure S10. *In silico* binding studies of NodD2 protein of *S. meliloti* and 10 flavonoids. Each NodD2-flavonoid complex form hydrogen (green dotted line between amino acid and flavonoid, with distances) and hydrophobic (red half circles) bond during NodD2-flavonoid interaction. Sagittain B1 and Sagittain B2 were merged into a single flavonoid since their comparison results in the PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) were identical.

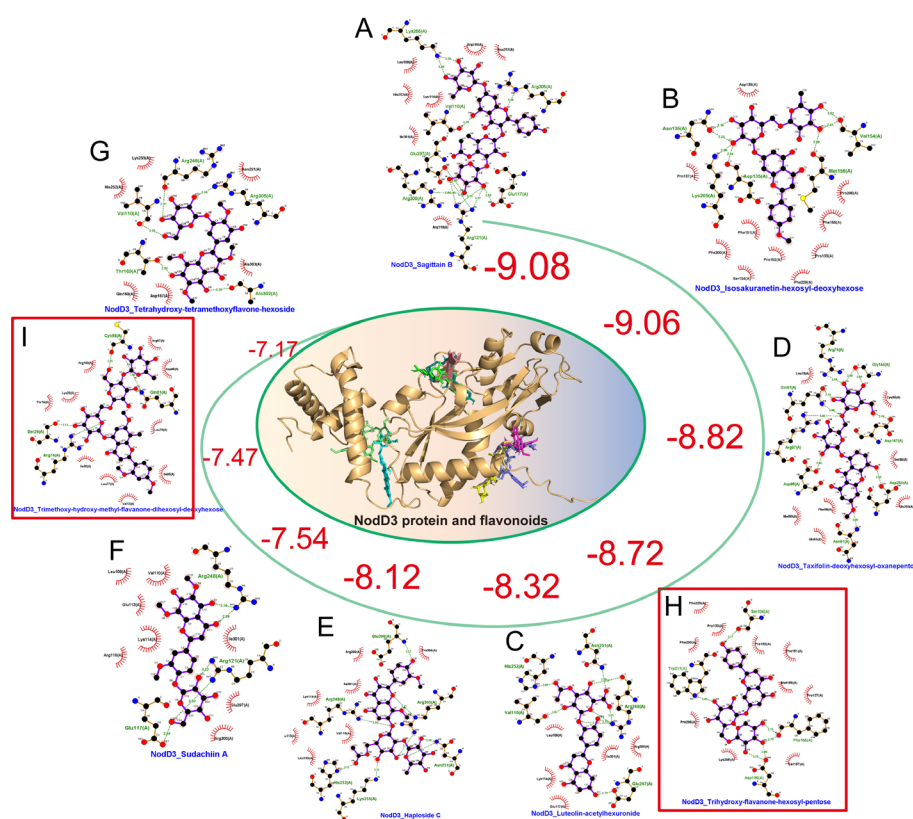


Figure S11. *In silico* binding studies between NodD3 protein of *S. meliloti* and 10 flavonoids. Here NodD3–flavonoid complexes were arranged based on their binding affinities (the more negative the values, (Kcal·mol⁻¹), the stronger the interactions). Each NodD3–flavonoid complex form hydrogen (green dotted line between amino acid and flavonoid) and hydrophobic (red half circles) bonds during NodD3–flavonoid interaction. The red-boxed NodD3–flavonoid indicates the flavonoid found exclusively in the RC *S. tuberosum*/HC+*R. irregularis* treatment. The central picture (inside the oval circle) predicts the binding sites of 9 flavonoids in NodD3 protein of *S. meliloti*. Individual figures are shown below. A: sagittain B1 and sagittain B2; B: isosakuranetin-hexosyl-deoxyhexose; C: luteolin-acetylhexuronide; D: taxifolin-deoxyhexosyl-oxanepentol; E: haploside C; F: sudachiin A; G: tetrahydroxy-tetramethoxyflavone-hexoside; H: trihydroxy-flavanone-hexosyl-pentose; I: trimethoxy-hydroxy-methyl-flavanone-dihexosyl-deoxyhexose. Sagittain B1 and Sagittain B2 were merged into a single flavonoid since their comparison results in the PubChem (<https://pubchem.ncbi.nlm.nih.gov>) were identical. The figure was designed according to Bag et al. (2022).

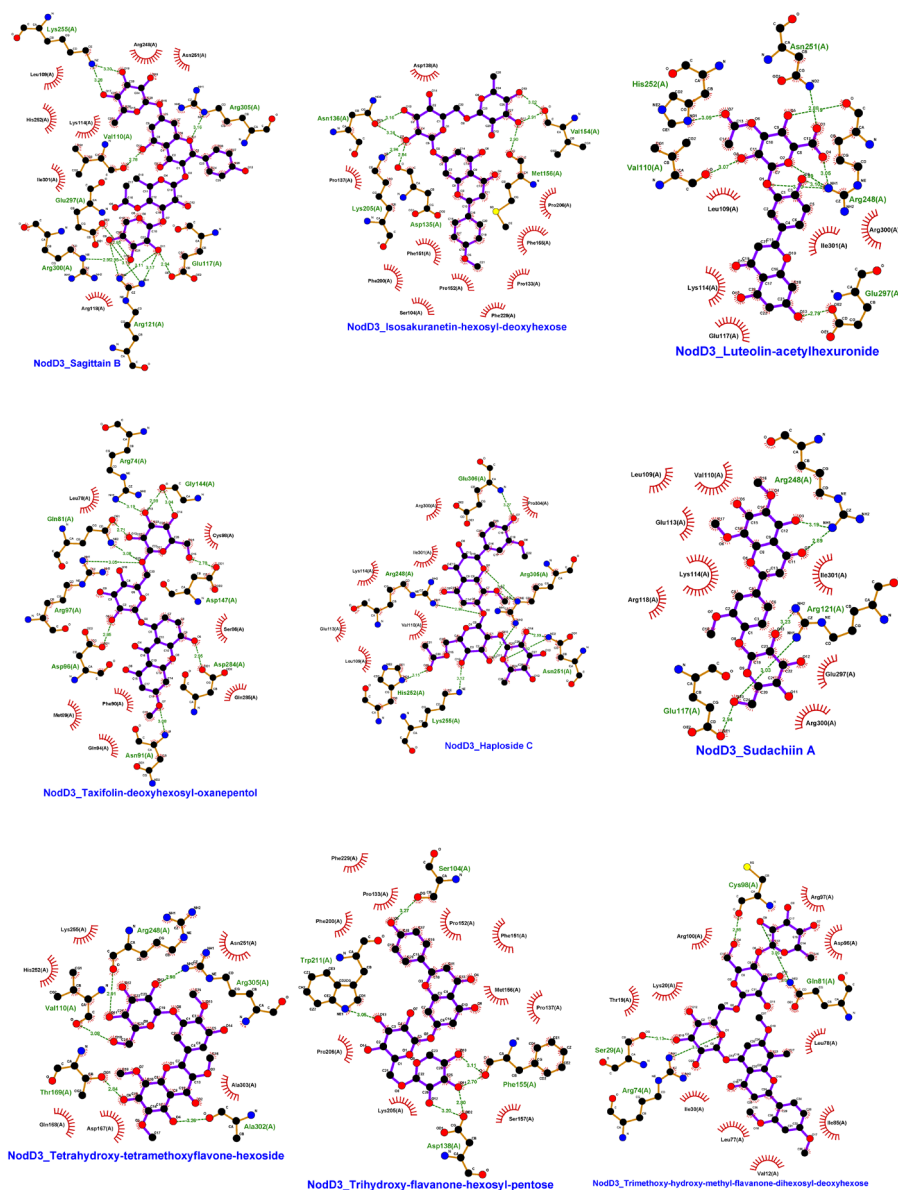


Figure S12. *In silico* binding studies of NodD3 protein of *S. meliloti* and 10 flavonoids. Each NodD3-flavonoid complex form hydrogen (green dotted line between amino acid and flavonoid, with distances) and hydrophobic (red half circles) bond during NodD3-flavonoid interaction. Sagittain B1 and Sagittain B2 were merged into a single flavonoid since their comparison results in the PubChem (<https://pubchem.ncbi.nlm.nih.gov>) were identical.

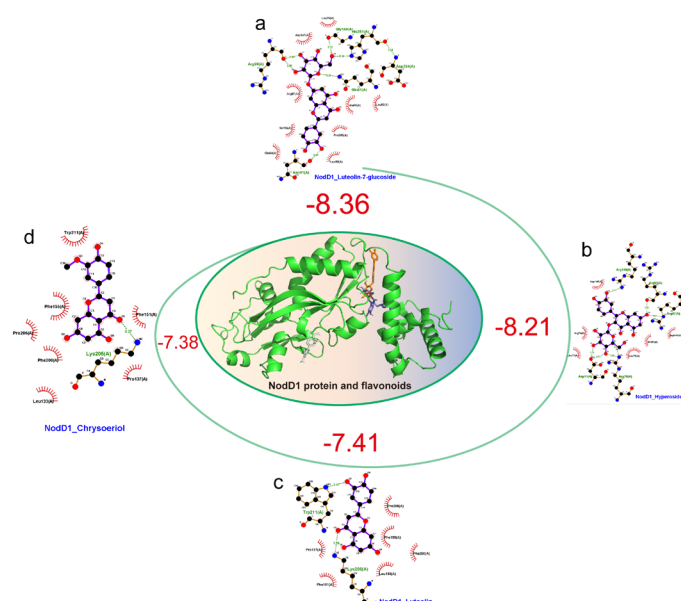


Figure S13. *In silico* binding studies between NodD1 protein of *S. meliloti* and 4 flavonoids from Compton et al.'s (2020) study. Here NodD1–flavonoid complexes were arranged based on their binding affinities (the more negative the values, ($\text{Kcal}\cdot\text{mol}^{-1}$), the stronger the interactions). Each NodD1–flavonoid complex form hydrogen (green dotted line between amino acid and flavonoid) and hydrophobic (red half circles) bonds during NodD1–flavonoid interaction. The red-boxed NodD1–flavonoid indicates the flavonoid found exclusively in the $\text{RC}^+S.tuberosum/\text{HC}^+R.irregularis$ treatment. The central picture (inside the oval circle) predicts the binding sites of 4 flavonoids in NodD1 protein of *S. meliloti*. Individual figures are shown below. a: luteolin-7-glucoside; b: hyperoside; c: luteolin; d: chrysoeriol. The figure was designed according to Bag et al. (2022).

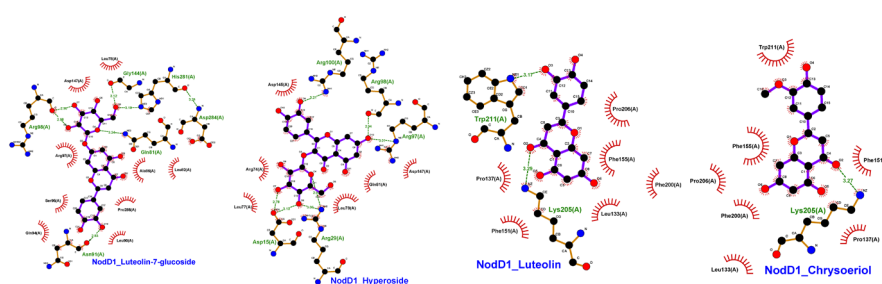


Figure S14. *In silico* binding studies of NodD1 protein of *S. meliloti* and 4 flavonoids from Compton et al.'s (2020) study. Each NodD1-flavonoid complex form hydrogen (green dotted line between amino acid and flavonoid, with distances) and hydrophobic (red half circles) bond during NodD1-flavonoid interaction.

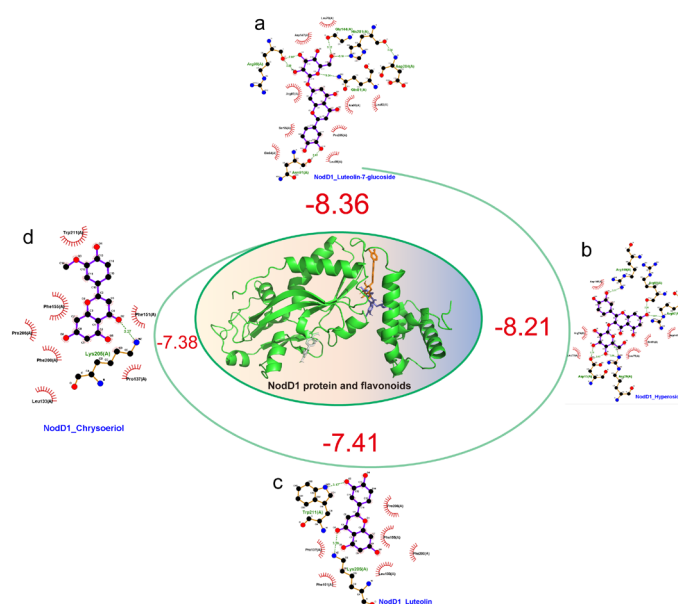


Figure S15. *In silico* binding studies between NodD2 protein of *S. meliloti* and 4 flavonoids from Compton et al.'s (2020) study. Here NodD2–flavonoid complexes were arranged based on their binding affinities (the more negative the values, ($\text{Kcal}\cdot\text{mol}^{-1}$), the stronger the interactions). Each NodD2–flavonoid complex form hydrogen (green dotted line between amino acid and flavonoid) and hydrophobic (red half circles) bonds during NodD2–flavonoid interaction. The red-boxed NodD2–flavonoid indicates the flavonoid found exclusively in the $\text{RC}^+S.tuberosum/\text{HC}^+R.irregularis$ treatment. The central picture (inside the oval circle) predicts the binding sites of 4 flavonoids in NodD2protein of *S. meliloti*. Individual figures are shown below. a: luteolin-7-glucoside; b: hyperoside; c: luteolin; d: chrysoeriol. The figure was designed according to Bag et al. (2022).

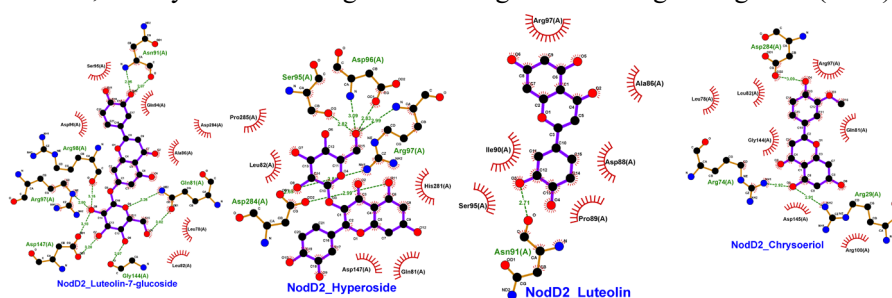


Figure S16. *In silico* binding studies of NodD2 protein of *S. meliloti* and 4 flavonoids from Compton et al.'s (2020) study. Each NodD2-flavonoid complex form hydrogen (green dotted line between amino acid and flavonoid, with distances) and hydrophobic (red half circles) bond during NodD2-flavonoid interaction.

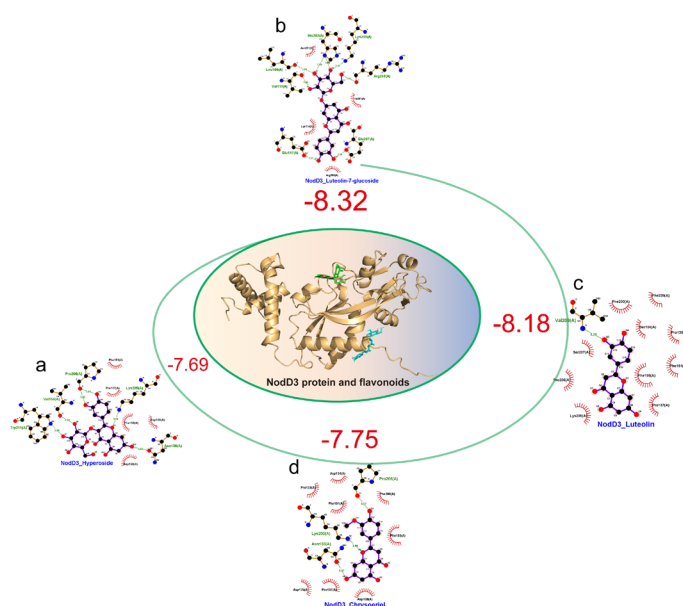


Figure S17. *In silico* binding studies between NodD3 protein of *S. meliloti* and 4 flavonoids from Compton et al.'s (2020) study. Here NodD3–flavonoid complexes were arranged based on their binding affinities (the more negative the values, (Kcal·mol⁻¹), the stronger the interactions). Each NodD3–flavonoid complex form hydrogen (green dotted line between amino acid and flavonoid) and hydrophobic (red half circles) bonds during NodD3–flavonoid interaction. The red-boxed NodD3–flavonoid indicates the flavonoid found exclusively in the RC*S.tuberosum*/HC⁺*R.irregularis* treatment. The central picture (inside the oval circle) predicts the binding sites of 4 flavonoids in NodD3 protein of *S. meliloti*. Individual figures are shown below. a: luteolin-7-glucoside; b: hyperoside; c: luteolin; d: chrysoeriol. The figure was designed according to Bag et al. (2022).

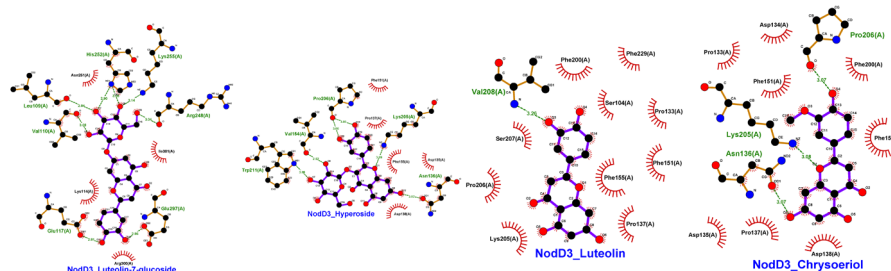


Figure S18. *In silico* binding studies of NodD3 protein of *S. meliloti* and 4 flavonoids from Compton et al.'s (2020) study. Each NodD3–flavonoid complex form hydrogen (green dotted line between amino acid and flavonoid, with distances) and hydrophobic (red half circles) bond during NodD3–flavonoid interaction.

