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The mycobiota: fungi take their place between plants and bacteria

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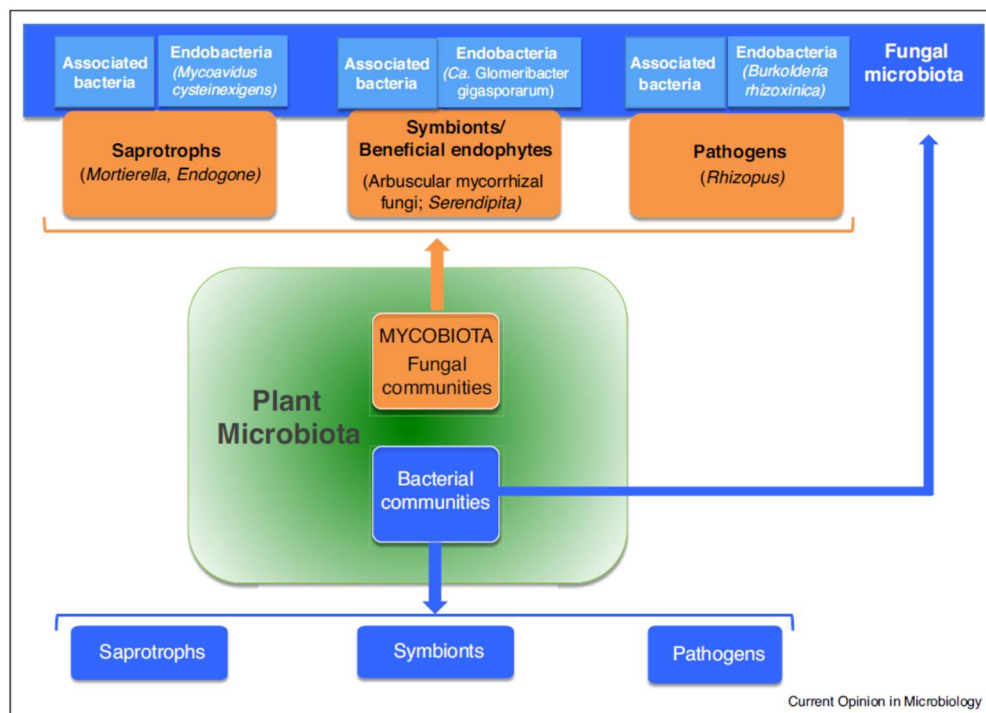
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Eukaryotes host numerous intracellular and associated microbes in their microbiota. Fungi, the so-called Mycobiota, are important members of both human and plant microbiota. Moreover, members of the plant mycobiota host their own microbiota on their surfaces and inside their hyphae. The microbiota of the mycobiota includes mycorrhizal helper bacteria (for mycorrhizal fungi) and fungal endobacteria, which are critical for the fungal host and, as such, likely affect the plant. This review discusses the contribution that these often overlooked members make to the composition and performance of the plant microbiota.

Introduction

The Fungal kingdom encompasses a plethora of eukaryotic species that proliferate in diverse environments; fungi also have important roles as components of the microbiota, where they act as symbionts, endophytes, parasites, or saprotrophs. Characterizing the microbiota of diverse species across kingdoms has revealed an unexpected double nature of the fungi in the microbiome: they colonize higher eukaryotes from humans to plants [1,2]. In the mean time, as with all other eukaryotes, fungi host their own microbiota, consisting of microbial communities that adhere to the hyphal surface, develop among the pseudotissues produced by hyphal aggregation, or colonize the fungal cytoplasm. In this review, we illustrate the double role played by fungi in the microbiota (Figure 1).



The flow chart offers a map to the Mycobiota as a component of the more complex Plant Microbiota. The fungal communities consist of saprotrophic, symbiotic and pathogenic fungi. On their own, these fungi may associate with soil bacteria or host endobacteria, which represent the more intimate form of interaction. These bacterial communities in their whole add a further level of complexity to the microbial component of the Plant Microbiota.