Annex III

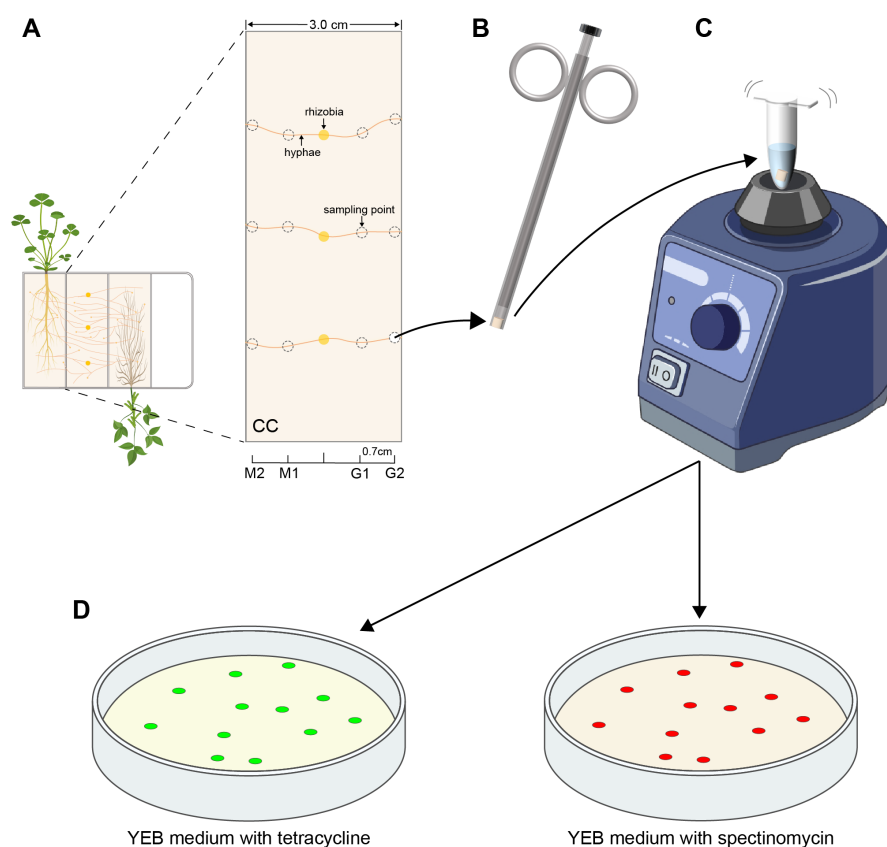


Figure S19. Workflow for sampling and quantifying bacterial migration along hyphae. This figure illustrates the key steps in assessing *S. meliloti* and *B. diazoefficiens* migration along *R. irregularis* hyphae connecting *M. truncatula* and *G. max* within a quadri-compartmented Petri plate system. **(A)** Diagram of sampling points at 0.7 cm and 1.4 cm from the inoculation site on hyphae in the central compartment. **(B)** Image of the 2 mm diameter cork borer used to collect medium samples. **(C)** Schematic of vortexing to homogenize bacterial suspensions from sampled medium. **(D)** Diagram of selective plating on YEB agar with species-specific antibiotics to enumerate colony-forming units (CFUs).



Figure S20. Picture of the three-compartment pot system.

Table S4. The information of eight flavonoids in silico NodD1-flavonoid binding studies

No.	Flavonoids	PubChem CID	m/z
1	DL-Liquiritigenin	1889	255.0656
2	Formononetin	439246	273.0756
3	Naringenin	73571	287.0910/287.0911
4	3,7-Dimethylquercetin	5281779	329.0660
5	Iriline	5281779	299.0548
6	Erylatissin A	11739635	353.1382
7	Daidzin	107971	417.1180
8	6''-O-Malonyldaidzin	14500869	503.1185

ANNEXES – ANNEX III

Table S5. LC-MS/MS analysis of chemical features and functions of non-flavonoid compounds in *Glycine max* and *Medicago truncatula* symbiotic systems with rhizobium: comparison of compounds detected exclusively in RC^{G.max}/HC^{+R.irregularis} treatment (Group A) and RC^{M.truncatula}/HC^{+R.irregularis} treatment (Group B).

Group	Ontology	Structure	m/z	Classification
A	Glutamic acid and derivatives	Tetrahydrofolic acid	446.18	Amino Acid Derivatives
A	N-acyl-alpha amino acids and derivatives	N-butyryl-L-homoserine lactone	172.1	Amino Acid Derivatives
A	Anthraquinones	aklanonic acid	395.08	Aromatic Compounds
A	Tricarboxylic acids and derivatives	Mumefural	301.06	Aromatic Compounds
A	Coumestans	Psoralidin	337.11	Aromatic Compounds
A	Pyridinecarboxylic acids	UNPD141826	167.04	Aromatic Compounds
A	Isocoumarins and derivatives	UNPD197888	451.07	Aromatic Compounds
A	Hydroxycinnamic acid glycosides	1-O-Sinapoylglucose	385.11	Glycosides
A	Cyanogenic glycosides	NCGC00380916-01_C16H27NO10_	416.15	Glycosides
A	Hexacarboxylic acids and derivatives	Taxezipidine L	755.29	Carboxylic Acids
A	Tricarboxylic acids and derivatives	Mumefural	301.06	Carboxylic Acids
A	Methoxyphenols	Sesamolinal	371.11	Phenols
A	Cyclohexenones	Panepoxydone;(-)-Panepoxydone	211.1	Ketones
A	5'-deoxyribonucleosides	aminodeoxyfutalosine	414.14	Nucleosides
A	Diarylethers	2-(1-[(6-{5-[4-(1H-1,2,4-triazol-1-yl)phenoxy]-1H-1,2,3,4-tetrazol-1-yl}-hexahydrofuro[3,2-b]furan-3-yl)carbamoyl)methyl}cyclopentyl)acetic acid	525.22	Heterocyclic Compounds
A	Pyranodioxins	N-[8-hydroxy-2,2-dimethyl-6-(4-nitrophenoxy)-hexahydro-2H-pyrano[3,2-d][1,3]dioxin-7-yl]acetamide	383.14	Heterocyclic Compounds
A	Sesquiterpenoids	9-Hydroxymegastigma-4,6,7-trien-3-one	207.14	Terpenoids
A	Triterpene saponins	Acetylsoyasaponin A3	1243.6	Terpenoids
A	Triterpene saponins	Acetylsoyasaponin A4	1363.6	Terpenoids

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A	Triterpene saponins	Assamsaponin G	1259.6	Terpenoids
A	Triterpene saponins	Lobatoside I	1393.6	Terpenoids
A	Terpene glycosides	NCGC00380501-01_C16H26O9_	385.15	Terpenoids
A	Terpene glycosides	Nepetaside	345.15	Terpenoids
A	Terpene glycosides	Oleoside 11-methyl ester	405.14	Terpenoids
A	Iridoid O-glycosides	Radiatoside	721.26	Terpenoids
A	Triterpene saponins	Theasaponin A3;(-)-Theasaponin A3	1273.6	Terpenoids
A	Germaacranolides and derivatives	Tulipinolide	291.16	Terpenoids
A	Triterpene saponins	UNPD185064	617.28	Terpenoids
A	Triterpene saponins	Vaccaroside G	1405.6	Terpenoids
A	Triterpenoids	Vaccaroside H	1393.6	Terpenoids
B	1-benzopyrans	3,4-Dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-carboxylic acid	249.11	Aromatic Compounds
B	Difurocoumarocyclopentenones	Aflatoxin B1	313.07	Aromatic Compounds
B	Steroidal glycosides	3beta-[(beta-D-glucopyranosyl)oxy]-17alpha-hydroxy-16beta-[(O-beta-D-xylopyranosyl)-(1->3)-2-O-acetyl-alpha-L-arabinopyranosyloxy]cholest-5-en-22-one	899.46	Glycosides
B	Oligosaccharides	UNPD131592	1017.3	Saccharides
B	Bile acids, alcohols and derivatives	UNPD64192	733.38	Steroids
B	Triterpene saponins	Polygalasaponin J;(+) -Polygalasaponin J	1251.6	Terpenoids



Scan the QR code or visit this link (<https://doi.org/10.1093/ismejo/wraf100>) to access the **Supplemental dataset 2**.

OVERVIEW OF THE SCIENTIFIC ACHIEVEMENTS

Scientific Publications

Research articles published

He Jiadong, Zhang Lin, Van Dingenen Judith, Desmet Sandrien, Goormachtig Sofie, Calonne-Salmon Maryline, and Declerck Stéphane. (2024). Arbuscular Mycorrhizal hyphae facilitate rhizobia dispersal and nodulation in legumes. The ISME Journal, 18 (1), wrae185. <https://doi.org/10.1093/ismejo/wrae185>

Impact factor: 10.8

He Jiadong, Van Dingenen Judith, Goormachtig Sofie, Calonne-Salmon Maryline, Declerck Stéphane. (2025). Legume-specific recruitment of rhizobia by hyphae of arbuscular mycorrhizal fungi. The ISME Journal, 19 (1), wraf100. <https://doi.org/10.1093/ismejo/wraf100>

Submitted to The ISME Journal.

Impact factor: 10.8

Conference Participation

International Conference on Advanced Materials, Belgium, 22–24 November 2022. Poster presentation. Title: Role of common mycelial networks of AMF on mobilizing free-living or symbiotic N-fixing bacteria and stimulating N acquisition by plants. Authors: He Jiadong, Calonne-Salmon Maryline, Van Dingenen Judith, Goormachtig Sofie, Declerck Stéphane.

The 6th International Molecular Mycorrhiza Meeting, Cambridge, UK, 25–27 September 2023. Poster presentation. Title: Role of common mycelial networks of AMF on mobilizing free-living or symbiotic N-fixing bacteria and stimulating N acquisition by plants. Authors: He Jiadong, Calonne-Salmon Maryline, Van Dingenen Judith, Goormachtig Sofie, Declerck Stéphane.

The 12th Edition of the International Mycological Congress, Maastricht, Netherlands, 11–15 August 2024. Poster presentation. Title: Rhizobia hitchhiking on arbuscular mycorrhizal hyphae allows nodulation in legumes. Authors: He

OVERVIEW OF THE SCIENTIFIC ACHIEVEMENTS

Jiadong, Zhang Lin, Van Dingenen Judith, Goormachtig Sofie, Calonne-Salmon Maryline, Declerck Stéphane.

Teaching and Student Supervision

International training on in vitro culture of Arbuscular Mycorrhizal Fungi, organized by the CESAMM laboratory, UCL, Prof. Stéphane Declerck: Plant systems (AMP+HAMP) Session. Louvain-la-Neuve, Belgium (2021, 2022, 2023, and 2024).

AUTHOR'S CONTRIBUTION

The work presented in this thesis was conducted during the course of my PhD. The **Introduction, State of the Art, General Discussion, Conclusions and Perspectives** sections were written entirely by myself and are not intended for publication. These sections were developed to provide the theoretical background, integrate the findings, and discuss the broader implications of the research.

Chapter I is a research article published in The ISME Journal (2024), with an estimated 90% contribution from me. The experimental design for this study, which focused on elucidating the role of AM fungal hyphae in facilitating the long-distance migration of rhizobia and the nodulation process in legumes, was developed through discussions with Prof. Stéphane Declerck (Université catholique de Louvain) and Dr. Maryline Calonne-Salmon (Université catholique de Louvain). Building on this collaborative design, I was responsible for conducting the experiments, including setting up and implementing *in vitro* and greenhouse-based experimental systems, performing time-lapse microscopy to analyze rhizobial migration on hyphae surface, and conducting statistical analysis and interpretation of the data. I also wrote the manuscript, synthesizing the findings and integrating them into a cohesive narrative. The flavonoid profiling of hyphal compartments colonized by *Rhizophagus irregularis* was conducted at the VIB Metabolomics Core Ghent (VIB-MCG) by Dr. Judith Van Dingenen (Ghent University), Dr. Sandrien Desmet (Ghent University), and Prof. Sofie Goormachtig (Ghent University). I collaborated closely with them to interpret the metabolomics data and link flavonoid signaling to rhizobial behavior. Throughout the research process, Prof. Stéphane Declerck and Dr. Maryline Calonne-Salmon provided critical guidance and valuable insights that supported the progression of the study. Prof. Lin Zhang (China Agricultural University) offered some conceptual inputs during the writing process. All co-authors participated in the review of the manuscript, providing highly specialized and constructive feedbacks to enhance the scientific clarity and rigor of the publication.

Chapter II is a research article published in The ISME Journal (2025), where my contribution was also estimated at 90%. This study expanded on the findings of Chapter I by investigating the legume-specific recruitment of rhizobia via common mycorrhizal networks. I developed and implemented the experimental systems, which included connecting *Medicago truncatula* and *Glycine max* using AM fungal networks, and conducted detailed assessments of selective rhizobial migration (*Sinorhizobium meliloti* and *Bradyrhizobium diazoefficiens*) and nodulation processes. I also collaborated with Sofie Goormachtig and Judith van

AUTHOR'S CONTRIBUTION

Dingene from the VIB Metabolomics Core Ghent for flavonoid profiling, while I performed the interpretation of the results to link specific flavonoids to rhizobial host preference. I wrote the manuscript and revised it based on the highly detailed and professional feedbacks provided by Prof. Stéphane Declerck and Dr. Maryline Calonne-Salmon. All co-authors reviewed the manuscript and provided expert feedback to strengthen its scientific accuracy and presentation.

REFERENCES

REFERENCES

- Abdel-Lateif K., Bogusz D., Hoche V., 2012. The role of flavonoids in the establishment of plant roots endosymbioses with arbuscular mycorrhiza fungi, rhizobia and frankia bacteria. *Plant Signal. Behav.* 7, 636–641.
- Abeysinghe, G., Kuchira, M., Kudo, G., Masuo, S., Ninomiya, A., Takahashi, K., Utada, A.S., Hagiwara, D., Nomura, N., Takaya, N., Obana, N., Takeshita, N., 2020. Fungal mycelia and bacterial thiamine establish a mutualistic growth mechanism. *Life Sci. Alliance* 3, e202000878.
- Adiji, O.A., Docampo-Palacios, M.L., Alvarez-Hernandez, A., Pasinetti, G.M., Wang, X., Dixon, R.A., 2021. UGT84F9 is the major flavonoid UDP-glucuronosyltransferase in *Medicago truncatula*. *Plant Physiol.* 185, 1617–1637.
- Agnolucci, M., Battini, F., Cristani, C., Giovannetti, M., 2015. Diverse bacterial communities are recruited on spores of different arbuscular mycorrhizal fungal isolates. *Biol. Fertil. Soils* 51, 379–389.
- Akiyama, K., 2007. Chemical identification and functional analysis of apocarotenoids involved in the development of arbuscular mycorrhizal symbiosis. *Biosci. Biotechnol. Biochem.* 71, 1405–1414.
- Akiyama, K., Matsuzaki, K., Hayashi, H., 2005. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. *Nature* 435, 824–827.
- Alaux, P.-L., Naveau, F., Declerck, S., Cranenbrouck, S., 2020. Common mycorrhizal network induced JA/ET genes expression in healthy potato plants connected to potato plants infected by *Phytophthora infestans*. *Front. Plant Sci.* 11.
- Aleklett, K., Kiers, E.T., Ohlsson, P., Shimizu, T.S., Caldas, V.E.A., Hammer, E.C., 2018. Build your own soil: Exploring microfluidics to create microbial habitat structures. *ISME J.* 12, 312–319.
- Anckaert, A., Declerck, S., Poussart, L.-A., Lambert, S., Helmus, C., Boubsi, F., Steels, S., Argüelles-Arias, A., Calonne-Salmon, M., Ongena, M., 2024. The biology and chemistry of a mutualism between a soil bacterium and a mycorrhizal fungus. *Curr. Biol.* 34, 4934-4950.e8.
- Andersen, O.M., Markham, K.R. (Eds.), 2005. *Flavonoids: Chemistry, biochemistry and applications*. CRC Press, Boca Raton.
- Andrade, D.S., Murphy, P.J., Giller, K.E., 2000. Diversity and abundance of populations of bean-nodulating rhizobia as a function of liming and cropping history in acidic brazilian soils, in: Pedrosa, F.O., Hungria, M., Yates, G., Newton, W.E. (Eds.), *Nitrogen Fixation: From Molecules to Crop Productivity*. Springer Netherlands, Dordrecht, p. 546.
- Andrade, S.A.L., Abreu, C.A., De Abreu, M.F., Silveira, A.P.D., 2004. Influence of lead additions on arbuscular mycorrhiza and rhizobium symbioses under

REFERENCES

- soybean plants. *Appl. Soil Ecol.* 26, 123–131.
- Antoun, H., Beauchamp, C.J., Goussard, N., Chabot, R., Lalande, R., 1998. Potential of rhizobium and *bradyrhizobium* species as plant growth promoting rhizobacteria on non-legumes: Effect on radishes (*raphanus sativus* L.). *Plant Soil* 204, 57–67.
- Antunes, P.M., De Varennes, A., Rajcan, I., Goss, M.J., 2006. Accumulation of specific flavonoids in soybean (*glycine max* (L.) merr.) as a function of the early tripartite symbiosis with arbuscular mycorrhizal fungi and *bradyrhizobium japonicum* (kirchner) jordan. *Soil Biol. Biochem.* 38, 1234–1242.
- Arora, N.K., Kang, S.C., Maheshwari, D.K., 2001. Isolation of siderophore-producing strains of rhizobium meliloti and their biocontrol potential against macrophomina phaseolina that causes charcoal rot of groundnut. *Curr. Sci.* 81, 673–677.
- Artursson, V., Jansson, J.K., 2003. Use of bromodeoxyuridine immunocapture to identify active bacteria associated with arbuscular mycorrhizal hyphae. *Appl. Environ. Microbiol.* 69, 6208–6215.
- Awaydul A., Zhu W., Yuan Y., Xiao J., Hu H., Chen X., Koide R.T., Cheng L., 2019. Common mycorrhizal networks influence the distribution of mineral nutrients between an invasive plant, *solidago canadensis*, and a native plant, *kummerowia striata*. *Mycorrhiza* 29, 29–38.
- Babikova, Z., Gilbert, L., Bruce, T.J.A., Birkett, M., Caulfield, J.C., Woodcock, C., Pickett, J.A., Johnson, D., 2013. Underground signals carried through common mycelial networks warn neighbouring plants of aphid attack. *Ecol. Lett.* 16, 835–843.
- Babikova, Z., Johnson, D., Bruce, T., Pickett, J., Gilbert, L., 2014. Underground allies: How and why do mycelial networks help plants defend themselves? *Bioessays* 36, 21–26.
- Badri, D.V., Vivanco, J.M., 2009. Regulation and function of root exudates. *Plant Cell Environ.* 32, 666–681.
- Bag, S., Mondal, A., Majumder, A., Mondal, S.K., Banik, A., 2022. Flavonoid mediated selective cross-talk between plants and beneficial soil microbiome. *Phytochem. Rev.* 21, 1739–1760.
- Bago, B., Azcón-Aguilar, C., Goulet, A., Piché, Y., 1998. Branched absorbing structures (BAS): A feature of the extraradical mycelium of symbiotic arbuscular mycorrhizal fungi. *New Phytol.* 139, 375–388.
- Bago, B., Pfeffer, P.E., Abubaker, J., Jun, J., Allen, J.W., Brouillette, J., Douds, D.D., Lammers, P.J., Shachar-Hill, Y., 2003. Carbon Export from Arbuscular Mycorrhizal Roots Involves the Translocation of Carbohydrate as well as Lipid. *Plant Physiol.* 131, 1496–1507.
- Bais, H.P., Park, S.-W., Weir, T.L., Callaway, R.M., Vivanco, J.M., 2004. How

REFERENCES

- plants communicate using the underground information superhighway. *Trends Plant Sci.* 9, 26–32.
- Bais, H.P., Weir, T.L., Perry, L.G., Gilroy, S., Vivanco, J.M., 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu. Rev. Plant Biol.* 57, 233–266.
- Bakhshandeh, S., Corneo, P.E., Mariotte, P., Kertesz, M.A., Dijkstra, F.A., 2017. Effect of crop rotation on mycorrhizal colonization and wheat yield under different fertilizer treatments. *Agric. Ecosyst. Environ.* 247, 130–136.
- Banasiak, J., Jamruszka, T., Murray, J.D., Jasiński, M., 2021. A roadmap of plant membrane transporters in arbuscular mycorrhizal and legume–rhizobium symbioses. *Plant Physiol.* 187, 2071–2091.
- Banfalvi, Z., Nieuwkoop, A., Schell, M., Besl, L., Stacey, G., 1988. Regulation of nod gene expression in *bradyrhizobium japonicum*. *Mol. Gen. Genet. MGG* 214, 420–424.
- Banitz, T., Johst, K., Wick, L.Y., Schamfuß, S., Harms, H., Frank, K., 2013. Highways versus pipelines: Contributions of two fungal transport mechanisms to efficient bioremediation. *Environ. Microbiol. Rep.* 5, 211–218.
- Barnett, M.J., Long, S.R., 2015. The *sinorhizobium meliloti* SyrM regulon: Effects on global gene expression are mediated by *SyrA* and *nodD3*. *J. Bacteriol.* 197, 1792–1806.
- Barto, E.K., Weidenhamer, J.D., Cipollini, D., Rillig, M.C., 2012. Fungal superhighways: do common mycorrhizal networks enhance below ground communication? *Trends Plant Sci.* 17, 633–637.
- Bécard, G., Kosuta, S., Tamasloukht, M., Séjalon-Delmas, N., Roux, C., 2004. Partner communication in the arbuscular mycorrhizal interaction. *Can. J. Bot.* 82, 1186–1197.
- Begum, A.A., Leibovitch, S., Migner, P., Zhang, F., 2001. Specific flavonoids induced nod gene expression and pre-activated nod genes of rhizobium leguminosarum increased pea (*pisum sativum* L.) and lentil (*lens culinaris* L.) nodulation in controlled growth chamber environments. *J. Exp. Bot.* 52, 1537–1543.
- Beilby, J.P., 1983. Effects of inhibitors on early protein, RNA, and lipid synthesis in germinating vesicular–arbuscular mycorrhizal fungal spores of *Glomus caledonium*. *Can. J. Microbiol.* 29, 596–601.
- Bell, C.D., Soltis, D.E., Soltis, P.S., 2010. The age and diversification of the angiosperms re-revisited. *Am. J. Bot.* 97, 1296–1303.
- Belmondo, S., Fiorilli, V., Pérez-Tienda, J., Ferrol, N., Marmeisse, R., Lanfranco, L., 2014. A dipeptide transporter from the arbuscular mycorrhizal fungus *rhizophagus irregularis* is upregulated in the intraradical phase. *Front. Plant Sci.* 5.

REFERENCES

- Besserer, A., Puech-Pagès, V., Kiefer, P., Gomez-Roldan, V., Jauneau, A., Roy, S., Portais, J.-C., Roux, C., Bécard, G., Séjalon-Delmas, N., 2006. Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria. *PLoS Biol.* 4, e226.
- Bethlenfalvay, G.J., Ames, R.N., 1987. Comparison of two methods for quantifying extraradical mycelium of vesicular-arbuscular mycorrhizal fungi. *Soil Sci. Soc. Am. J.* 51, 834–837.
- Bever, J.D., Simms, E.L., 2000. Evolution of nitrogen fixation in spatially structured populations of rhizobium. *Heredity* 85, 366–372.
- Bhattacharyya, P.N., Jha, D.K., 2012. Plant growth-promoting rhizobacteria (PGPR): Emergence in agriculture. *World J. Microbiol. Biotechnol.* 28, 1327–1350.
- Bianciotto, V., Andreotti, S., Balestrini, R., Bonfante, P., Perotto, S., 2001. Mucoid mutants of the biocontrol strain *Pseudomonas fluorescens* CHA0 show increased ability in biofilm formation on mycorrhizal and nonmycorrhizal carrot roots. *Mol. Plant. Microbe Interact.* 14, 255–260.
- Bianciotto, V., Genre, A., Jargeat, P., Lumini, E., Bécard, G., Bonfante, P., 2004. Vertical transmission of endobacteria in the arbuscular mycorrhizal fungus *Gigaspora margarita* through generation of vegetative spores. *Appl. Environ. Microbiol.* 70, 3600–3608.
- Bonfante, P., 2003. Plants, mycorrhizal fungi and endobacteria: A dialog among cells and genomes. *Biol. Bull.* 204, 215–220.
- Bonfante, P., Genre, A., 2010. Mechanisms underlying beneficial plant–fungus interactions in mycorrhizal symbiosis. *Nat. Commun.* 1, 48.
- Bonneau, L., Huguet, S., Wipf, D., Pauly, N., Truong, H.-N., 2013. Combined phosphate and nitrogen limitation generates a nutrient stress transcriptome favorable for arbuscular mycorrhizal symbiosis in *Medicago truncatula*. *New Phytol.* 199, 188–202.
- Bravo, A., Brands, M., Wewer, V., Dörmann, P., Harrison, M.J., 2017. Arbuscular mycorrhiza-specific enzymes FatM and RAM2 fine-tune lipid biosynthesis to promote development of arbuscular mycorrhiza. *New Phytol.* 214, 1631–1645.
- Brencic, A., Winans, S.C., 2005. Detection of and response to signals involved in host-microbe interactions by plant-associated bacteria. *Microbiol. Mol. Biol. Rev.* 69, 155–194.
- Breuillin-Sessoms, F., Floss, D.S., Gomez, S.K., Pumplin, N., Ding, Y., Levesque-Tremblay, V., Noar, R.D., Daniels, D.A., Bravo, A., Eaglesham, J.B., Benedito, V.A., Udvardi, M.K., Harrison, M.J., 2015. Suppression of arbuscule degeneration in *Medicago truncatula* phosphate transporter4 mutants is dependent on the ammonium transporter 2 family protein AMT2;3. *Plant Cell* 27, 1352–1366.

REFERENCES

- Brockwell, J., Bottomley, P.J., Thies, J.E., 1995. Manipulation of rhizobia microflora for improving legume productivity and soil fertility: A critical assessment. *Plant Soil* 174, 143–180.
- Broughton, W.J., Jabbouri, S., Perret, X., 2000. Keys to symbiotic harmony. *J. Bacteriol.* 182, 5641–5652.
- Brundrett, M.C., Tedersoo, L., 2018. Evolutionary history of mycorrhizal symbioses and global host plant diversity. *New Phytol.* 220, 1108–1115.
- Bücking, H., Liepold, E., Ambilwade, P., Bücking, H., Liepold, E., Ambilwade, P., 2012. The role of the mycorrhizal symbiosis in nutrient uptake of plants and the regulatory mechanisms underlying these transport processes, in: *Plant Science*. IntechOpen.
- Buée, M., Rossignol, M., Jauneau, A., Ranjeva, R., Bécard, G., 2000. The pre-symbiotic growth of arbuscular mycorrhizal fungi is induced by a branching factor partially purified from plant root exudates. *Mol. Plant. Microbe Interact.* 13, 693–698.
- Bukovská, P., Bonkowski, M., Konvalinková, T., Beskid, O., Hujšlová, M., Püschel, D., Řezáčová, V., Gutiérrez-Núñez, M.S., Gryndler, M., Jansa, J., 2018. Utilization of organic nitrogen by arbuscular mycorrhizal fungi—is there a specific role for protists and ammonia oxidizers? *Mycorrhiza* 28, 465–465.
- Bukovská, P., Gryndler, M., Gryndlerová, H., Püschel, D., Jansa, J., 2016. Organic nitrogen-driven stimulation of arbuscular mycorrhizal fungal hyphae correlates with abundance of ammonia oxidizers. *Front. Microbiol.* 7.
- Bukovská, P., Rozmoš, M., Kotianová, M., Gančarčíková, K., Dudáš, M., Hřelová, H., Jansa, J., 2021. Arbuscular mycorrhiza mediates efficient recycling from soil to plants of nitrogen bound in chitin. *Front. Microbiol.* 12.
- Bulgarelli, D., Schlaeppli, K., Spaepen, S., Themaat, E.V.L. van, Schulze-Lefert, P., 2013. Structure and Functions of the Bacterial Microbiota of Plants. *Annu. Rev. Plant Biol.* 64, 807–838.
- Calabrese, S., Pérez-Tienda, J., Ellerbeck, M., Arnould, C., Chatagnier, O., Boller, T., Schüßler, A., Brachmann, A., Wipf, D., Ferrol, N., Courty, P.-E., 2016. GintAMT3 – a low-affinity ammonium transporter of the arbuscular mycorrhizal *rhizophagus irregularis*. *Front. Plant Sci.* 7.
- Cangioli, L., Vaccaro, F., Fini, M., Mengoni, A., Fagorzi, C., 2022. Scent of a symbiont: The personalized genetic relationships of rhizobium—plant interaction. *Int. J. Mol. Sci.* 23, 3358.
- Capoen, W., Sun, J., Wysham, D., Otegui, M.S., Venkateshwaran, M., Hirsch, S., Miwa, H., Downie, J.A., Morris, R.J., Ané, J.-M., Oldroyd, G.E.D., 2011. Nuclear membranes control symbiotic calcium signaling of legumes. *Proc. Natl. Acad. Sci.* 108, 14348–14353.

REFERENCES

- Cappellazzo, G., Lanfranco, L., Fitz, M., Wipf, D., Bonfante, P., 2008. Characterization of an amino acid permease from the endomycorrhizal fungus *glomus mosseae*. *Plant Physiol.* 147, 429–437.
- Casieri, L., Ait Lahmidi, N., Doidy, J., Veneault-Fourrey, C., Migeon, A., Bonneau, L., Courty, P.-E., Garcia, K., Charbonnier, M., Delteil, A., Brun, A., Zimmermann, S., Plassard, C., Wipf, D., 2013. Biotrophic transportome in mutualistic plant–fungal interactions. *Mycorrhiza* 23, 597–625.
- Catford, J.G., Staehelin, C., Larose, G., Piché, Y., Vierheilig, H., 2006. Systemically suppressed isoflavonoids and their stimulating effects on nodulation and mycorrhization in alfalfa split-root systems. *Plant Soil* 285, 257–266.
- Catoira, R., Galera, C., de Billy, F., Penmetsa, R.V., Journet, E.-P., Maillet, F., Rosenberg, C., Cook, D., Gough, C., Dénarié, J., 2000. Four Genes of *Medicago truncatula* Controlling Components of a Nod Factor Transduction Pathway. *Plant Cell* 12, 1647–1665.
- Cerri, M.R., Wang, Q., Stolz, P., Folgmann, J., Frances, L., Katzer, K., Li, X., Heckmann, A.B., Wang, T.L., Downie, J.A., Klingl, A., de Carvalho-Niebel, F., Xie, F., Parniske, M., 2017. The 1 transcription factor gene is a target of the CCaMK/CYCLOPS complex and controls rhizobial infection in *lotus japonicus*. *New Phytol.* 215, 323–337.
- Cesco, S., Neumann, G., Tomasi, N., Pinton, R., Weisskopf, L., 2010. Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition. *Plant Soil* 329, 1–25.
- Charpentier, M., Sun, J., Martins, T.V., Radhakrishnan, G.V., Findlay, K., Soumpourou, E., Thouin, J., Véry, A.-A., Sanders, D., Morris, R.J., Oldroyd, G.E.D., 2016. Nuclear-localized cyclic nucleotide-gated channels mediate symbiotic calcium oscillations. *Science* 352, 1102–1105.
- Chen, T., Zhu, H., Ke, D., Cai, K., Wang, C., Gou, H., Hong, Z., Zhang, Z., 2012. A MAP kinase kinase interacts with SymRK and regulates nodule organogenesis in *lotus japonicus*. *Plant Cell* 24, 823–838.
- Chen, W.F., Wang, E.T., Ji, Z.J., Zhang, J.J., 2021. Recent development and new insight of diversification and symbiosis specificity of legume rhizobia: mechanism and application. *J. Appl. Microbiol.* 131, 553–563.
- Cheng, H.-P., Walker, G.C., 1998. Succinoglycan is required for initiation and elongation of infection threads during nodulation of alfalfa by *Rhizobium meliloti*. *J. Bacteriol.* 180, 5183–5191.
- Cheng, L., Booker, F.L., Tu, C., Burkey, K.O., Zhou, L., Shew, H.D., Rufty, T.W., Hu, S., 2012. Arbuscular mycorrhizal fungi increase organic carbon decomposition under elevated CO₂. *Science* 337, 1084–1087.
- Chieb, M., Gachomo, E.W., 2023. The role of plant growth promoting rhizobacteria in plant drought stress responses. *BMC Plant Biol.* 23, 407.

REFERENCES

- Chin, E., Slupsky, C.M., 2013. 10 - applications of metabolomics in food science: Food composition and quality, sensory and nutritional attributes, in: Weimer, B.C., Slupsky, C. (Eds.), *Metabolomics in Food and Nutrition*, Woodhead Publishing Series in Food Science, Technology and Nutrition. Woodhead Publishing, pp. 217–230.
- Christensen, J.R., Reck-Peterson, S.L., 2022. Hitchhiking across kingdoms: Cotransport of cargos in fungal, animal, and plant cells. *Annu. Rev. Cell Dev. Biol.* 38, 155–178.
- Cloutier, M.L., Murrell, E., Barbercheck, M., Kaye, J., Finney, D., García-González, I., Bruns, M.A., 2020. Fungal community shifts in soils with varied cover crop treatments and edaphic properties. *Sci. Rep.* 10, 6198.
- Coba de la Peña, T., Fedorova, E., Pueyo, J.J., Lucas, M.M., 2018. The symbiosome: Legume and rhizobia co-evolution toward a nitrogen-fixing organelle? *Front. Plant Sci.* 8.
- Compton, K.K., Hildreth, S.B., Helm, R.F., Scharf, B.E., 2020. An updated perspective on *Sinorhizobium meliloti* chemotaxis to alfalfa flavonoids. *Front. Microbiol.* 11, 581482.
- Compton, K.K., Hildreth, S.B., Helm, R.F., Scharf, B.E., 2018. *Sinorhizobium meliloti* chemoreceptor McpV senses short-chain carboxylates via direct binding. *J. Bacteriol.* 200, 10.1128/jb.519-18.
- Courty, P.E., Smith, P., Koegel, S., Redecker, D., Wipf, D., 2015. Inorganic nitrogen uptake and transport in beneficial plant root-microbe interactions. *Crit. Rev. Plant Sci.* 34, 4–16.
- Crosino, A., and Genre, A., 2022. Peace talks: symbiotic signaling molecules in arbuscular mycorrhizas and their potential application. *J. Plant Interact.* 17, 824–839.
- Dakora, F.D., Joseph, C.M., Phillips, D.A., 1993. Alfalfa (*medicago sativa* L.) root exudates contain isoflavonoids in the presence of *rhizobium meliloti*. *Plant Physiol.* 101, 819–824.
- Dalpé, Y., de Souza, F.A., Declerck, S., 2005. Life cycle of *glomus* species in monoxenic culture, in: Declerck, S., Fortin, J.A., Strullu, D.-G. (Eds.), *In Vitro Culture of Mycorrhizas*. Springer, Berlin, Heidelberg, pp. 49–71.
- Daniel, R.G.M., 2024. In silico methods for the study of the interactions between drugs and their protein targets. *Mex. J. Med. Res. ICSA* 12, 67–71.
- Das, D., Varshney, K., Ogawa, S., Torabi, S., Hüttel, R., Nelson, D.C., Gutjahr, C., 2025. Ethylene promotes SMAX1 accumulation to inhibit arbuscular mycorrhiza symbiosis. *Nat. Commun.* 16, 2025.
- Day, D.A., Whitehead, L.F., Kaiser, B.N., Udvardi, M.K., Mouritzen, P., Rosendahl, L., 1998. Ammonium transport from the bacteroid to the plant, in: Elmerich, C., Kondorosi, A., Newton, W.E. (Eds.), *Biological Nitrogen Fixation for the 21st Century: Proceedings of the 11th International Congress*

REFERENCES

- on Nitrogen Fixation, Institut Pasteur, Paris, France, July 20–25, 1997. Springer Netherlands, Dordrecht, pp. 449–452.
- de Andrade, L.A., Santos, C.H.B., Frezarin, E.T., Sales, L.R., Rigobelo, E.C., 2023a. Plant Growth-Promoting Rhizobacteria for Sustainable Agricultural Production. *Microorganisms* 11, 1088.
- de Andrade, L.A., Santos, C.H.B., Frezarin, E.T., Sales, L.R., Rigobelo, E.C., 2023b. Plant Growth-Promoting Rhizobacteria for Sustainable Agricultural Production. *Microorganisms* 11, 1088.
- de Novais, C.B., Sbrana, C., da Conceição Jesus, E., Rouws, L.F.M., Giovannetti, M., Avio, L., Siqueira, J.O., Saggin Júnior, O.J., da Silva, E.M.R., de Faria, S.M., 2020. Mycorrhizal networks facilitate the colonization of legume roots by a symbiotic nitrogen-fixing bacterium. *Mycorrhiza* 30, 389–396.
- Debellé, F., 2013. Nod factors, in: Maloy, S., Hughes, K. (Eds.), *Brenner's Encyclopedia of Genetics* (Second Edition). Academic Press, San Diego, pp. 74–77.
- Declerck, S., D'Or, D., Bivort, C., de Souza, F.A., 2004. Development of extraradical mycelium of *scutellospora reticulata* under root-organ culture: Spore production and function of auxiliary cells. *Mycol. Res.* 108, 84–92.
- Declerck, S., Dupré de Boulois, H., Bivort, C., Delvaux, B., 2003. Extraradical mycelium of the arbuscular mycorrhizal fungus *Glomus lamellosum* can take up, accumulate and translocate radiocaesium under root-organ culture conditions. *Environ. Microbiol.* 5, 510–516.
- Declerck, S., Strullu, D.-G., Fortin, J.A., 2005. In vitro culture of mycorrhizas, *Soil biology*. Springer, Berlin [etc.].
- Declerck, S., Strullu, D.G., Plenchette, C., 1998. Monoxenic culture of the intraradical forms of *Glomus* sp. isolated from a tropical ecosystem: A proposed methodology for germplasm collection. *Mycologia* 90, 579–585.
- del Cerro, P., Rolla-Santos, A.A.P., Gomes, D.F., Marks, B.B., Espuny, M. del R., Rodríguez-Carvajal, M.Á., Soria-Díaz, M.E., Nakatani, A.S., Hungria, M., Ollero, F.J., Megías, M., 2015. Opening the “black box” of *nodD3*, *nodD4* and *nodD5* genes of rhizobium tropici strain CIAT 899. *BMC Genomics* 16, 864.
- Del Valle, I., Webster, T.M., Cheng, H.-Y., Thies, J.E., Kessler, A., Miller, M.K., Ball, Z.T., MacKenzie, K.R., Masiello, C.A., Silberg, J.J., Lehmann, J., 2020. Soil organic matter attenuates the efficacy of flavonoid-based plant-microbe communication. *Sci. Adv.* 6, eaax8254.
- Delaux, P.-M., Radhakrishnan, G., Oldroyd, G., 2015. Tracing the evolutionary path to nitrogen-fixing crops. *Curr. Opin. Plant Biol.* 26, 95–99.
- Dénarié, J., Debellé, F., Promé, J.-C., 1996. RHIZOBIUM LIPO-CHITOOLIGOSACCHARIDE NODULATION FACTORS: Signaling molecules mediating recognition and morphogenesis. *Annu. Rev. Biochem.*

REFERENCES

- 65, 503–535.
- Dickson, S., 2004. The arum–paris continuum of mycorrhizal symbioses. *New Phytol.* 163, 187–200.
- Ding, X., Zhang, S., Wang, R., Li, S., Liao, X., 2016. AM fungi and rhizobium regulate nodule growth, phosphorous (P) uptake, and soluble sugar concentration of soybeans experiencing P deficiency. *J. Plant Nutr.* 39, 1915–1925.
- Dini-Andreote, F., van Elsas, J.D., 2013. Back to the basics: The need for ecophysiological insights to enhance our understanding of microbial behaviour in the rhizosphere. *Plant Soil* 373, 1–15.
- Dixon, R.A., Steele, C.L., 1999. Flavonoids and isoflavonoids – a gold mine for metabolic engineering. *Trends Plant Sci.* 4, 394–400.
- Dong, W., Song, Y., 2020. The significance of flavonoids in the process of biological nitrogen fixation. *Int. J. Mol. Sci.* 21, 5926.
- Downie, J.A., 2010. The roles of extracellular proteins, polysaccharides and signals in the interactions of rhizobia with legume roots. *FEMS Microbiol. Rev.* 34, 150–170.
- Drechsler, N., Courty, P.-E., Brulé, D., Kunze, R., 2018. Identification of arbuscular mycorrhiza-inducible nitrate transporter 1/peptide transporter family (NPF) genes in rice. *Mycorrhiza* 28, 93–100.
- Duan, S., Jin, Z., Zhang, L., Declerck, S., 2025. Mechanisms of cooperation in the plants-arbuscular mycorrhizal fungi-bacteria continuum. *ISME J.* 19, wraf023.
- Dunn, M.F., 2015. Key roles of microsymbiont amino acid metabolism in rhizobia-legume interactions. *Crit. Rev. Microbiol.* 41, 411–451.
- Dupré De Boulois, H., Voets, L., Delvaux, B., Jakobsen, I., Declerck, S., 2006. Transport of radiocaesium by arbuscular mycorrhizal fungi to *Medicago truncatula* under *in vitro* conditions. *Environ. Microbiol.* 8, 1926–1934.
- Durant, E., Hoysted, G.A., Howard, N., Sait, S.M., Childs, D.Z., Johnson, D., Field, K.J., 2023. Herbivore-driven disruption of arbuscular mycorrhizal carbon-for-nutrient exchange is ameliorated by neighboring plants. *Curr. Biol.* 33, 2566–2573.e4.
- Dusha, I., Austin, S., Dixon, R., 1999. The upstream region of the *nodD3* gene of *sinorhizobium meliloti* carries enhancer sequences for the transcriptional activator NtrC. *FEMS Microbiol. Lett.* 179, 491–499.
- Eberhardt, J., Santos-Martins, D., Tillack, A.F., Forli, S., 2021. AutoDock Vina 1.2.0: New Docking Methods, Expanded Force Field, and Python Bindings. *J. Chem. Inf. Model.* 61, 3891–3898.
- Emmett, B.D., Lévesque-Tremblay, V., Harrison, M.J., 2021. Conserved and reproducible bacterial communities associate with extraradical hyphae of arbuscular mycorrhizal fungi. *ISME J.* 15, 2276–2288.