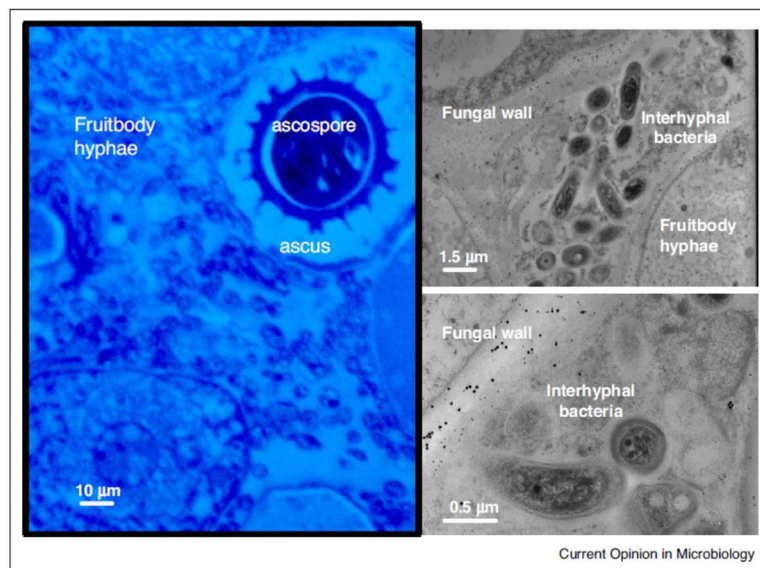
Mycobiota: fungi of the plant microbiota

The knowledge that fungi live strictly associated with plants in diverse niches, particularly in the rhizosphere, dates back more than 100 years [3]. However, only a minor part of the mycobiome is cultivable, in line with what is known about fungal diversity, where only a small portion of the estimated 3.8 million species [4] are in collections (<http://www.wfcc.info/ccinfo/home/>). Most of our knowledge of plant-associated fungi therefore comes from molecular analysis, where the internal transcribed spacer (ITS) of the nuclear rRNA operon is used as the official taxonomic barcode for fungi [5], providing species-level taxonomic delineation for most groups. Emerging ‘omics’ techniques, as well as the concept of the microbiota as an additional plant genome (alongside the nuclear and organellar genomes), offer new views of fungal diversity. The numbers of operational taxonomic units (OTUs) increase with sequencing and sampling depth [6], suggesting that many members of the mycobiota remain to be discovered. Fungi proliferate in different environments (soil, air, water) and with different nutritional strategies (saprotrophic, biotrophic, parasitic), but the highest numbers of fungi are found to be plant-associated and in the soil. These findings suggested a remarkable fungal/plant species ratio of 17/1. Moreover, these approaches revealed a variety of fungal communities associated with myriad plants in diverse environments [7]. Pioneering reports on the plant microbiota focused on identifying *Arabidopsis thaliana* bacterial assemblages [8], but recent papers consider eukaryotic and prokaryotic components of the microbiota. For example, Bergelson *et al.* [9] grew a worldwide panel of *A. thaliana* accessions and found that fungi influence root microbiota structure. Ascomycota and Basidiomycota are more common in leaves than in roots, whereas Mortierellomycota are moderately enriched in the root microbiota. Irrespective of their qualitative differences, the leaf and root microbiotas had similar fungal richness. Moving from identity to functions, Almario *et al.* [10] identified 15 fungal taxa consistently present in the root of *Arabis alpina*, including a Helotiales taxon that colonizes the root endosphere and transfers phosphate to the plant. Similar functions have been described for the endophyte *Colletotrichum tofieldiae* [11] and an endophytic strain of *Fusarium solani* was found to protect against root and foliar pathogens [12]. Emerging work is therefore discovering novel, beneficial members of the mycobiota in addition to long-standing studies on mycorrhizal fungi. Living in association with plants and exploring the soil with their network of extraradical hyphae make mycorrhizal fungi a perfect example of the plant microbiota. Their diversity in the most different environments has been deeply investigated [13]. Notwithstanding some pitfalls and potential biases when applied to fungal communities [7], high-throughput sequencing has shown that fungi are unexpectedly diverse, important members of the plant microbiota. The challenge for the future will be to unravel the complex interactions among fungi and neighboring bacteria, and their effects on host physiology and metabolism [14].

From nutrient transfer to truffles: fungal-associated bacterial communities

Mycobiota-associated bacteria have diverse effects on their interacting fungi and plants, from nutrient transfer to production of aromatic metabolites. Mycorrhiza helper bacteria (MHB) were the first to be acknowledged for their positive effects; identified by Garbaye [15], they interact with ecto- and arbuscular mycorrhizal fungi (AMF), and belong to very diverse taxa including Proteobacteria such as *Pseudomonas* and Oxalobacteraceae, Actinomycetes such as *Streptomyces*, and Firmicutes such as *Bacillus* [16,17]. MHB may enhance mycorrhizal functions, provide nutrients to the fungus and plant, and promote defenses. For example, the fructose exuded by the AMF *Rhizophagus irregularis* stimulates phosphatase expression and secretion in the MHB *Rahnella aquatilis*, thus promoting the mineralization of organic phosphorus (i.e. phytate) into inorganic phosphorus [18]. Even if established in fully artificial conditions, this system reveals an interesting cooperation between AMF

and bacteria. High-throughput sequencing gave a wider description of MHB communities: Iffis *et al.* [19] identified the dominant AMF-associated bacterial OTUs in the roots of plants growing in hydrocarbon-polluted soils. Vik *et al.* [20] explored the bacterial community composition of the ectomycorrhizal roots of *Bistorta vivipara* and concluded that Actinobacteria were significantly more abundant in ectomycorrhizas than in soil. A detailed profile of bacterial communities associated with *Pinus sylvestris* roots colonized by different fungi demonstrated that each ectomycorrhizal root harboured distinct bacterial communities [21]. Other root-associated fungi have their own microbiota. Glancing at a truffle under an electron microscope in the pre-microbiota era was a shocking experience that revealed diverse bacteria proliferating around the aggregating hyphae forming the fruiting body (Figure 2).



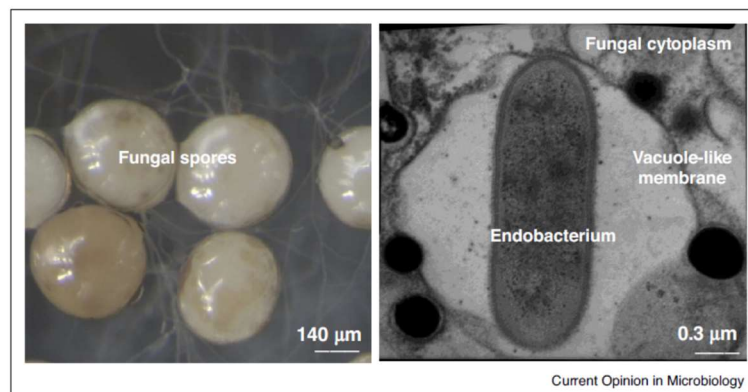
An example of fungal-associated bacteria: on the left, a section from a fruiting body of a truffle, an ectomycorrhizal fungus, is seen under light microscope. The fruiting body consists of aggregating sterile hyphae which surround ascospore-containing asci. When seen at transmission electron microscope (on the right), the inter-hyphal space appears to be filled up by bacterial colonies, very diverse for shape and size. At higher magnification, the bacterial ultrastructure becomes apparent. The fungal wall is labeled by wheat germ agglutinin linked to colloidal gold particles, which detect the chitinous component of the truffle hyphal wall. Pictures by courtesy of Raffaella Balestrini.