REFERENCES

- Endre, G., Kereszt, A., Kevei, Z., Mihacea, S., Kaló, P., Kiss, G.B., 2002. A receptor kinase gene regulating symbiotic nodule development. *Nature* 417, 962–966.
- Etemadi, M., Gutjahr, C., Couzigou, J.-M., Zouine, M., Lauressergues, D., Timmers, A., Audran, C., Bouzayen, M., Bécard, G., Combier, J.-P., 2014. Auxin perception is required for arbuscule development in arbuscular mycorrhizal symbiosis. *Plant Physiol.* 166, 281–292.
- Faghihinia, M., Jansa, J., Halverson, L.J., Staddon, P.L., 2023. Hyphosphere microbiome of arbuscular mycorrhizal fungi: A realm of unknowns. *Biol. Fertil. Soils* 59, 17–34.
- Fahde, S., Boughribil, S., Sijilmassi, B., Amri, A., 2023. Rhizobia: A Promising Source of Plant Growth-Promoting Molecules and Their Non-Legume Interactions: Examining Applications and Mechanisms. *Agriculture* 13, 1279.
- Farag, M.A., Huhman, D.V., Lei, Z., Sumner, L.W., 2007. Metabolic profiling and systematic identification of flavonoids and isoflavonoids in roots and cell suspension cultures of *medicago truncatula* using HPLC–UV–ESI–MS and GC–MS. *Phytochemistry* 68, 342–354.
- Fellbaum, C.R., Gachomo, E.W., Beesetty, Y., Choudhari, S., Strahan, G.D., Pfeffer, P.E., Kiers, E.T., Bücking, H., 2012. Carbon availability triggers fungal nitrogen uptake and transport in arbuscular mycorrhizal symbiosis. *Proc. Natl. Acad. Sci.* 109, 2666–2671.
- Feng, H., Fu, R., Hou, X., Lv, Y., Zhang, N., Liu, Y., Xu, Z., Miao, Y., Krell, T., Shen, Q., Zhang, R., 2021. Chemotaxis of beneficial rhizobacteria to root exudates: The first step towards root–microbe rhizosphere interactions. *Int. J. Mol. Sci.* 22, 6655.
- Feng, Z., Liu, X., Zhu, H., Yao, Q., 2020. Responses of Arbuscular Mycorrhizal Symbiosis to Abiotic Stress: A Lipid-Centric Perspective. *Front. Plant Sci.* 11.
- Ferguson, B.J., Indrasumunar, A., Hayashi, S., Lin, M.-H., Lin, Y.-H., Reid, D.E., Gresshoff, P.M., 2010. Molecular analysis of legume nodule development and autoregulation. *J. Integr. Plant Biol.* 52, 61–76.
- Ferguson, B.J., Mens, C., Hastwell, A.H., Zhang, M., Su, H., Jones, C.H., Chu, X., Gresshoff, P.M., 2019. Legume nodulation: The host controls the party. *Plant Cell Environ.* 42, 41–51.
- Fester, T., Strack, D., Hause, B., 2001. Reorganization of tobacco root plastids during arbuscule development. *Planta* 213, 864–868.
- Frankenberger, W.T., Arshad, M., 2020. *Phytohormones in Soils: Microbial Production and Function*, 1st ed. CRC Press, Boca Raton.
- Gage, D.J., 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol. Mol. Biol. Rev.* 68, 280–300.

REFERENCES

- Gahan, J., Schmalenberger, A., 2015. Arbuscular mycorrhizal hyphae in grassland select for a diverse and abundant hyphospheric bacterial community involved in sulfonate desulfurization. *Appl. Soil Ecol.* 89, 113–121.
- Gamper, H.A., Walker, C., Schüßler, A., 2009. *Diversispora celata* sp. nov: Molecular ecology and phylotaxonomy of an inconspicuous arbuscular mycorrhizal fungus. *New Phytol.* 182, 495–506.
- Gao, J.-P., Liang, W., Liu, C.-W., Xie, F., Murray, J.D., 2024. Unraveling the rhizobial infection thread. *J. Exp. Bot.* 75, 2235–2245.
- Gao, J.-P., Xu, P., Wang, M., Zhang, X., Yang, J., Zhou, Y., Murray, J.D., Song, C.-P., Wang, E., 2021. Nod factor receptor complex phosphorylates GmGEF2 to stimulate ROP signaling during nodulation. *Curr. Biol.* 31, 3538–3550.e5.
- Gao, Q., Gao, Y., Liu, X., Zhou, W., Wang, Q., 2018. Effects of inoculation with arbuscular mycorrhizal fungi and rhizobia on growth of *medicago sativa* under saline-alkaline stress. *Acta Ecol. Sin.* 38.
- Garcia, K., Doidy, J., Zimmermann, S.D., Wipf, D., Courty, P.-E., 2016. Take a trip through the plant and fungal transportome of mycorrhiza. *Trends Plant Sci.* 21, 937–950.
- Garg, N., Geetanjali, 2007. Symbiotic nitrogen fixation in legume nodules: Process and signaling. A review. *Agron. Sustain. Dev.* 27, 59–68.
- Gautrat, P., Laffont, C., Frugier, F., 2020. Compact root architecture 2 promotes root competence for nodulation through the miR2111 systemic effector. *Curr. Biol.* 30, 1339–1345.e3.
- Genre, A., Bonfante, P., 2010. The making of symbiotic cells in arbuscular mycorrhizal roots, in: Koltai, H., Kapulnik, Y. (Eds.), *Arbuscular Mycorrhizas: Physiology and Function*. Springer Netherlands, Dordrecht, pp. 57–71.
- Genre, A., Chabaud, M., Balzergue, C., Puech-Pagès, V., Novero, M., Rey, T., Fournier, J., Rochange, S., Bécard, G., Bonfante, P., Barker, D.G., 2013. Short-chain chitin oligomers from arbuscular mycorrhizal fungi trigger nuclear Ca^{2+} spiking in *Medicago truncatula* roots and their production is enhanced by strigolactone. *New Phytol.* 198, 190–202.
- Gholami, A., Geyter, N.D., Pollier, J., Goormachtig, S., Goossens, A., 2014. Natural product biosynthesis in medicago species. *Nat. Prod. Rep.* 31, 356–380.
- Gibson, K.E., Kobayashi, H., Walker, G.C., 2008. Molecular determinants of a symbiotic chronic infection. *Annu. Rev. Genet.* 42, 413–441.
- Gilbert, L., Johnson, D., 2017. Chapter four - plant–plant communication through common mycorrhizal networks, in: Becard, G. (Ed.), *Advances in Botanical Research, How Plants Communicate with Their Biotic Environment*. Academic Press, pp. 83–97.

REFERENCES

- Gilbert, L., Johnson, D., 2015. Plant-mediated ‘apparent effects’ between mycorrhiza and insect herbivores. *Curr. Opin. Plant Biol.* 26, 100–105.
- Giovannetti, M., 2000. Spore germination and pre-symbiotic mycelial growth, in: Kapulnik, Y., Douds, D.D. (Eds.), *Arbuscular Mycorrhizas: Physiology and Function*. Springer Netherlands, Dordrecht, pp. 47–68.
- Giovannetti, M., Sbrana, C., Avio, L., Strani, P., 2004. Patterns of below-ground plant interconnections established by means of arbuscular mycorrhizal networks. *New Phytol.* 164, 175–181.
- Giraud, E., Moulin, L., Vallenet, D., Barbe, V., Cytryn, E., Avarre, J.-C., Jaubert, M., Simon, D., Cartieaux, F., Prin, Y., Bena, G., Hannibal, L., Fardoux, J., Kojadinovic, M., Vuillet, L., Lajus, A., Cruveiller, S., Rouy, Z., Mangenot, S., Segurens, B., Dossat, C., Franck, W.L., Chang, W.-S., Saunders, E., Bruce, D., Richardson, P., Normand, P., Dreyfus, B., Pignol, D., Stacey, G., Emerich, D., Verméglio, A., Médigue, C., Sadowsky, M., 2007. Legumes symbioses: Absence of nod genes in photosynthetic bradyrhizobia. *Science* 316, 1307–1312.
- Gleadow, R.M., Woodrow, I.E., 2002. Mini-Review: Constraints on Effectiveness of Cyanogenic Glycosides in Herbivore Defense. *J. Chem. Ecol.* 28, 1301–1313.
- Gleason, C., Chaudhuri, S., Yang, T., Muñoz, A., Poovaiah, B.W., Oldroyd, G.E.D., 2006. Nodulation independent of rhizobia induced by a calcium-activated kinase lacking autoinhibition. *Nature* 441, 1149–1152.
- Glick, B.R., 2012. Plant Growth-Promoting Bacteria: Mechanisms and Applications. *Scientifica* 2012, 963401.
- Govindarajulu, M., Pfeffer, P.E., Jin, H., Abubaker, J., Douds, D.D., Allen, J.W., Bücking, H., Lammers, P.J., Shachar-Hill, Y., 2005. Nitrogen transfer in the arbuscular mycorrhizal symbiosis. *Nature* 435, 819–823.
- Griesmann, M., Chang, Y., Liu, X., Song, Y., Haberer, G., Crook, M.B., Billault-Penneteau, B., Lauretsergues, D., Keller, J., Imanishi, L., Roswanjaya, Y.P., Kohlen, W., Pujic, P., Battenberg, K., Alloisio, N., Liang, Y., Hilhorst, H., Salgado, M.G., Hoher, V., Gherbi, H., Svistoonoff, S., Doyle, J.J., He, S., Xu, Y., Xu, S., Qu, J., Gao, Q., Fang, X., Fu, Y., Normand, P., Berry, A.M., Wall, L.G., Ané, J.-M., Pawlowski, K., Xu, X., Yang, H., Spannagl, M., Mayer, K.F.X., Wong, G.K.-S., Parniske, M., Delaux, P.-M., Cheng, S., 2018. Phylogenomics reveals multiple losses of nitrogen-fixing root nodule symbiosis. *Science* 361, eaat1743.
- Groth, M., Takeda, N., Perry, J., Uchida, H., Dräxl, S., Brachmann, A., Sato, S., Tabata, S., Kawaguchi, M., Wang, T.L., Parniske, M., 2010. NENA, a *lotus japonicus* homolog of Sec13, is required for rhizodermal infection by arbuscular mycorrhiza fungi and rhizobia but dispensable for cortical endosymbiotic development. *Plant Cell* 22, 2509–2526.

REFERENCES

- Gubry-Rangin, C., Garcia, M., Béna, G., 2010. Partner choice in *Medicago truncatula*–*Sinorhizobium* symbiosis. *Proc. R. Soc. B-Biol. Sci.* 277, 1947–1951.
- Guyonnet, J.P., Guillemet, M., Dubost, A., Simon, L., Ortet, P., Barakat, M., Heulin, T., Achouak, W., Haichar, F. el Z., 2018. Plant nutrient resource use strategies shape active rhizosphere microbiota through root exudation. *Front. Plant Sci.* 9.
- Halder, A.K., Chakrabartty, P.K., 1993. Solubilization of inorganic phosphate by *rhizobium*. *Folia Microbiol. (Praha)* 38, 325–330.
- Halder, A.K., Mishra, A.K., Bhattacharyya, P., Chakrabartty, P.K., 1990. Solubilization of rock phosphate by *rhizobium* and *bradyrhizobium*. *J. Gen. Appl. Microbiol.* 36, 81–92.
- Han, S., Na, L., Rongchao, Z., Xiuqin, H., Wenyu, Z., Bo, Z., Xinpeng, L., Zhen, W., Jie, X., 2023. Study on signal transmission mechanism of arbuscular mycorrhizal hyphal network against root rot of salvia miltiorrhiza. *Sci. Rep.* 13, 16936.
- Hao, Z., Xie, W., Jiang, X., Wu, Z., Zhang, X., Chen, B., 2019. Arbuscular mycorrhizal fungus improves rhizobium–*glycyrrhiza* seedling symbiosis under drought stress. *Agronomy* 9, 572.
- Hartmann, R.M., Schaepe, S., Nübel, D., Petersen, A.C., Bertolini, M., Vasilev, J., Küster, H., Hohnjec, N., 2019. Insights into the complex role of GRAS transcription factors in the arbuscular mycorrhiza symbiosis. *Sci. Rep.* 9, 3360.
- Hartwig, U.A., Joseph, C.M., Phillips, D.A., 1991. Flavonoids released naturally from alfalfa seeds enhance growth rate of *rhizobium meliloti*. *Plant Physiol.* 95, 797–803.
- Hartwig, U.A., Maxwell, C.A., Joseph, C.M., Phillips, D.A., 1989. Interactions among flavonoid *nod* gene inducers released from alfalfa seeds and roots. *Plant Physiol.* 91, 1138–1142.
- Hasegawa, M., Mitsuhashi, I., Seo, S., Okada, K., Yamane, H., Iwai, T., Ohashi, Y., 2014. Analysis on blast fungus-responsive characters of a flavonoid phytoalexin sakuranetin; accumulation in infected rice leaves, antifungal activity and detoxification by fungus. *Molecules* 19, 11404–11418.
- Hassan, S., Mathesius, U., 2012. The role of flavonoids in root-rhizosphere signalling: Opportunities and challenges for improving plant-microbe interactions. *J. Exp. Bot.* 63, 3429–3444.
- Hawkins, H.-J., Cargill, R.I.M., Van Nuland, M.E., Hagen, S.C., Field, K.J., Sheldrake, M., Soudzilovskaia, N.A., Kiers, E.T., 2023. Mycorrhizal mycelium as a global carbon pool. *Curr. Biol.* 33, R560–R573.
- Hawkins, J.P., Oresnik, I.J., 2022. The Rhizobium-Legume Symbiosis: Co-opting Successful Stress Management. *Front. Plant Sci.* 12, 796045.

REFERENCES

- He, J., Zhang, L., Van Dingenen, J., Desmet, S., Goormachtig, S., Calonne-Salmon, M., Declerck, S., 2024. Arbuscular mycorrhizal hyphae facilitate rhizobia dispersal and nodulation in legumes. *ISME J.* 18, wrae185.
- He, S., Guo, L., Li, J., Wang, Y., Liu, Z., Cheng, Y., Hu, T., Long, M., 2017. Advances in arbuscular mycorrhizal fungi and legumes symbiosis research. *Acta Prataculturae Sin.* 26, 187–194.
- Helber, N., Wippel, K., Sauer, N., Schaarschmidt, S., Hause, B., Requena, N., 2011. A versatile monosaccharide transporter that operates in the arbuscular mycorrhizal fungus *glomus* sp is crucial for the symbiotic relationship with plants. *Plant Cell* 23, 3812–3823.
- Hestrin, R., Weber, P.K., Pett-Ridge, J., Lehmann, J., 2021. Plants and mycorrhizal symbionts acquire substantial soil nitrogen from gaseous ammonia transport. *New Phytol.* 231, 1746–1757.
- Hirsch, P.R., 1996. Population dynamics of indigenous and genetically modified rhizobia in the field. *New Phytol.* 133, 159–171.
- Hobbie, E.A., 2006. Carbon allocation to ectomycorrhizal fungi correlates with belowground allocation in culture studies. *Ecology* 87, 563–569.
- Hodge, A., Campbell, C.D., Fitter, A.H., 2001. An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. *Nature* 413, 297–299.
- Honma, M.A., Ausubel, F.M., 1987. *Rhizobium meliloti* has three functional copies of the *nodD* symbiotic regulatory gene. *Proc. Natl. Acad. Sci.* 84, 8558–8562.
- Hontoria, C., García-González, I., Quemada, M., Roldán, A., Alguacil, M.M., 2019. The cover crop determines the AMF community composition in soil and in roots of maize after a ten-year continuous crop rotation. *Sci. Total Environ.* 660, 913–922.
- Horiuchi, J., Prithiviraj, B., Bais, H.P., Kimball, B.A., Vivanco, J.M., 2005. Soil nematodes mediate positive interactions between legume plants and rhizobium bacteria. *Planta* 222, 848–857.
- Hydbom, S., Ernfors, M., Birgander, J., Hollander, J., Jensen, E.S., Olsson, P.A., 2017. Reduced tillage stimulated symbiotic fungi and microbial saprotrophs, but did not lead to a shift in the saprotrophic microorganism community structure. *Appl. Soil Ecol.* 119, 104–114.
- Ivanov, S., Harrison, M.J., 2019. Accumulation of phosphoinositides in distinct regions of the periarbuscular membrane. *New Phytol.* 221, 2213–2227.
- Jämtgård, S., Näsholm, T., Huss-Danell, K., 2010. Nitrogen compounds in soil solutions of agricultural land. *Soil Biol. Biochem.* 42, 2325–2330.
- Jansa, J., Hodge, A., 2021. Swimming, gliding, or hyphal riding? On microbial migration along the arbuscular mycorrhizal hyphal highway and functional consequences thereof. *New Phytol.* 230, 14–16.