The existence of this complex system of bacteria and fungi opened the question of whether we are smelling and tasting bacteria or the precious truffles. Indeed, the *Tuber borchii* bacterial communities are dominated by α -proteobacteria and β -proteobacteria [22], which produce sulphur-containing volatiles such as thiophene derivatives, characteristic of the captivating aroma of truffles. Many other bacterial communities associate with Basidiomycota fruiting bodies, from *Chantarellus* to *Tricholoma* [23,24]; soil is a major source of associated taxa, but the fungal host has a strong effect. Bacteria inhabiting fungal fruiting bodies may be selected based on their metabolic functions and habitat requirements [23]; for example, growth-promoting bacteria such as *Dietzia*, *Ewingella*, *Pseudomonas*, *Paenibacillus*, and *Rhodococcus* could be positively selected for their beneficial effects on fungal growth. Bacterial–fungal interactions (BFIs) occur in many niches and affect biogeochemical cycles, plant and animal health, as well as drug, food, and toxin production (reviewed in Deveau *et al.* [25]). In the fungal microbiota, ‘fungiphiles’ explore soil niches using saprotrophic fungi as substrates [26] while others use mycorrhizal fungi as a highway to reach plant organs [27]. The phyllosphere microbial community is also strongly influenced by the interaction between microorganisms: particular taxa, including fungi, act as ‘hub microbes’ due to their relevance in

shaping the plant microbiota [28]. In another special environment, cheese rinds, *Serratia* isolates disperse on fungal networks by swimming in the liquid layers formed on *Mucor* hyphae [29]. Indeed, by mechanisms including flagella-mediated motility, fungal-associated bacterial dispersal can shift the cheese rind microbiota composition by promoting the growth of motile over non-motile community members.

Life on the inside: endobacteria of fungi

In addition to their surface bacteria, numerous fungi have cytoplasmic endobacteria. They represent the most extreme and specialized type of BFI since, in many cases, these bacteria have lost their capacity to live independently, have experienced a strong genome reduction, and exploit the fungal cytoplasm as a niche to complete their life cycles [25,30,31,32]. Bacteria with this intracellular habit are transmitted by diverse strategies: *Listeria* pathogens in human cells and *Phytoplasma* in plant cells often colonize their host cells by horizontal transmission [33,34]. By contrast, the beneficial bacteria that live in insect tissues and complement the host diet with essential nutrients [35] are often transmitted vertically and have been maintained by co-evolution events. Fungi offer a wide range of examples of endobacteria. Endobacteria have been detected in Ascomycota and Basidiomycota [25], even if their presence seems to be transient, and often only supported by detection of their 16S ribosomal DNA. For these reasons, and because they are cultivable, they are described as facultative endobacteria. A good example is given by the *Rhizobium radiobacter* strain F4 detected inside *Serendipita indica*, but able to induce plant growth like the conventional plant growth-promoting rhizobacteria [36]. In contrast to the Ascomycota and Basidiomycota, the basal group of Mucoromycota [37] contains endobacteria that have been consistently detected and are vertically transmitted. The endobacteria include rod-shaped Beta-proteobacteria (*Burkholderia*-related endobacteria, BRE) and coccoid-shaped Mollicutes (*Mycoplasma*-related endobacteria, MRE). These microbes have been detected in *Gigaspora* (Figure 3), *Diversispora*, *Rhizophagus*, *Geosiphon pyriforme* (Glomeromycotina), *Rhizopus*, *Endogone* (Mucoromycotina), and *Mortierella* (Mortierellomycotina).



An example of fungal endobacteria: on the left, a group of *Gigaspora margarita* spores + during the germination process, as seen under the stereomicroscope $\times 100$. On the right, the endobacterium *Candidatus Glomeribacter gigasporarum* is detected inside a fungal-like vacuole.

Some BRE and MRE genomes have been sequenced (Table 1) [38–44] revealing common features: reduced genomes (BRE: 3.7–1.8 Mbp; MRE: 0.6–1.3 Mbp), loss of biosynthetic capabilities related to primary metabolism, and specialization in fungal metabolite uptake.