REFERENCES

- Jasiński, M., Kachlicki, P., Rodziewicz, P., Figlerowicz, M., Stobiecki, M., 2009. Changes in the profile of flavonoid accumulation in *Medicago truncatula* leaves during infection with fungal pathogen *Phoma medicaginis*. *Plant Physiol. Biochem.* 47, 847–853.
- Javot, H., Penmetsa, R.V., Terzaghi, N., Cook, D.R., Harrison, M.J., 2007. A *medicago truncatula* phosphate transporter indispensable for the arbuscular mycorrhizal symbiosis. *Proc. Natl. Acad. Sci.* 104, 1720–1725.
- Jiang, F., Zhang, L., Zhou, J., George, T.S., Feng, G., 2021. Arbuscular mycorrhizal fungi enhance mineralisation of organic phosphorus by carrying bacteria along their extraradical hyphae. *New Phytol.* 230, 304–315.
- Jiang, L., Zhang, X., Zhao, Y., Zhu, H., Fu, Q., Lu, X., Huang, W., Yang, X., Zhou, X., Wu, L., Yang, A., He, Xie, Dong, M., Peng, Z., Yang, J., Guo, L., Wen, J., Huang, H., Xie, Y., Zhu, S., Li, C., He, Xiahong, Zhu, Y., Friml, J., Du, Y., 2024. Phytoalexin sakuranetin attenuates endocytosis and enhances resistance to rice blast. *Nat. Commun.* 15, 3437.
- Jiang, Y., Wang, W., Xie, Q., Liu, N., Liu, L., Wang, D., Zhang, X., Yang, C., Chen, X., Tang, D., Wang, E., 2017. Plants transfer lipids to sustain colonization by mutualistic mycorrhizal and parasitic fungi. *Science* 356, 1172–1175.
- Jin, H., Pfeffer, P.E., Douds, D.D., Piotrowski, E., Lammers, P.J., Shachar-Hill, Y., 2005. The uptake, metabolism, transport and transfer of nitrogen in an arbuscular mycorrhizal symbiosis. *New Phytol.* 168, 687–696.
- Jin, Y., Liu, H., Luo, D., Yu, N., Dong, W., Wang, C., Zhang, X., Dai, H., Yang, J., Wang, E., 2016. DELLA proteins are common components of symbiotic rhizobial and mycorrhizal signalling pathways. *Nat. Commun.* 7, 12433.
- Jin, Z., Jiang, F., Wang, L., Declerck, S., Feng, G., Zhang, L., 2024. Arbuscular mycorrhizal fungi and streptomyces: Brothers in arms to shape the structure and function of the hyphosphere microbiome in the early stage of interaction. *Microbiome* 12, 83.
- Johnson, D., Gilbert, L., 2015. Interplant signalling through hyphal networks. *New Phytol.* 205, 1448–1453.
- Joner, E.J., Jakobsen, I., 1995. Growth and extracellular phosphatase activity of arbuscular mycorrhizal hyphae as influenced by soil organic matter. *Soil Biol. Biochem.* 27, 1153–1159.
- Jones, K.M., Kobayashi, H., Davies, B.W., Taga, M.E., Walker, G.C., 2007. How rhizobial symbionts invade plants: the Sinorhizobium–Medicago model. *Nat. Rev. Microbiol.* 5, 619–633.
- Journet E.-P., El-Gachtouli N., Vernoud V., de Billy F., Pichon M., Dedieu A., Arnould C., Morandi D., Barker D.G., Gianinazzi-Pearson V., 2001. *Medicago truncatula* *ENOD11* : A novel RPRP-encoding early nodulin gene expressed during mycorrhization in arbuscule-containing cells. *Mol. Plant. Microbe Interact.* 14, 737–748.

REFERENCES

- Juge, C., Samson, J., Bastien, C., Vierheilig, H., Coughlan, A., Piché, Y., 2002. Breaking dormancy in spores of the arbuscular mycorrhizal fungus *Glomus intraradices*: A critical cold-storage period. *Mycorrhiza* 12, 37–42.
- Junier, P., Cailleau, G., Palmieri, I., Vallotton, C., Trautschold, O.C., Junier, T., Paul, C., Bregnard, D., Palmieri, F., Estoppey, A., Buffi, M., Lohberger, A., Robinson, A., Kelliher, J.M., Davenport, K., House, G.L., Morales, D., Gallegos-Graves, L.V., Dichosa, A.E.K., Lupini, S., Nguyen, H.N., Young, J.D., Rodrigues, D.F., Parra-Vasquez, A.N.G., Bindschedler, S., Chain, P.S.G., 2021. Democratization of fungal highway columns as a tool to investigate bacteria associated with soil fungi. *FEMS Microbiol. Ecol.* 97, fiab003.
- Kajić, S., Hulak, N., Sikora, S., 2016. Environmental stress response and adaptation mechanisms in rhizobia. *Agric. Conspec. Sci.* 81, 15–19.
- Kameoka, H., Tsutsui, I., Saito, K., Kikuchi, Y., Handa, Y., Ezawa, T., Hayashi, H., Kawaguchi, M., Akiyama, K., 2019. Stimulation of asymbiotic sporulation in arbuscular mycorrhizal fungi by fatty acids. *Nat. Microbiol.* 4, 1654–1660.
- Kanamori, N., Madsen, L.H., Radutoiu, S., Frantescu, M., Quistgaard, E.M.H., Miwa, H., Downie, J.A., James, E.K., Felle, H.H., Haaning, L.L., Jensen, T.H., Sato, S., Nakamura, Y., Tabata, S., Sandal, N., Stougaard, J., 2006. A nucleoporin is required for induction of Ca^{2+} spiking in legume nodule development and essential for rhizobial and fungal symbiosis. *Proc. Natl. Acad. Sci.* 103, 359–364.
- Karst, J., Jones, M.D., Hoeksema, J.D., 2023. Positive citation bias and overinterpreted results lead to misinformation on common mycorrhizal networks in forests. *Nat. Ecol. Evol.* 7, 501–511.
- Kaschuk, G., Leffelaar, P.A., Giller, K.E., Alberton, O., Hungria, M., Kuyper, T.W., 2010. Responses of legumes to rhizobia and arbuscular mycorrhizal fungi: A meta-analysis of potential photosynthate limitation of symbioses. *Soil Biol. Biochem.* 42, 125–127.
- Ke, D., Fang, Q., Chen, C., Zhu, H., Chen, T., Chang, X., Yuan, S., Kang, H., Ma, L., Hong, Z., Zhang, Z., 2012. The small GTPase ROP6 interacts with NFR5 and is involved in nodule formation in *lotus japonicus*. *Plant Physiol.* 159, 131–143.
- Kennedy, I.R., Pereg-Gerk, L.L., Wood, C., Deaker, R., Gilchrist, K., Katupitiya, S., 1997. Biological nitrogen fixation in non-leguminous field crops: Facilitating the evolution of an effective association between azospirillum and wheat. *Plant Soil* 194, 65–79.
- Kevei, Z., Loughnon, G., Mergaert, P., Horváth, G.V., Kereszt, A., Jayaraman, D., Zaman, N., Marcel, F., Regulski, K., Kiss, G.B., Kondorosi, A., Endre, G., Kondorosi, E., Ané, J.-M., 2007. 3-hydroxy-3-methylglutaryl coenzyme a

REFERENCES

- Reductase1 interacts with NORK and is crucial for nodulation in *medicago truncatula*. *Plant Cell* 19, 3974–3989.
- Kidston, R., Lang, W.H., 1921. XXXII.—on old red sandstone plants showing structure, from the rhynie chert bed, aberdeenshire. Part IV. Restorations of the vascular cryptogams, and discussion of their bearing on the general morphology of the pteridophyta and the origin of the organisation of land-plants. *Earth Environ. Sci. Trans. R. Soc. Edinb.* 52, 831–854.
- Kikuchi, Y., Hijikata, N., Yokoyama, K., Ohtomo, R., Handa, Y., Kawaguchi, M., Saito, K., Ezawa, T., 2014. Polyphosphate accumulation is driven by transcriptome alterations that lead to near-synchronous and near-equivalent uptake of inorganic cations in an arbuscular mycorrhizal fungus. *New Phytol.* 204, 638–649.
- Kiriachek, S.G., Azevedo, L.C.B. de, Peres, L.E.P., Lambais, M.R., 2009. Regulação do desenvolvimento de micorrizas arbusculares. *Rev. Bras. Ciênc. Solo* 33, 1–16.
- Knights, H.E., Ramachandran, V.K., Jorin, B., Ledermann, R., Parsons, J.D., Aroney, S.T.N., Poole, P.S., 2024. *Rhizobium* determinants of rhizosphere persistence and root colonization. *ISME J.* 18, wrac072.
- Kobae, Y., Tamura, Y., Takai, S., Banba, M., Hata, S., 2010. Localized expression of arbuscular mycorrhiza-inducible ammonium transporters in soybean. *Plant Cell Physiol.* 51, 1411–1415.
- Kobayashi, H., Graven, Y.N., Broughton, W.J., Perret, X., 2004. Flavonoids induce temporal shifts in gene-expression of *nod*-box controlled loci in *rhizobium* sp. NGR234. *Mol. Microbiol.* 51, 335–347.
- Koegel, S., Ait Lahmidi, N., Arnould, C., Chatagnier, O., Walder, F., Ineichen, K., Boller, T., Wipf, D., Wiemken, A., Courty, P.-E., 2013. The family of ammonium transporters (AMT) in *orghum bicolor*: Two AMT members are induced locally, but not systemically in roots colonized by arbuscular mycorrhizal fungi. *New Phytol.* 198, 853–865.
- Koegel, S., Brulé, D., Wiemken, A., Boller, T., Courty, P.-E., 2015. The effect of different nitrogen sources on the symbiotic interaction between *sorghum bicolor* and *glomus intraradices*: Expression of plant and fungal genes involved in nitrogen assimilation. *Soil Biol. Biochem.* 86, 159–163.
- Koegel, S., Mieulet, D., Baday, S., Chatagnier, O., Lehmann, M.F., Wiemken, A., Boller, T., Wipf, D., Bernèche, S., Guiderdoni, E., Courty, P.-E., 2017. Phylogenetic, structural, and functional characterization of AMT3;1, an ammonium transporter induced by mycorrhization among model grasses. *Mycorrhiza* 27, 695–708.
- Kohlmeier, S., Smits, T.H.M., Ford, R.M., Keel, C., Harms, H., Wick, L.Y., 2005. Taking the fungal highway: mobilization of pollutant-degrading bacteria by fungi. *Environ. Sci. Technol.* 39, 4640–4646.

REFERENCES

- Kotik, M., Kulik, N., Valentová, K., 2023. Flavonoids as Aglycones in Retaining Glycosidase-Catalyzed Reactions: Prospects for Green Chemistry. *J. Agric. Food Chem.* 71, 14890–14910.
- Kuga, Y., Saito, K., Nayuki, K., Peterson, R.L., Saito, M., 2008. Ultrastructure of rapidly frozen and freeze-substituted germ tubes of an arbuscular mycorrhizal fungus and localization of polyphosphate. *New Phytol.* 178, 189–200.
- Kumar, G.A., Kumar, S., Bhardwaj, R., Swapnil, P., Meena, M., Seth, C.S., Yadav, A., 2024. Recent advancements in multifaceted roles of flavonoids in plant–rhizomicrobiome interactions. *Front. Plant Sci.* 14.
- Kuyper, T.W., Jansa, J., 2023. Arbuscular mycorrhiza: advances and retreats in our understanding of the ecological functioning of the mother of all root symbioses. *Plant Soil* 489, 41–88.
- Lanfranco, L., Novero, M., Bonfante, P., 2005. The mycorrhizal fungus *gigaspora margarita* possesses a CuZn superoxide dismutase that is up-regulated during symbiosis with legume hosts. *Plant Physiol.* 137, 1319–1330.
- Larimer, A.L., Clay, K., Bever, J.D., 2014. Synergism and context dependency of interactions between arbuscular mycorrhizal fungi and rhizobia with a prairie legume. *Ecology* 95, 1045–1054.
- Larose, G., Chênevert, R., Moutoglis, P., Gagné, S., Piché, Y., Vierheilig, H., 2002. Flavonoid levels in roots of *Medicago sativa* are modulated by the developmental stage of the symbiosis and the root colonizing arbuscular mycorrhizal fungus. *J. Plant Physiol.* 159, 1329–1339.
- Laskowski, R.A., Swindells, M.B., 2011. LigPlot+: Multiple ligand–protein interaction diagrams for drug discovery. *J. Chem. Inf. Model.* 51, 2778–2786.
- Ledermann, R., Bartsch, I., Remus-Emsermann, M.N., Vorholt, J.A., Fischer, H.-M., 2015. Stable fluorescent and enzymatic tagging of *Bradyrhizobium diazoefficiens* to analyze host-plant infection and colonization. *Mol. Plant. Microbe Interact.* 28, 959–967.
- Lee, J.-H., Dean, M., Austin, J.R., Burdette, J.E., Murphy, B.T., 2018. Irlone from red clover (*Trifolium pratense*) potentiates progesterone signaling. *J. Nat. Prod.* 81, 1962–1967.
- Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J.C., Dénarié, J., 1990. Symbiotic host-specificity of rhizobium *meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. *Nature* 344, 781–784.
- Leveau, J.H.J., Preston, G.M., 2008. Bacterial mycophagy: Definition and diagnosis of a unique bacterial–fungal interaction. *New Phytol.* 177, 859–876.
- Lévy, J., Bres, C., Geurts, R., Chalhoub, B., Kulikova, O., Duc, G., Journet, E.-P., Ané, J.-M., Lauber, E., Bisseling, T., Dénarié, J., Rosenberg, C., Debelle, F.,

REFERENCES

2004. A putative Ca^{2+} and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. *Science* 303, 1361–1364.
- Li, Y., Pei, Y., Shen, Y., Zhang, R., Kang, M., Ma, Y., Li, D., Chen, Y., 2022. Progress in the self-regulation system in legume nodule development-AON (autoregulation of nodulation). *Int. J. Mol. Sci.* 23, 6676.
- Li, Y., Tian, C.F., Chen, W.F., Wang, L., Sui, X.H., Chen, W.X., 2013. High-resolution transcriptomic analyses of *sinorhizobium* sp. NGR234 bacteroids in determinate nodules of *vigna unguiculata* and indeterminate nodules of *leucaena leucocephala*. *PLoS One* 8, e70531.
- Liao, D., Wang, S., Cui, M., Liu, J., Chen, A., Xu, G., 2018. Phytohormones Regulate the Development of Arbuscular Mycorrhizal Symbiosis. *Int. J. Mol. Sci.* 19, 3146.
- Limpens, E., Bisseling, T., 2009. Nod factor signal transduction in the rhizobium–legume symbiosis, in: Emons, A.M.C., Ketelaar, T. (Eds.), *Root Hairs*. Springer, Berlin, Heidelberg, pp. 249–276.
- Lin, J., Li, X., Luo, Z., Mysore, K.S., Wen, J., Xie, F., 2018. NIN interacts with NLPs to mediate nitrate inhibition of nodulation in *medicago truncatula*. *Nat. Plants* 4, 942–952.
- Lira, M.A., Nascimento, L.R.S., Fracetto, G.G.M., 2015. Legume-rhizobia signal exchange: promiscuity and environmental effects. *Front. Microbiol.* 6.
- Liu, C.-W., Breakspear, A., Stacey, N., Findlay, K., Nakashima, J., Ramakrishnan, K., Liu, M., Xie, F., Endre, G., De Carvalho-Niebel, F., Oldroyd, G.E.D., Udvardi, M.K., Fournier, J., Murray, J.D., 2019. A protein complex required for polar growth of rhizobial infection threads. *Nat. Commun.* 10, 2848.
- Liu, C.-W., Murray, J., 2016. The role of flavonoids in nodulation host-range specificity: An update. *Plants* 5, 33.
- Liu, Y., Xu, Z., Chen, L., Xun, W., Shu, X., Chen, Y., Sun, X., Wang, Z., Ren, Y., Shen, Q., Zhang, R., 2024. Root colonization by beneficial rhizobacteria. *FEMS Microbiol. Rev.* 48, fuad066.
- Liu, Y., Yuan, L., 2020. Effects of rhizobia and arbuscular mycorrhizal fungi on nodulation, yield and quality of *medicago sativa*. *Acta Pedol. Sin.* 57, 1292–1298.
- Liu, Y.H., Wang, E.T., Jiao, Y.S., Tian, C.F., Wang, L., Wang, Z.J., Guan, J.J., Singh, R.P., Chen, W.X., Chen, W.F., 2018. Symbiotic characteristics of *Bradyrhizobium diazoefficiens* USDA 110 mutants associated with shrubby sophora (*Sophora flavescens*) and soybean (*Glycine max*). *Microbiol. Res.* 214, 19–27.
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta\text{CT}}$ method. *Methods* 25, 402–408.
- Loh, J., Stacey, G., 2003. Nodulation gene regulation in *Bradyrhizobium*

REFERENCES

- japonicum*: a unique integration of global regulatory circuits. Appl. Environ. Microbiol. 69, 10–17.
- Lohar, D., Stiller, J., Kam, J., Stacey, G., Gresshoff, P.M., 2009. Ethylene insensitivity conferred by a mutated arabidopsis ethylene receptor gene alters nodulation in transgenic *Lotus japonicus*. Ann. Bot. 104, 277–285.
- López-Gutiérrez, J.C., Malcolm, G.M., Koide, R.T., Eissenstat, D.M., 2008. Ectomycorrhizal fungi from alaska and pennsylvania: Adaptation of mycelial respiratory response to temperature? New Phytol. 180, 741–744.
- López-Pedrosa, A., González-Guerrero, M., Valderas, A., Azcón-Aguilar, C., Ferrol, N., 2006. *GintAMT1* encodes a functional high-affinity ammonium transporter that is expressed in the extraradical mycelium of *Glomus intraradices*. Fungal Genet. Biol. 43, 102–110.
- Luna, L., Miralles, I., Andrenelli, M.C., Gispert, M., Pellegrini, S., Vignozzi, N., Solé-Benet, A., 2016. Restoration techniques affect soil organic carbon, glomalin and aggregate stability in degraded soils of a semiarid mediterranean region. Catena 143, 256–264.
- Luo, Z., Lin, J., Zhu, Y., Fu, M., Li, X., Xie, F., 2021. NLP1 reciprocally regulates nitrate inhibition of nodulation through SUNN-CRA2 signaling in *medicago truncatula*. Plant Commun. 2.
- Luthfiana, N., Inamura, N., Tantriani, Sato, T., Saito, K., Oikawa, A., Chen, W., Tawaray, K., 2021. Metabolite profiling of the hyphal exudates of *rhizophagus clarus* and *rhizophagus irregularis* under phosphorus deficiency. Mycorrhiza 31, 403–412.
- Machado, D., Pueppke, S.G., Vinardel, J.M., Ruiz-Sainz, J.E., Krishnan, H.B., 1998. Expression of *nodD1* and *nodD2* in *sinorhizobium meliloti*, a nitrogen-fixing symbiont of soybean and other legumes. Mol. Plant. Microbe Interact. 11, 375–382.
- Magkourilou, E., Bell, C.A., Daniell, T.J., Field, K.J., 2024. The functionality of arbuscular mycorrhizal networks across scales of experimental complexity and ecological relevance. Funct. Ecol. n/a, 1–16.
- Magne, K., Massot, S., Folletti, T., Sauviac, L., Ait-Salem, E., Pires, I., Saad, M.M., Eida, A.A., Bougouffa, S., Jugan, A., Rolli, E., Forquet, R., Puech-Pages, V., Maillet, F., Bernal, G., Gibelin, C., Hirt, H., Gruber, V., Peyraud, R., Vailleau, F., Gourion, B., Ratet, P., 2024. Atypical rhizobia trigger nodulation and pathogenesis on the same legume hosts. Nat. Commun. 15, 9246.
- Maillet F., Poinot V., André O., Puech-Pagès V., Haouy A., Gueunier M., Cromer L., Giraudet D., Formey D., Niebel A., Martinez E.A., Driguez H., Bécard G., Dénarié J., 2011. Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. Nature 469, 58–63.
- Maj, D., Wielbo, J., Marek-Kozaczuk, M., Skorupska, A., 2010. Response to

REFERENCES

- flavonoids as a factor influencing competitiveness and symbiotic activity of *rhizobium leguminosarum*. Microbiol. Res. 165, 50–60.
- Marais, J.P.J., Deavours, B., Dixon, R.A., Ferreira, D., 2006. The stereochemistry of flavonoids, in: Grotewold, E. (Ed.), The Science of Flavonoids. Springer, New York, NY, pp. 1–46.
- Margalef, O., Sardans, J., Fernández-Martínez, M., Molowny-Horas, R., Janssens, I.A., Ciais, P., Goll, D., Richter, A., Obersteiner, M., Asensio, D., Peñuelas, J., 2017. Global patterns of phosphatase activity in natural soils. Sci. Rep. 7, 1337.
- Martin, F.M., Uroz, S., Barker, D.G., 2017. Ancestral alliances: Plant mutualistic symbioses with fungi and bacteria. Science 356.
- Marx, J., 2004. The roots of plant-microbe collaborations. Science 304, 234–236.
- Masson-Boivin, C., Giraud, E., Perret, X., Batut, J., 2009. Establishing nitrogen-fixing symbiosis with legumes: How many rhizobium recipes? Trends Microbiol. 17, 458–466.
- Masson-Boivin, C., Sachs, J.L., 2018. Symbiotic nitrogen fixation by rhizobia - the roots of a success story. Curr. Opin. Plant Biol., Biotic Interactions 44, 7–15.
- Matekwor Ahulu, E., Nakata, M., Nonaka, M., 2005. Arum- and paris-type arbuscular mycorrhizas in a mixed pine forest on sand dune soil in niigata prefecture, central honshu, japan. Mycorrhiza 15, 129–136.
- Mathesius U., 2020. The role of the flavonoid pathway in *medicago truncatula* in root nodule formation. A review, in: de Bruijn F. (Ed.), The Model Legume Medicago truncatula. Wiley, pp. 434–438.
- Mazur, A., Koper, P., 2012. Rhizobial plasmids — replication, structure and biological role. Open Life Sci. 7, 571–586.
- McGonigle, T.P., Miller, M.H., Evans, D.G., Fairchild, G.L., Swan, J.A., 1990. A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytol. 115, 495–501.
- Meixner, C., Ludwig-Müller, J., Miersch, O., Gresshoff, P., Staehelin, C., Vierheilig, H., 2005. Lack of mycorrhizal autoregulation and phytohormonal changes in the supernodulating soybean mutant *nts1007*. Planta 222, 709–715.
- Mendoza-Suárez, M.A., Geddes, B.A., Sánchez-Cañizares, C., Ramírez-González, R.H., Kirchhelle, C., Jorin, B., Poole, P.S., 2020. Optimizing *rhizobium*-legume symbioses by simultaneous measurement of rhizobial competitiveness and N₂ fixation in nodules. Proc. Natl. Acad. Sci. 117, 9822–9831.
- Mikkelsen, B.L., Rosendahl, S., Jakobsen, I., 2008. Underground resource allocation between individual networks of mycorrhizal fungi. New Phytol. 180, 890–898.

REFERENCES

- Miller, R.M., Jastrow, J.D., Reinhardt, D.R., 1995. External hyphal production of vesicular-arbuscular mycorrhizal fungi in pasture and tallgrass prairie communities. *Oecologia* 103, 17–23.
- Miyauchi, S., Kiss, E., Kuo, A., Drula, E., Kohler, A., Sánchez-García, M., Morin, E., Andreopoulos, B., Barry, K.W., Bonito, G., Buée, M., Carver, A., Chen, C., Cichocki, N., Clum, A., Culley, D., Crous, P.W., Fauchery, L., Girlanda, M., Hayes, R.D., Kéri, Z., LaButti, K., Lipzen, A., Lombard, V., Magnuson, J., Maillard, F., Murat, C., Nolan, M., Ohm, R.A., Pangilinan, J., Pereira, M. de F., Perotto, S., Peter, M., Pfister, S., Riley, R., Sitrit, Y., Stielow, J.B., Szöllősi, G., Žifčáková, L., Štursová, M., Spatafora, J.W., Tedersoo, L., Vaario, L.-M., Yamada, A., Yan, M., Wang, P., Xu, J., Bruns, T., Baldrian, P., Vilgalys, R., Dunand, C., Henrissat, B., Grigoriev, I.V., Hibbett, D., Nagy, L.G., Martin, F.M., 2020. Large-scale genome sequencing of mycorrhizal fungi provides insights into the early evolution of symbiotic traits. *Nat. Commun.* 11, 5125.
- Morris, G.M., Huey, R., Lindstrom, W., Sanner, M.F., Belew, R.K., Goodsell, D.S., Olson, A.J., 2009. AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *J. Comput. Chem.* 30, 2785–2791.
- Moscatiello, R., Squartini, A., Mariani, P., Navazio, L., 2010. Flavonoid-induced calcium signalling in rhizobium leguminosarum bv. viciae. *New Phytol.* 188, 814–823.
- Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* 15, 473–497.
- Murray, J.D., Muni, R.R.D., Torres-Jerez, I., Tang, Y., Allen, S., Andriankaja, M., Li, G., Laxmi, A., Cheng, X., Wen, J., Vaughan, D., Schultze, M., Sun, J., Charpentier, M., Oldroyd, G., Tadege, M., Ratet, P., Mysore, K.S., Chen, R., Udvardi, M.K., 2011. Vapyrin, a gene essential for intracellular progression of arbuscular mycorrhizal symbiosis, is also essential for infection by rhizobia in the nodule symbiosis of medicago truncatula. *Plant J.* 65, 244–252.
- Nair, A., and Bhargava, S., 2012. Reduced mycorrhizal colonization (rmc) tomato mutant lacks expression of SymRK signaling pathway genes. *Plant Signal. Behav.* 7, 1578–1583.
- Naoumkina, M., Dixon, R.A., 2008. Subcellular localization of flavonoid natural products: A signaling function? *Plant Signal. Behav.* 3, 573–575.
- Ngwene, B., George, E., Claussen, W., Neumann, E., 2010. Phosphorus uptake by cowpea plants from sparingly available or soluble sources as affected by nitrogen form and arbuscular-mycorrhiza-fungal inoculation. *J. Plant Nutr. Soil Sci.* 173, 353–359.
- Nishida, H., Nosaki, S., Suzuki, T., Ito, M., Miyakawa, T., Nomoto, M., Tada, Y., Miura, K., Tanokura, M., Kawaguchi, M., Suzuki, T., 2021. Different DNA-

REFERENCES

- binding specificities of NLP and NIN transcription factors underlie nitrate-induced control of root nodulation. *Plant Cell* 33, 2340–2359.
- Noë, R., Kiers, E.T., 2018. Mycorrhizal markets, firms, and co-ops. *Trends Ecol. Evol.* 33, 777–789.
- Novak, V., Andeer, P.F., Bowen, B.P., Ding, Y., Zhalnina, K., Hofmockel, K.S., Tomaka, C., Harwood, T.V., Van Winden, M.C.M., Golini, A.N., Kosina, S.M., Northen, T.R., 2024. Reproducible growth of *brachypodium* in EcoFAB 2.0 reveals that nitrogen form and starvation modulate root exudation. *Sci. Adv.* 10, eadg7888.
- Nuccio, E.E., Hodge, A., Pett-Ridge, J., Herman, D.J., Weber, P.K., Firestone, M.K., 2013. An arbuscular mycorrhizal fungus significantly modifies the soil bacterial community and nitrogen cycling during litter decomposition. *Environ. Microbiol.* 15, 1870–1881.
- Oehl, F., de Souza, F.A., Sieverding, E., 2008. Revision of scutellospora and description of five new genera and three new families in the arbuscular mycorrhiza-forming glomeromycetes. *Mycotaxon* 106, 311–360.
- Oehl, F., Sieverding, E., 2004. Pacispora, a new vesicular arbuscular mycorrhizal fungal genus in the glomeromycetes. *J. Appl. Bot.* 78, 72–82.
- Oelmüller, R., 2019. Interplant communication via hyphal networks. *Plant Physiol. Rep.* 24, 463–473.
- Oldroyd, G.E.D., 2013. Speak, friend, and enter: Signalling systems that promote beneficial symbiotic associations in plants. *Nat. Rev. Microbiol.* 11, 252–263.
- Oldroyd, G.E.D., Downie, J.A., 2008. Coordinating nodule morphogenesis with rhizobial infection in legumes. *Annu. Rev. Plant Biol.* 59, 519–546.
- Oldroyd, G.E.D., Long, S.R., 2003. Identification and characterization of nodulation-signaling pathway 2, a gene of *medicago truncatula* involved in nod factor signaling. *Plant Physiol.* 131, 1027–1032.
- Ossler, J.N., Zielinski, C.A., Heath, K.D., 2015. Tripartite mutualism: Facilitation or trade-offs between rhizobial and mycorrhizal symbionts of legume hosts. *Am. J. Bot.* 102, 1332–1341.
- Otto, S., Bruni, E.P., Harms, H., Wick, L.Y., 2017. Catch me if you can: Dispersal and foraging of *bdellovibrio bacteriovorus* 109J along mycelia. *ISME J.* 11, 386–393.
- Owoloye, A.J., Ligali, F.C., Enejoh, O.A., Musa, A.Z., Aina, O., Idowu, E.T., Oyebola, K.M., 2022. Molecular docking, simulation and binding free energy analysis of small molecules as PfHT1 inhibitors. *PLoS One* 17, e0268269.
- Palmero, F., Fernandez, J.A., Garcia, F.O., Haro, R.J., Prasad, P.V.V., Salvagiotti, F., Ciampitti, I.A., 2022. A quantitative review into the contributions of biological nitrogen fixation to agricultural systems by grain legumes. *Eur. J. Agron.* 136, 126514.

REFERENCES

- Pan, B., Smith, D.L., 2000. Preincubation of *B. japonicum* cells with genistein reduces the inhibitory effects of mineral nitrogen on soybean nodulation and nitrogen fixation under field conditions. *Plant Soil* 223, 237–244.
- Panda, S.K., da Silva, L.C.N., 2025. Editorial: Understanding biofilms: recent trends and developments. *Front. Cell. Infect. Microbiol.* 15.
- Panna, D., Highland, K., 2010. Mycorrhizal colonization and distribution of arbuscular mycorrhizal fungi associated with *Michelia champaca* L. under plantation system in northeast India. *J. For. Res.* 21, 137–142.
- Parihar, M., Rakshit, A., Meena, V.S., Gupta, V.K., Rana, K., Choudhary, M., Tiwari, G., Mishra, P.K., Pattanayak, A., Bisht, J.K., Jatav, S.S., Khatri, P., Jatav, H.S., 2020. The potential of arbuscular mycorrhizal fungi in C cycling: A review. *Arch. Microbiol.* 202, 1581–1596.
- Parniske, M., 2018. Uptake of bacteria into living plant cells, the unifying and distinct feature of the nitrogen-fixing root nodule symbiosis. *Curr. Opin. Plant Biol., Biotic Interactions* 44, 164–174.
- Parniske, M., 2008. Arbuscular mycorrhiza: The mother of plant root endosymbioses. *Nat. Rev. Microbiol.* 6, 763–775.
- Parr, M., Griffith, E., Grossman, J., 2017. Drivers of promiscuous soybean associated rhizobia diversity in un-inoculated soil in Malawi. *Symbiosis* 71, 129–141.
- Paszkowski, U., Gutjahr, C., 2013. Multiple control levels of root system remodeling in arbuscular mycorrhizal symbiosis. *Front. Plant Sci.* 4.
- Patten, C.L., Glick, B.R., 1996. Bacterial biosynthesis of indole-3-acetic acid. *Can. J. Microbiol.* 42, 207–220.
- Pawlowski, K., Demchenko, K.N., 2012. The diversity of actinorhizal symbiosis. *Protoplasma* 249, 967–979.
- Peck, M.C., Fisher, R.F., Bliss, R., Long, S.R., 2013. Isolation and characterization of mutant *Sinorhizobium meliloti* NodD1 proteins with altered responses to luteolin. *J. Bacteriol.* 195, 3714–3723.
- Peck M.C., Fisher R.F., Long S.R., 2006. Diverse flavonoids stimulate NodD1 binding to *nod* gene promoters in *Sinorhizobium meliloti*. *J. Bacteriol.* 188, 5417–5427.
- Pereira, S., Mucha, Â., Gonçalves, B., Bacelar, E., Látr, A., Ferreira, H., Oliveira, I., Rosa, E., Marques, G., 2019. Improvement of some growth and yield parameters of faba bean (*Vicia faba*) by inoculation with rhizobium laguerreae and arbuscular mycorrhizal fungi. *Crop Pasture Sci.* 70, 595.
- Pérez-Tienda, J., Testillano, P.S., Balestrini, R., Fiorilli, V., Azcón-Aguilar, C., Ferrol, N., 2011. GintAMT2, a new member of the ammonium transporter family in the arbuscular mycorrhizal fungus *Glomus intraradices*. *Fungal Genet. Biol.* 48, 1044–1055.
- Perret, X., Staehelin, C., Broughton, W.J., 2000. Molecular basis of symbiotic

REFERENCES

- promiscuity. *Microbiol. Mol. Biol. Rev.* 64, 180–201.
- Peters, N.K., Frost, J.W., Long, S.R., 1986. A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. *Science* 233, 977–980.
- Pfeffer, P.E., Douds, D.D., Jr., Bécard, G., Shachar-Hill, Y., 1999. Carbon uptake and the metabolism and transport of lipids in an arbuscular Mycorrhizal. *Plant Physiol.* 120, 587–598.
- Pickles, B.J., Wilhelm, R., Asay, A.K., Hahn, A.S., Simard, S.W., Mohn, W.W., 2017. Transfer of ¹³C between paired douglas-fir seedlings reveals plant kinship effects and uptake of exudates by ectomycorrhizas. *New Phytol.* 214, 400–411.
- Pieterse, C.M.J., Zamioudis, C., Berendsen, R.L., Weller, D.M., Wees, S.C.M.V., Bakker, P.A.H.M., 2014. Induced Systemic Resistance by Beneficial Microbes. *Annu. Rev. Phytopathol.* 52, 347–375.
- Pimprikar, P., Carbonnel, S., Paries, M., Katzer, K., Klingl, V., Bohmer, M.J., Karl, L., Floss, D.S., Harrison, M.J., Parniske, M., Gutjahr, C., 2016. A CCaMK-CYCLOPS-DELLA complex activates transcription of RAM1 to regulate arbuscule branching. *Curr. Biol.* 26, 987–998.
- Pirozynski, K.A., Dalpe, Y., 1989. Geological history of the glomaceae with particular reference to mycorrhizal symbiosis. *Symbiosis* 07, 1–36.
- Pons S., Fournier S., Chervin C., Bécard G., Rochange S., Frei Dit Frey N., Puech Pagès V., 2020. Phytohormone production by the arbuscular mycorrhizal fungus *rhizophagus irregularis*. *PLOS ONE* 15, e0240886.
- Poole, P., 2024. Symbiosis for rhizobia is not an easy ride. *Nat. Microbiol.* 9, 314–315.
- Poole, P., Ramachandran, V., Terpolilli, J., 2018. Rhizobia: From saprophytes to endosymbionts. *Nat. Rev. Microbiol.* 16, 291–303.
- Porcel, R., Aroca, R., Cano, C., Bago, A., Ruiz-Lozano, J.M., 2006. Identification of a gene from the arbuscular mycorrhizal fungus *Glomus intraradices* encoding for a 14-3-3 protein that is up-regulated by drought stress during the AM symbiosis. *Microb. Ecol.* 52, 575–582.
- Pozo, M.J., López-Ráez, J.A., Azcón-Aguilar, C., García-Garrido, J.M., 2015. Phytohormones as integrators of environmental signals in the regulation of mycorrhizal symbioses. *New Phytol.* 205, 1431–1436.
- Priyadharsini, P., Muthukumar, T., 2015. Insight into the role of arbuscular mycorrhizal fungi in sustainable agriculture, in: Thangavel, P., Sridevi, G. (Eds.), *Environmental Sustainability: Role of Green Technologies*. Springer India, New Delhi, pp. 3–37.
- Pueppke, S.G., Broughton, W.J., 1999. *Rhizobium* sp. Strain NGR234 and *R. fredii* USDA257 share exceptionally broad, nested host ranges. *Mol. Plant. Microbe Interact.* 12, 293–318.
- Pumplin, N., Harrison, M.J., 2009. Live-cell imaging reveals periarbuscular

REFERENCES

- membrane domains and organelle location in *medicago truncatula* roots during arbuscular mycorrhizal symbiosis. *Plant Physiol.* 151, 809–819.
- Püschel, D., Janoušková, M., Voříšková, A., Gryndlerová, H., Vosátka, M., Jansa, J., 2017. Arbuscular mycorrhiza stimulates biological nitrogen fixation in two medicago spp. through improved phosphorus acquisition. *Front. Plant Sci.* 8.
- Qiu, X., Wang, W., Yang, J., Li, D., Jiao, J., Wang, E., Yuan, H., 2024. Fulvic Acid Promotes Legume–Rhizobium Symbiosis by Stimulating Endogenous Flavonoids Synthesis and Secretion. *J. Agric. Food Chem.* 72, 6133–6142.
- Rajkumar, M., Ae, N., Prasad, M.N.V., Freitas, H., 2010. Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. *Trends Biotechnol.* 28, 142–149.
- Ramos, A.C., Façanha, A.R., Feijó, J.A., 2008. Ion dynamics during the polarized growth of arbuscular mycorrhizal fungi: From presymbiosis to symbiosis, in: Varma, A. (Ed.), *Mycorrhiza: State of the Art, Genetics and Molecular Biology, Eco-Function, Biotechnology, Eco-Physiology, Structure and Systematics*. Springer, Berlin, Heidelberg, pp. 241–260.
- Ramos, A.C., Lima, P.T., Dias, P.N., Kasuya, M.C.M., Feijó, J.A., 2009. A pH signaling mechanism involved in the spatial distribution of calcium and anion fluxes in ectomycorrhizal roots. *New Phytol.* 181, 448–462.
- Ratu, S.T.N., Teulet, A., Miwa, H., Masuda, S., Nguyen, H.P., Yasuda, M., Sato, S., Kaneko, T., Hayashi, M., Giraud, E., Okazaki, S., 2021. Rhizobia use a pathogenic-like effector to hijack leguminous nodulation signalling. *Sci. Rep.* 11, 2034.
- Rausch, C., Bucher, M., 2002. Molecular mechanisms of phosphate transport in plants. *Planta* 216, 23–37.
- Redecker, D., Kodner, R., Graham, L.E., 2000. Glomalean fungi from the ordovician. *Science* 289, 1920–1921.
- Redmond, J.W., Batley, M., Djordjevic, M.A., Innes, R.W., Kuempel, P.L., Rolfe, B.G., 1986. Flavones induce expression of nodulation genes in rhizobium. *Nature* 323, 632–635.
- Reid, D.E., Ferguson, B.J., Hayashi, S., Lin, Y.-H., Gresshoff, P.M., 2011. Molecular mechanisms controlling legume autoregulation of nodulation. *Ann. Bot.* 108, 789–795.
- Ren, G., 2018. The evolution of determinate and indeterminate nodules within the papilionoideae subfamily. Wageningen University.
- Requena, N., Breuninger, M., Franken, P., Ocón, A., 2003. Symbiotic status, phosphate, and sucrose regulate the expression of two plasma membrane H⁺-ATPase genes from the mycorrhizal fungus *glomus mosseae*. *Plant Physiol.* 132, 1540–1549.
- Requena, N., Serrano, E., Ocón, A., Breuninger, M., 2007. Plant signals and

REFERENCES

- fungal perception during arbuscular mycorrhiza establishment. *Phytochemistry, Molecular Basics of Mycorrhizal Symbiosis* 68, 33–40.
- Richardson, A.E., Hadobas, P.A., 1997. Soil isolates of *Pseudomonas* spp. that utilize inositol phosphates. *Can. J. Microbiol.* 43, 509–516.
- Rillig, M.C., 2004. Arbuscular mycorrhizae and terrestrial ecosystem processes. *Ecol. Lett.* 7, 740–754.
- Rillig, M.C., Lehmann, A., Lanfranco, L., Caruso, T., Johnson, D., 2024. Clarifying the definition of common mycorrhizal networks. *Funct. Ecol.* 1365–2435.14545.
- Rillig, M.C., Ramsey, P.W., Morris, S., Paul, E.A., 2003. Glomalin, an arbuscular-mycorrhizal fungal soil protein, responds to land-use change. *Plant Soil* 253, 293–299.
- Rillig, M.C., Wright, S.F., Nichols, K.A., Schmidt, W.F., Torn, M.S., 2001. Large contribution of arbuscular mycorrhizal fungi to soil carbon pools in tropical forest soils. *Plant Soil* 233, 167–177.
- Rodríguez-Fernández, J.L., Cabañas, L.G., 2013. Chemoattraction: Basic Concepts and Role in the Immune Response, in: *Els. John Wiley & Sons, Ltd.*
- Rolfe, S.A., Griffiths, J., Ton, J., 2019. Crying out for help with root exudates: adaptive mechanisms by which stressed plants assemble health-promoting soil microbiomes. *Curr. Opin. Microbiol., Environmental Microbiology* 49, 73–82.
- Rose, C.M., Venkateshwaran, M., Volkening, J.D., Grimsrud, P.A., Maeda, J., Bailey, D.J., Park, K., Howes-Podoll, M., Os, D. den, Yeun, L.H., Westphall, M.S., Sussman, M.R., Ané, J.-M., Coon, J.J., 2012. Rapid phosphoproteomic and transcriptomic changes in the rhizobia-legume symbiosis *. *Mol. Cell. Proteomics* 11, 724–744.
- Rosner, K., Bodner, G., Hage-Ahmed, K., Steinkellner, S., 2018. Long-term soil tillage and cover cropping affected arbuscular mycorrhizal fungi, nutrient concentrations, and yield in sunflower. *Agron. J.* 110, 2664–2672.
- Rozmoš, M., Bukovská, P., Hřelová, H., Kotianová, M., Dudáš, M., Gančarčíková, K., Jansa, J., 2022. Organic nitrogen utilisation by an arbuscular mycorrhizal fungus is mediated by specific soil bacteria and a protist. *ISME J.* 16, 676–685.
- Rübsam, H., Krönauer, C., Abel, N.B., Ji, H., Lironi, D., Hansen, S.B., Nadzieja, M., Kolte, M.V., Abel, D., de Jong, N., Madsen, L.H., Liu, H., Stougaard, J., Radutoiu, S., Andersen, K.R., 2023. Nanobody-driven signaling reveals the core receptor complex in root nodule symbiosis. *Science* 379, 272–277.
- Saeki, K., 2011. Rhizobial measures to evade host defense strategies and endogenous threats to persistent symbiotic nitrogen fixation: A focus on two legume-rhizobium model systems. *Cell. Mol. Life Sci.* 68, 1327–1339.