Table 1

List of fungal endobacteria, whose genomes have been so far sequenced, together with their fungal host. When compared to the cultivable *Rhizobium (Agrobacterium) radiobacter*, hosted by a Basidiomycete, the genomes of endobacteria hosted by Mucoromycota are reduced, as well as their free-living capacities

Endobacterium	Bacterial taxonomy	Endobacterium genome size (Mb)	Cultivability	Fungal host	Fungal nutritional strategy	Fungal taxonomy	Ref.
<i>Rhizobium (Agrobacterium) radiobacter</i>	Gram - Rhizobiaceae	5.6	Cultivable	<i>Piriformospora indica</i>	Endophyte	Basidiomycota	[36]
<i>Burkholderia rhizoxinica</i>	Gram - Burkholderiaceae	3.75	Limited	<i>Rhizopus microsporus</i>	Opportunistic plant/human pathogen	Mucoromycotina	[44]
<i>Mycoavidus cysteinexigens</i> B1-EB	Gram - Burkholderiaceae	2.79	Limited	<i>Mortierella elongata</i>	Saprotroph	Mortierellomycotina	[50]
<i>M. cysteinexigens</i> AG77	Gram - Burkholderiaceae	2.64	Limited	<i>Mortierella elongata</i>	Saprotroph	Mortierellomycotina	[39]
<i>Candidatus Glomeribacter gigasporarum</i>	Gram - Burkholderiaceae	1.8–2.00	Uncultivable	<i>Gigaspora margarita</i> BEG 34	Obligate symbiont (AMF)	Glomeromycotina	[42]
Mycoplasma-related endobacteria (MRE)	<i>Mycoplasmataceae</i>	2.9 (3 phylotypes)	Uncultivable	<i>Endogone</i> sp. FLAS F-59071	Saprotroph/symbiont	Mucoromycotina	[55]
Mycoplasma-related endobacteria (MRE)	<i>Mycoplasmataceae</i>	1.8 (2 phylotypes)	Uncultivable	<i>Jimgerdemannia lactiflua</i>	Saprotroph/symbiont	Mucoromycotina	[55]
Mycoplasma-related endobacteria (MRE)	<i>Mycoplasmataceae</i>	0.9	Uncultivable	<i>Jimgerdemannia flammicorona</i> AD002	Saprotroph/symbiont	Mucoromycotina	[55]
<i>Candidatus Moenioplasma glomeromycotinum</i> (MRE)	<i>Mycoplasmataceae</i>	0.7–1.3	Uncultivable	<i>Claroideoglossum etunicatum</i>	Obligate symbiont (AMF)	Glomeromycotina	[40]
<i>Candidatus Moenioplasma glomeromycotinum</i> (MRE)	<i>Mycoplasmataceae</i>	0.7–1.3	Uncultivable	<i>Racocetra verrucosa</i>	Obligate symbiont (AMF)	Glomeromycotina	[40]
<i>Candidatus Moenioplasma glomeromycotinum</i> (MRE)	<i>Mycoplasmataceae</i>	0.7–1.3	Uncultivable	<i>Rhizophagus clarus</i>	Obligate symbiont (AMF)	Glomeromycotina	[40]
Mycoplasma-related endobacteria (Dh MRE)	<i>Mycoplasmataceae</i>	0.71	Uncultivable	<i>Denticulata heterogama</i> FL65	Obligate symbiont (AMF)	Glomeromycotina	[41]
Mycoplasma-related endobacteria (De MREI-1)	<i>Mycoplasmataceae</i>	0.63	Uncultivable	<i>Diversispora epigea</i>	Obligate symbiont (AMF)	Glomeromycotina	[38]
Mycoplasma-related endobacteria (De MRE-2)	<i>Mycoplasmataceae</i>	0.61	Uncultivable	<i>Diversispora epigea</i>	Obligate symbiont (AMF)	Glomeromycotina	[38]