REFERENCES

- Saito, K., Yoshikawa, M., Yano, K., Miwa, H., Uchida, H., Asamizu, E., Sato, S., Tabata, S., Imaizumi-Anraku, H., Umehara, Y., Kouchi, H., Murooka, Y., Szczyglowski, K., Downie, J.A., Parniske, M., Hayashi, M., Kawaguchi, M., 2007. NUCLEOPORIN85 is required for calcium spiking, fungal and bacterial symbioses, and seed production in *lotus japonicus*. *Plant Cell* 19, 610–624.
- Sakamoto, K., Ogiwara, N., Kaji, T., Sugimoto, Y., Ueno, M., Sonoda, M., Matsui, A., Ishida, J., Tanaka, M., Totoki, Y., Shinozaki, K., Seki, M., 2019. Transcriptome analysis of soybean (*glycine max*) root genes differentially expressed in rhizobial, arbuscular mycorrhizal, and dual symbiosis. *J. Plant Res.* 132, 541–568.
- Santi, C., Bogusz, D., Franche, C., 2013. Biological nitrogen fixation in non-legume plants. *Ann. Bot.* 111, 743–767.
- Sasaki, T., Suzaki, T., Soyano, T., Kojima, M., Sakakibara, H., Kawaguchi, M., 2014. Shoot-derived cytokinins systemically regulate root nodulation. *Nat. Commun.* 5, 4983.
- Scervino, J.M., Ponce, M.A., Erra-Bassells, R., Bompadre, J., Vierheilig, H., Ocampo, J.A., Godeas, A., 2007. The effect of flavones and flavonols on colonization of tomato plants by arbuscular mycorrhizal fungi of the genera *Gigaspora* and *Glomus*. *Can. J. Microbiol.* 53, 702–709.
- Scervino, J.M., Ponce, M.A., Erra-Bassells, R., Vierheilig, H., Ocampo, J.A., Godeas, A., 2005a. Flavonoids exhibit fungal species and genus specific effects on the presymbiotic growth of *gigaspora* and *glomus*. *Mycol. Res.* 109, 789–794.
- Scervino, J.M., Ponce, M.A., Erra-Bassells, R., Vierheilig, H., Ocampo, J.A., Godeas, A., 2005b. Arbuscular mycorrhizal colonization of tomato by *gigaspora* and *glomus* species in the presence of root flavonoids. *J. Plant Physiol.* 162, 625–633.
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: An open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682.
- Schmieder, S.S., Stanley, C.E., Rzepiela, A., van Swaay, D., Sabotič, J., Nørrelykke, S.F., deMello, A.J., Aebi, M., Künzler, M., 2019. Bidirectional propagation of signals and nutrients in fungal networks via specialized hyphae. *Curr. Biol.* 29, 217–228.e4.
- Schrödinger, LLC, 2015. The PyMOL molecular graphics system, version 1.8.
- Schultze, M., Kondorosi, A., 1998. Regulation of symbiotic root nodule development. *Annu. Rev. Genet.* 32, 33–57.
- Schüßler, A., Martin, H., Cohen, D., Fitz, M., Wipf, D., 2006. Characterization of

REFERENCES

- a carbohydrate transporter from symbiotic glomeromycotan fungi. *Nature* 444, 933–936.
- Schüßler, A., Schwarzott, D., Walker, C., 2001. A new fungal phylum, the *glomeromycota*: Phylogeny and evolution. *Mycol. Res.* 105, 1413–1421.
- Shen, J., Yuan, L., Zhang, J., Li, H., Bai, Z., Chen, X., Zhang, W., Zhang, F., 2011. Phosphorus Dynamics: From Soil to Plant. *Plant Physiol.* 156, 997–1005.
- Shen, S.C., Wang, S.P., Yu, G.Q., Zhu, J.B., 1989. Expression of the nodulation and nitrogen fixation genes in rhizobium meliloti during development. *Genome* 31, 354–360.
- Shi, J., Wang, X., Wang, E., 2023. Mycorrhizal symbiosis in plant growth and stress adaptation: From genes to ecosystems. *Annu. Rev. Plant Biol.* 74, 569–607.
- Shi J., Zhao B., Zheng S., Zhang X., Wang X., Dong W., Xie Q., Wang G., Xiao Y., Chen F., Yu N., Wang E., 2021. A phosphate starvation response-centered network regulates mycorrhizal symbiosis. *Cell* 184, 5527-5540.e18.
- Simard, S.W., Beiler, K.J., Bingham, M.A., Deslippe, J.R., Philip, L.J., Teste, F.P., 2012. Mycorrhizal networks: mechanisms, ecology and modelling. *Fungal Biol. Rev.*, Hyphal networks: mechanisms, modelling and ecology 26, 39–60.
- Simon, A., Bindschedler, S., Job, D., Wick, L.Y., Filippidou, S., Kooli, W.M., Verrecchia, E.P., Junier, P., 2015. Exploiting the fungal highway: development of a novel tool for the *in situ* isolation of bacteria migrating along fungal mycelium. *FEMS Microbiol. Ecol.* 91, fiv116.
- Simon, A., Hervé, V., Al-Dourobi, A., Verrecchia, E., Junier, P., 2017. An in situ inventory of fungi and their associated migrating bacteria in forest soils using fungal highway columns. *FEMS Microbiol. Ecol.* 93, fiw217.
- Sindhu, S., Dahiya, A., Gera, R., Sindhu, S.S., 2020. Mitigation of abiotic stress in legume-nodulating rhizobia for sustainable crop production. *Agric. Res.* 9, 444–459.
- Singh, S., Katzer, K., Lambert, J., Cerri, M., Parniske, M., 2014. CYCLOPS, a DNA-binding transcriptional activator, orchestrates symbiotic root nodule development. *Cell Host Microbe* 15, 139–152.
- Singla, P., Garg, N., 2017. Plant flavonoids: Key players in signaling, establishment, and regulation of rhizobial and mycorrhizal endosymbioses, in: Varma, A., Prasad, R., Tuteja, N. (Eds.), *Mycorrhiza - Function, Diversity, State of the Art*. Springer International Publishing, Cham, pp. 133–176.
- Siqueira, J.O., Sylvia, D.M., Gibson, J., Hubbell, D.H., 1985. Spores, germination, and germ tubes of vesicular–arbuscular mycorrhizal fungi. *Can. J. Microbiol.* 31, 965–972.
- Skorupska, A., Wielbo, J., Kidaj, D., Marek-Kozaczuk, M., 2010. Enhancing rhizobium–legume symbiosis using signaling factors, in: Khan, M.S.,

REFERENCES

- Musarrat, J., Zaidi, A. (Eds.), *Microbes for Legume Improvement*. Springer, Vienna, pp. 27–54.
- Smit, G., Puvanesarajah, V., Carlson, R.W., Barbour, W.M., Stacey, G., 1992. *Bradyrhizobium japonicum* nodD1 can be specifically induced by soybean flavonoids that do not induce the nodYABCSUIJ operon. *J. Biol. Chem.* 267, 310–318.
- Smit, P., Raedts, J., Portyanko, V., Debellé, F., Gough, C., Bisseling, T., Geurts, R., 2005. NSP1 of the GRAS protein family is essential for rhizobial nod factor-induced transcription. *Science* 308, 1789–1791.
- Smith, F.A., Smith, S.E., 1997. Structural diversity in (vesicular)–arbuscular mycorrhizal symbioses. *New Phytol.* 137, 373–388.
- Smith, S.E., 2010. *Mycorrhizal Symbiosis*, 3rd ed. ed, *Mycorrhizal Symbiosis*. Elsevier Science & Technology, San Diego.
- Smith, S.E., Jakobsen, I., Grønlund, M., Smith, F.A., 2011. Roles of arbuscular mycorrhizas in plant phosphorus nutrition: interactions between pathways of phosphorus uptake in arbuscular mycorrhizal roots have important implications for understanding and manipulating plant phosphorus acquisition. *Plant Physiol.* 156, 1050–1057.
- Smith, S.E., Read, D., 2008. 2 - colonization of roots and anatomy of arbuscular mycorrhizas, in: Smith, S.E., Read, D. (Eds.), *Mycorrhizal Symbiosis* (Third Edition). Academic Press, London, pp. 42–90.
- Song, Y.Y., Zeng, R.S., Xu, J.F., Li, J., Shen, X., Yihdego, W.G., 2010. Interplant communication of tomato plants through underground common mycorrhizal networks. *PLoS ONE* 5, e13324.
- Soudzilovskaia, N.A., van Bodegom, P.M., Terrer, C., Zelfde, M. van't, McCallum, I., Luke McCormack, M., Fisher, J.B., Brundrett, M.C., de Sá, N.C., Tedersoo, L., 2019. Global mycorrhizal plant distribution linked to terrestrial carbon stocks. *Nat. Commun.* 10, 5077.
- Souza, T., 2015. AMF's main structures, in: Souza, T. (Ed.), *Handbook of Arbuscular Mycorrhizal Fungi*. Springer International Publishing, Cham, pp. 43–63.
- Spaepen, S., Vanderleyden, J., 2011. Auxin and plant-microbe interactions. *Cold Spring Harb. Perspect. Biol.* 3, a001438.
- Spaepen, S., Vanderleyden, J., Remans, R., 2007. Indole-3-acetic acid in microbial and microorganism-plant signaling. *FEMS Microbiol. Rev.* 31, 425–448.
- Spain, J.L., Sieverding, E., Oehl, F., 2006. *Appendicispora*: A new genus in the arbuscular mycorrhiza-forming glomeromycetes, with a discussion of the genus *archaeospora*. *Mycotaxon* 97, 163.
- Spaink, H.P., 2000. Root nodulation and infection factors produced by rhizobial bacteria. *Annu. Rev. Microbiol.* 54, 257–288.

REFERENCES

- Spini, G., Decorosi, F., Cerboneschi, M., Tegli, S., Mengoni, A., Viti, C., Giovannetti, L., 2016. Effect of the plant flavonoid luteolin on ensifer meliloti 3001 phenotypic responses. *Plant Soil* 399, 159–178.
- Sprent, J.I., 2009. Legume nodulation: A global perspective. John Wiley & Sons.
- St-Arnaud, M., Hamel, C., Vimard, B., Caron, M., Fortin, J.A., 1996. Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *glomus intraradices* in an *in vitro* system in the absence of host roots. *Mycol. Res.* 100, 328–332.
- Steinauer, K., Thakur, M.P., Emilia Hannula, S., Weinhold, A., Uthe, H., van Dam, N.M., Martijn Bezemer, T., 2023. Root exudates and rhizosphere microbiomes jointly determine temporal shifts in plant-soil feedbacks. *Plant Cell Environ.* 46, 1885–1899.
- Stracke, S., Kistner, C., Yoshida, S., Mulder, L., Sato, S., Kaneko, T., Tabata, S., Sandal, N., Stougaard, J., Szczygłowski, K., Parniske, M., 2002. A plant receptor-like kinase required for both bacterial and fungal symbiosis. *Nature* 417, 959–962.
- Strullu-Derrien, C., 2018. Fossil filamentous microorganisms associated with plants in early terrestrial environments. *Curr. Opin. Plant Biol., Biotic Interactions* 44, 122–128.
- Stürmer, S.L., Bever, J.D., Morton, J.B., 2018. Biogeography of arbuscular mycorrhizal fungi (glomeromycota): A phylogenetic perspective on species distribution patterns. *Mycorrhiza* 28, 587–603.
- Subramanian, S., Stacey, G., Yu, O., 2007. Distinct, crucial roles of flavonoids during legume nodulation. *Trends Plant Sci.* 12, 282–285.
- Suominen, L., Luukkainen, R., Roos, C., Lindström, K., 2003. Activation of the *nodA* promoter by the *nodD* genes of *rhizobium galegae* induced by synthetic flavonoids or *galega orientalis* root exudate. *FEMS Microbiol. Lett.* 219, 225–232.
- Szczygłowski, K., Amyot, L., 2003. Symbiosis, inventiveness by recruitment? *Plant Physiol.* 131, 935–940.
- Takeda, N., Maekawa, T., Hayashi, M., 2012. Nuclear-localized and deregulated calcium- and calmodulin-dependent protein kinase activates rhizobial and mycorrhizal responses in *lotus japonicus*. *Plant Cell* 24, 810–822.
- Tamasloukht, M., Séjalon-Delmas, N., Kluever, A., Jauneau, A., Roux, C., Bécard, G., Franken, P., 2003. Root factors induce mitochondrial-related gene expression and fungal respiration during the developmental switch from asymbiosis to presymbiosis in the arbuscular mycorrhizal Fungus *Gigaspora rosea*. *Plant Physiol.* 131, 1468–1478.
- Tate, R., 1995. Soil microbiology (symbiotic nitrogen fixation) new york.
- Tawaraya, K., Hashimoto, K., Wagatsuma, T., 1998. Effect of root exudate fractions from P-deficient and P-sufficient onion plants on root colonisation

REFERENCES

- by the arbuscular mycorrhizal fungus *gigaspora margarita*. *Mycorrhiza* 8, 67–70.
- Taylor, L.P., Grotewold, E., 2005. Flavonoids as developmental regulators. *Curr. Opin. Plant Biol., Physiology and metabolism* 8, 317–323.
- Thomas, V.E., Antony-Babu, S., 2024. Core hyphosphere microbiota of *Fusarium oxysporum* f. sp. *niveum*. *Environ. Microbiome* 19, 14.
- Tian, G., Kong, Q., Lai, L., Ray-Chaudhury, A., Lin, C.G., 2010. Increased expression of cholesterol 24S-hydroxylase results in disruption of glial glutamate transporter EAAT2 association with lipid rafts: A potential role in alzheimer's disease. *J. Neurochem.* 113, 978–989.
- Tirichine, L., Imaizumi-Anraku, H., Yoshida, S., Murakami, Y., Madsen, L.H., Miwa, H., Nakagawa, T., Sandal, N., Albrechtsen, A.S., Kawaguchi, M., Downie, A., Sato, S., Tabata, S., Kouchi, H., Parniske, M., Kawasaki, S., Stougaard, J., 2006. Deregulation of a Ca²⁺/calmodulin-dependent kinase leads to spontaneous nodule development. *Nature* 441, 1153–1156.
- Tisserant, E., Malbreil, M., Kuo, A., Kohler, A., Symeonidi, A., Balestrini, R., Charron, P., Duensing, N., Frei dit Frey, N., Gianinazzi-Pearson, V., Gilbert, L.B., Handa, Y., Herr, J.R., Hijri, M., Koul, R., Kawaguchi, M., Krajinski, F., Lammers, P.J., Masclaux, F.G., Murat, C., Morin, E., Ndikumana, S., Pagni, M., Petitpierre, D., Requena, N., Rosikiewicz, P., Riley, R., Saito, K., San Clemente, H., Shapiro, H., van Tuinen, D., Bécard, G., Bonfante, P., Paszkowski, U., Shachar-Hill, Y.Y., Tuskan, G.A., Young, J.P.W., Sanders, I.R., Henrissat, B., Rensing, S.A., Grigoriev, I.V., Corradi, N., Roux, C., Martin, F., 2013. Genome of an arbuscular mycorrhizal fungus provides insight into the oldest plant symbiosis. *Proc. Natl. Acad. Sci.* 110, 20117–20122.
- Toljander, J.F., Artursson, V., Paul, L.R., Jansson, J.K., Finlay, R.D., 2006. Attachment of different soil bacteria to arbuscular mycorrhizal fungal extraradical hyphae is determined by hyphal vitality and fungal species. *FEMS Microbiol. Lett.* 254, 34–40.
- Toljander, J.F., Lindahl, B.D., Paul, L.R., Elfstrand, M., Finlay, R.D., 2007. Influence of arbuscular mycorrhizal mycelial exudates on soil bacterial growth and community structure: Effects of AM mycelial exudates on soil bacteria. *FEMS Microbiol. Ecol.* 61, 295–304.
- Tominaga, T., Yao, L., Saito, H., Kaminaka, H., 2022. Conserved and Diverse Transcriptional Reprogramming Triggered by the Establishment of Symbioses in Tomato Roots Forming Arum-Type and Paris-Type Arbuscular Mycorrhizae. *Plants* 11, 747.
- Toyota, M., Spencer, D., Sawai-Toyota, S., Jiaqi, W., Zhang, T., Koo, A.J., Howe, G.A., Gilroy, S., 2018. Glutamate triggers long-distance, calcium-based plant defense signaling. *Science* 361, 1112–1115.

REFERENCES

- Treseder, K.K., Allen, M.F., 2000. Mycorrhizal fungi have a potential role in soil carbon storage under elevated CO₂ and nitrogen deposition. *New Phytol.* 147, 189–200.
- Trivedi, P., Leach, J.E., Tringe, S.G., Sa, T., Singh, B.K., 2020. Plant–microbiome interactions: From community assembly to plant health. *Nat. Rev. Microbiol.* 18, 607–621.
- Trott, O., Olson, A.J., 2010. AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* 31, 455–461.
- Tsimilli-Michael, M., Eggenberg, P., Biro, B., Köves-Pechy, K., Vörös, I., Strasser, R.J., 2000. Synergistic and antagonistic effects of arbuscular mycorrhizal fungi and azospirillum and rhizobium nitrogen-fixers on the photosynthetic activity of alfalfa, probed by the polyphasic chlorophyll a fluorescence transient O-J-I-P. *Appl. Soil Ecol.* 15, 169–182.
- Tsugawa, H., Kind, T., Nakabayashi, R., Yukihiro, D., Tanaka, W., Cajka, T., Saito, K., Fiehn, O., Arita, M., 2016. Hydrogen rearrangement rules: Computational MS/MS fragmentation and structure elucidation using MS-FINDER software. *Anal. Chem.* 88, 7946–7958.
- Udvardi, M.K., Day, D.A., 1997. Metabolite transport across symbiotic membranes of legume nodules. *Annu. Rev. Plant Biol.* 48, 493–523.
- van Velzen, R., Holmer, R., Bu, F., Rutten, L., van Zeijl, A., Liu, W., Santuari, L., Cao, Q., Sharma, T., Shen, D., Roswanjaya, Y., Wardhani, T.A.K., Kalhor, M.S., Jansen, J., van den Hoogen, J., Güngör, B., Hartog, M., Hontelez, J., Verver, J., Yang, W.-C., Schijlen, E., Repin, R., Schilthuizen, M., Schranz, M.E., Heidstra, R., Miyata, K., Fedorova, E., Kohlen, W., Bisseling, T., Smit, S., Geurts, R., 2018. Comparative genomics of the nonlegume parasponia reveals insights into evolution of nitrogen-fixing rhizobium symbioses. *Proc. Natl. Acad. Sci.* 115, E4700–E4709.
- Venkateshwaran, M., Jayaraman, D., Chabaud, M., Genre, A., Balloon, A.J., Maeda, J., Forshey, K., den Os, D., Kwiecien, N.W., Coon, J.J., Barker, D.G., Ané, J.-M., 2015. A role for the mevalonate pathway in early plant symbiotic signaling. *Proc. Natl. Acad. Sci.* 112, 9781–9786.
- Verdin, A., Lounès-Hadj Sahraoui, A., Fontaine, J., Grandmougin-Ferjani, A., Durand, R., 2006. Effects of anthracene on development of an arbuscular mycorrhizal fungus and contribution of the symbiotic association to pollutant dissipation. *Mycorrhiza* 16, 397–405.
- Veresoglou, S.D., Verbruggen, E., Makarova, O., Mansour, I., Sen, R., Rillig, M.C., 2019. Arbuscular mycorrhizal fungi alter the community structure of ammonia oxidizers at high fertility via competition for soil NH₄⁺. *Microb. Ecol.* 78, 147–158.
- Vergara, C., Araujo, K.E.C., 2024. Arbuscular mycorrhizal symbiosis: From

REFERENCES

- infection signaling to bidirectional nutrient exchanges, in: Parihar, M., Rakshit, A., Adholeya, A., Chen, Y. (Eds.), *Arbuscular Mycorrhizal Fungi in Sustainable Agriculture: Inoculum Production and Application*. Springer Nature, Singapore, pp. 401–418.
- Vicario, J.C., Dardanelli, M.S., Giordano, W., 2015. Swimming and swarming motility properties of peanut-nodulating rhizobia. *FEMS Microbiol. Lett.* 362, 1–6.
- Vieira, C.K., Marascalchi, M.N., Rozmoš, M., Benada, O., Belova, V., Jansa, J., 2025. Arbuscular mycorrhizal fungal highways – what, how and why? *Soil Biol. Biochem.* 202, 109702.
- Vierheilig, H., 2004. Further root colonization by arbuscular mycorrhizal fungi in already mycorrhizal plants is suppressed after a critical level of root colonization. *J. Plant Physiol.* 161, 339–341.
- Vierheilig, H., Bago, B., Albrecht, C., Poulin, M.-J., Piché, Y., 1998a. Flavonoids and arbuscular-mycorrhizal fungi, in: Manthey, J.A., Buslig, B.S. (Eds.), *Flavonoids in The Living System*. Springer US, Boston, MA, pp. 9–33.
- Vierheilig, H., Bago, B., Albrecht, C., Poulin, M.-J., Piché, Y., 1998b. Plant phenolics: Recent advances on their biosynthesis, genetics, and ecophysiology, in: Manthey, J.A., Buslig, B.S. (Eds.), *Flavonoids in The Living System*. Springer US, Boston, MA, pp. 9–33.
- Vigneron, N., Radhakrishnan, G.V., Delaux, P.-M., 2018. What have we learnt from studying the evolution of the arbuscular mycorrhizal symbiosis? *Curr. Opin. Plant Biol., Biotic Interactions* 44, 49–56.
- Vishwakarma, K., Upadhyay, N., Kumar, N., Verma, R., Singh, J., Verma, P., Mishra, M., Jain, S., Tripathi, D.K., Mishra, R.K., Kumar, V., Sharma, S., 2017. Microbial interactions in *Litchi* rhizosphere, in: Kumar, M., Kumar, V., Bhalla-Sarin, N., Varma, A. (Eds.), *Lychee Disease Management*. Springer, Singapore, pp. 27–44.
- Vives-Peris, V., de Ollas, C., Gómez-Cadenas, A., Pérez-Clemente, R.M., 2020. Root exudates: From plant to rhizosphere and beyond. *Plant Cell Rep.* 39, 3–17.
- Voets, L., De La Providencia, I.E., Declerck, S., 2006. *Glomeraceae* and *Gigasporaceae* differ in their ability to form hyphal networks. *New Phytol.* 172, 185–188.
- Voets, L., De La Providencia, I.E., Fernandez, K., IJdo, M., Cranenbrouck, S., Declerck, S., 2009. Extraradical mycelium network of arbuscular mycorrhizal fungi allows fast colonization of seedlings under *in vitro* conditions. *Mycorrhiza* 19, 347–356.
- Voets, L., Dupré De Boulois, H., Renard, L., Strullu, D.-G., Declerck, S., 2005. Development of an autotrophic culture system for the *in vitro* mycorrhization of potato plantlets. *FEMS Microbiol. Lett.* 248, 111–118.

REFERENCES

- Voets, L., Goubau, I., Olsson, P.A., Merckx, R., Declerck, S., 2008. Absence of carbon transfer between *medicago truncatula* plants linked by a mycorrhizal network, demonstrated in an experimental microcosm: AMF do not transfer carbon between autotrophic plants. *FEMS Microbiol. Ecol.* 65, 350–360.
- Wahbi, S., Maghraoui, T., Hafidi, M., Sanguin, H., Oufdou, K., Prin, Y., Duponnois, R., Galiana, A., 2016. Enhanced transfer of biologically fixed N from faba bean to intercropped wheat through mycorrhizal symbiosis. *Appl. Soil Ecol.* 107, 91–98.
- Walker, C., 2005. A simple blue staining technique for arbuscular mycorrhizal and other root-inhabiting fungi. *Inoculum* 56, 68–69.
- Walker, C., Harper, C.J., Brundrett, M.C., Krings, M., 2018. Chapter 20 - looking for arbuscular mycorrhizal fungi in the fossil record: An illustrated guide, in: Krings, M., Harper, C.J., Cúneo, N.R., Rothwell, G.W. (Eds.), *Transformative Paleobotany*. Academic Press, pp. 481–517.
- Wang, D., Dong, W., Murray, J., Wang, E., 2022. Innovation and appropriation in mycorrhizal and rhizobial symbioses. *Plant Cell* 34, 1573–1599.
- Wang, D., Yang, S., Tang, F., Zhu, H., 2012. Symbiosis specificity in the legume – rhizobial mutualism. *Cell. Microbiol.* 14, 334–342.
- Wang, E., Schornack, S., Marsh, J.F., Gobbato, E., Schwessinger, B., Eastmond, P., Schultze, M., Kamoun, S., Oldroyd, G.E.D., 2012. A common signaling process that promotes mycorrhizal and oomycete colonization of plants. *Curr. Biol.* 22, 2242–2246.
- Wang, F., Kertesz, M.A., Feng, G., 2019. Phosphorus forms affect the hyphosphere bacterial community involved in soil organic phosphorus turnover. *Mycorrhiza* 29, 351–362.
- Wang, F., Shi, N., Jiang, R., Zhang, F., Feng, G., 2016. In situ stable isotope probing of phosphate-solubilizing bacteria in the hyphosphere. *J. Exp. Bot.* 67, 1689–1701.
- Wang, F., Zhang, L., Zhou, J., Rengel, Z., George, T.S., Feng, G., 2022. Exploring the secrets of hyphosphere of arbuscular mycorrhizal fungi: Processes and ecological functions. *Plant Soil* 481, 1–22.
- Wang, G., Sheng, L., Zhao, D., Sheng, J., Wang, X., Liao, H., 2016. Allocation of nitrogen and carbon is regulated by nodulation and mycorrhizal networks in soybean/maize intercropping system. *Front. Plant Sci.* 7.
- Wang, H., Moore, M.J., Soltis, P.S., Bell, C.D., Brockington, S.F., Alexandre, R., Davis, C.C., Latvis, M., Manchester, S.R., Soltis, D.E., 2009. Rosid radiation and the rapid rise of angiosperm-dominated forests. *Proc. Natl. Acad. Sci.* 106, 3853–3858.
- Wang, J., Liao, L., Wang, G., Liu, H., Wu, Y., Liu, G., Zhang, C., 2022. N-induced root exudates mediate the rhizosphere fungal assembly and affect species coexistence. *Sci. Total Environ.* 804, 150148.

REFERENCES

- Wang, X., Fang, L., Beiyuan, J., Cui, Y., Peng, Q., Zhu, S., Wang, M., Zhang, X., 2021. Improvement of alfalfa resistance against cd stress through rhizobia and arbuscular mycorrhiza fungi co-inoculation in cd-contaminated soil. *Environ. Pollut.* 277, 116758.
- Wasson, A.P., Pellerone, F.I., Mathesius, U., 2006. Silencing the flavonoid pathway in *Medicago truncatula* inhibits root nodule formation and prevents auxin transport regulation by rhizobia. *Plant Cell* 18, 1617–1629.
- Westhoek, A., Field, E., Rehling, F., Mulley, G., Webb, I., Poole, P.S., Turnbull, L.A., 2017. Policing the legume-rhizobium symbiosis: A critical test of partner choice. *Sci. Rep.* 7, 1419.
- Wewer, V., Brands, M., Dörmann, P., 2014. Fatty acid synthesis and lipid metabolism in the obligate biotrophic fungus *Rhizophagus irregularis* during mycorrhization of *Lotus japonicus*. *Plant J.* 79, 398–412.
- Winkel-Shirley, B., 2001. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant Physiol.* 126, 485–493.
- Wipf, D., Krajinski, F., Tuinen, D., Recorbet, G., Courty, P., 2019. Trading on the arbuscular mycorrhiza market: From arbuscules to common mycorrhizal networks. *New Phytol.* 223, 1127–1142.
- Wong, J.E.M.M., Nadzieja, M., Madsen, L.H., Bücherl, C.A., Dam, S., Sandal, N.N., Couto, D., Derbyshire, P., Uldum-Berentsen, M., Schroeder, S., Schwämmle, V., Nogueira, F.C.S., Asmussen, M.H., Thirup, S., Radutoiu, S., Blaise, M., Andersen, K.R., Menke, F.L.H., Zipfel, C., Stougaard, J., 2019. A *lotus japonicus* cytoplasmic kinase connects nod factor perception by the NFR5 LysM receptor to nodulation. *Proc. Natl. Acad. Sci.* 116, 14339–14348.
- Wu, Q., Zhang, Y.-C., Zhang, Z.-Z., Srivastava, A., 2017. Underground communication of root hormones by common mycorrhizal network between trifoliate orange and white clover. *Arch. Agron. Soil Sci.* 63, 1187–1197.
- Wu, Z., Chen, H., Pan, Y., Feng, H., Fang, D., Yang, J., Wang, Y., Yang, J., Sahu, S.K., Liu, J., Xing, Y., Wang, X., Liu, M., Luo, X., Gao, P., Li, L., Liu, Z., Yang, H., Liu, X., Xu, X., Liu, H., Wang, E., 2022. Genome of *hippophae rhamnoides* provides insights into a conserved molecular mechanism in actinorhizal and rhizobial symbioses. *New Phytol.* 235, 276–291.
- Xiao, X., Liao, X., Yan, Q., Xie, Y., Chen, J., Liang, G., Chen, M., Xiao, S., Chen, Y., Liu, J., 2023. Arbuscular mycorrhizal fungi improve the growth, water status, and nutrient uptake of *cinnamomum migao* and the soil nutrient stoichiometry under drought stress and recovery. *J. Fungi* 9, 321.
- Xie, Z.P., Staehelin, C., Vierheilig, H., Wiemken, A., Jabbouri, S., Broughton, W.J., Vogeli-Lange, R., Boller, T., 1995. Rhizobial nodulation factors stimulate mycorrhizal colonization of nodulating and nonnodulating soybeans. *Plant Physiol.* 108, 1519–1525.
- Yamato, M., Iwasaki, M., 2002. Morphological types of arbuscular mycorrhizal

REFERENCES

- fungi in roots of understory plants in japanese deciduous broadleaved forests. *Mycorrhiza* 12, 291–296.
- Yan, H., Freschet, G.T., Wang, H., Hogan, J.A., Li, S., Valverde-Barrantes, O.J., Fu, X., Wang, R., Dai, X., Jiang, L., Meng, S., Yang, F., Zhang, M., Kou, L., 2022. Mycorrhizal symbiosis pathway and edaphic fertility frame root economics space among tree species. *New Phytol.* 234, 1639–1653.
- Yano, K., Yoshida, S., Müller, J., Singh, S., Banba, M., Vickers, K., Markmann, K., White, C., Schuller, B., Sato, S., Asamizu, E., Tabata, S., Murooka, Y., Perry, J., Wang, T.L., Kawaguchi, M., Imaizumi-Anraku, H., Hayashi, M., Parniske, M., 2008. CYCLOPS, a mediator of symbiotic intracellular accommodation. *Proc. Natl. Acad. Sci.* 105, 20540–20545.
- Yoneyama, Kaori, Xie, X., Kusumoto, D., Sekimoto, H., Sugimoto, Y., Takeuchi, Y., Yoneyama, Koichi, 2007. Nitrogen deficiency as well as phosphorus deficiency in sorghum promotes the production and exudation of 5-deoxystrigol, the host recognition signal for arbuscular mycorrhizal fungi and root parasites. *Planta* 227, 125–132.
- Young, I.M., Crawford, J.W., 2004. Interactions and self-organization in the soil-microbe complex. *Science* 304, 1634–1637.
- Yu, N., Luo, D., Zhang, X., Liu, Junzhong, Wang, W., Jin, Y., Dong, W., Liu, Jiyun, Liu, H., Yang, W., Zeng, L., Li, Q., He, Z., Oldroyd, G.E.D., Wang, E., 2014. A DELLA protein complex controls the arbuscular mycorrhizal symbiosis in plants. *Cell Res.* 24, 130–133.
- Yuhashi, K.-I., Ichikawa, N., Ezura, H., Akao, S., Minakawa, Y., Nukui, N., Yasuta, T., Minamisawa, K., 2000. Rhizobitoxine production by *bradyrhizobium elkanii* enhances nodulation and competitiveness on *macroptilium atropurpureum*. *Appl. Environ. Microbiol.* 66, 2658–2663.
- Zaynab, M., Sharif, Y., Abbas, S., Afzal, M.Z., Qasim, M., Khalofah, A., Ansari, M.J., Khan, K.A., Tao, L., Li, S., 2021. Saponin toxicity as key player in plant defense against pathogens. *Toxicon* 193, 21–27.
- Zengler, K., Hofmockel, K., Baliga, N.S., Behie, S.W., Bernstein, H.C., Brown, J.B., Dinneny, J.R., Floge, S.A., Forry, S.P., Hess, M., Jackson, S.A., Jansson, C., Lindemann, S.R., Pett-Ridge, J., Maranas, C., Venturelli, O.S., Wallenstein, M.D., Shank, E.A., Northen, T.R., 2019. EcoFABs: Advancing microbiome science through standardized fabricated ecosystems. *Nat. Methods* 16, 567–571.
- Zhalnina, K., Louie, K.B., Hao, Z., Mansoori, N., da Rocha, U.N., Shi, S., Cho, H., Karaoz, U., Loqué, D., Bowen, B.P., Firestone, M.K., Northen, T.R., Brodie, E.L., 2018. Dynamic root exudate chemistry and microbial substrate preferences drive patterns in rhizosphere microbial community assembly. *Nat. Microbiol.* 3, 470–480.
- Zhang, F., Smith, D.L., 1996. Inoculation of soybean (*Glycine max.* (L.) Merr.)

REFERENCES

- with genistein-preincubated *Bradyrhizobium japonicum* or genistein directly applied into soil increases soybean protein and dry matter yield under short season conditions. *Plant Soil* 179, 233–241.
- Zhang, J., Subramanian, S., Stacey, G., Yu, O., 2009. Flavones and flavonols play distinct critical roles during nodulation of *medicago truncatula* by *sinorhizobium meliloti*. *Plant J.* 57, 171–183.
- Zhang, L., Fan, J., Ding, X., He, X., Zhang, F., Feng, G., 2014. Hyphosphere interactions between an arbuscular mycorrhizal fungus and a phosphate solubilizing bacterium promote phytate mineralization in soil. *Soil Biol. Biochem.* 74, 177–183.
- Zhang, L., Feng, G., Declerck, S., 2018a. Signal beyond nutrient, fructose, exuded by an arbuscular mycorrhizal fungus triggers phytate mineralization by a phosphate solubilizing bacterium. *ISME J.* 12, 2339–2351.
- Zhang, L., Shi, N., Fan, J., Wang, F., George, T.S., Feng, G., 2018b. Arbuscular mycorrhizal fungi stimulate organic phosphate mobilization associated with changing bacterial community structure under field conditions. *Environ. Microbiol.* 20, 2639–2651.
- Zhang, L., Zhou, J., George, T.S., Limpens, E., Feng, G., 2022. Arbuscular mycorrhizal fungi conducting the hyphosphere bacterial orchestra. *Trends Plant Sci.* 27, 402–411.
- Zhang, W., Li, X.-G., Sun, K., Tang, M.-J., Xu, F.-J., Zhang, M., Dai, C.-C., 2020. Mycelial network-mediated rhizobial dispersal enhances legume nodulation. *ISME J.* 14, 1015–1029.
- Zhao, Y., Cartabia, A., Lalaymia, I., Declerck, S., 2022. Arbuscular mycorrhizal fungi and production of secondary metabolites in medicinal plants. *Mycorrhiza* 32, 221–256.
- Zhou, J., Chai, X., Zhang, L., George, T.S., Wang, F., Feng, G., 2020. Different arbuscular mycorrhizal fungi cocolonizing on a single plant root system recruit distinct microbiomes. *mSystems* 5, 10.1128/msystems.929-20.
- Zhou, Y., Wang, H., Xu, S., Liu, K., Qi, H., Wang, M., Chen, X., Berg, G., Ma, Z., Cernava, T., Chen, Y., 2022. Bacterial-fungal interactions under agricultural settings: From physical to chemical interactions. *Stress Biol.* 2, 22.
- Zhu, Y., Miller, R.M., 2003. Carbon cycling by arbuscular mycorrhizal fungi in soil–plant systems. *Trends Plant Sci.* 8, 407–409.
- Zsögön, A., Lambais, M.R., Benedito, V.A., Figueira, A.V. de O., Peres, L.E.P., 2008. Reduced arbuscular mycorrhizal colonization in tomato ethylene mutants. *Sci. Agric.* 65, 259–267.
- Zuanazzi, J.A.S., Clergeot, P.H., Quirion, J.-C., Husson, H.-P., Kondorosi, A., Ratet, P., 1998. Production of *sinorhizobium meliloti* *nod* gene activator and repressor flavonoids from *medicago sativa* roots. *Mol. Plant. Microbe Interact.* 11, 784–794.

REFERENCES