Burkholderia rhizoxinica, the BRE of *Rhizopus microsporus*, uses host-derived lipids for energy, but *Mycoavidus cysteinexigens* and *Candidatus Glomeribacter Gigasporarum* (*CaGg*), the BRE of *Mortierella elongata* and *Gigaspora margarita*, respectively, import fungal amino acids and use fungal organic acids for energy. Most BRE have retained secondary metabolite gene clusters and secretion systems. *B. rhizoxinica*, which has limited free-living capacities, relies on a Type II secretion system (TISS) to re-invade fungal hyphae and diffuse horizontally [45]; other endobacteria that are thought to be exclusively vertically transmitted retain TISS and TIISS for unknown functions [39,42]. All known BREs retained several toxin/antitoxin operons in their genomes, but this has not been documented in MREs [46]. At least for *CaGg*, these gene clusters are finely regulated across the life cycle of *G. margarita*: the bacterium overexpresses toxin in the spores and expresses more of the antitoxin during AMF symbiosis. Therefore, endobacteria likely adapted to survive inside their hosts by modulating potentially dangerous activities. Lastly, and remarkably, despite being strongly reduced, MRE genomes contain a number of horizontally transferred genes of fungal origin. The impact of the MRE on the fungal host remains mostly undiscovered, but recent findings indicate that MRE may have adopted a non-lethal parasitic lifestyle in Mortierellomycotina [47]. By contrast, BRE and Mucor-omycota fungi have been more deeply characterized [39,48–50]. *B. rhizoxinica* supports the pathogenic ability of *R. microsporus* by synthesizing a powerful toxin, rhizoxin, which affects rice health [51]. *M. cysteinexigens* has been detected in many *Mortierella* isolates; surprisingly it decreases the growth of its host under laboratory conditions, probably due to lipid depletion. However, it may increase fungal competitiveness through secondary metabolite biosynthesis, including a toxin predicted to have insecticidal activity [52]. Some *Mortierella* species grow on insect exoskeletons; they may have been the ancestral hosts of MRE currently hosted by Mucoromycota [47]. These observations shed light on the origin of this association and on the potential contribution of *M. cysteinexigens* to its host's ecological success, challenging the hypothesis of an exclusively parasitic interaction. *CaGg*, closely related to *M. cysteinexigens*, lives inside the AMF *G. margarita*, where it positively influences pre-symbiotic growth and increases lipid storage [53,54]. Omics and biochemical analyses revealed that *CaGg* leads to higher ATP production and more efficient responses to oxidative stress [49,55,56]. Evidence emerging from these studies suggests that the fungal counterpart can survive without its endobacterium, and not all individuals from the same

species harbor endobacteria. The same is not true for the endobacteria, which likely have strategies for maintenance inside the host population, avoiding transmission bottlenecks and genetic drift [32]. In *B. rhizoxinica* these strategies include horizontal transmission and dispersal through manipulation of host sexuality. BRE and MRE have been useful for exploring the origin of Mucoromycota–endobacteria interactions [38,43,47]. On the basis of examination of endobacteria diversification, which seems to be encompassed by the diversity of their Mucoromycota hosts, Bonfante and Desiro` [30], suggested an invasion event that predates the diversification of Mucoromycota (550–700 MYA). The striking distribution of endobacteria in Mucoromycota may be due to their aseptate mycelium, which could favour diffusion and transmission [30]. Moreover, the Mucoromycota genomes possess up to 3% 6-methyladenine (6 mA) [57], a DNA modification that regulates gene expression and is rather common in bacteria. The shared use of 6 mA may have allowed the endobacteria to manipulate the Mucoromycota genome [32]. Endobacteria are emerging as more widespread than expected in fungal isolates. To better decipher their role, the next step will be to set up metagenomics protocols that detect their presence in natural environments, as suggested by the pioneering results of Bodenhausen *et al.* and Lastovetsky *et al.* [58,59] Indeed, these new results suggest that fungi may act as vectors of bacteria that increase plant microbiota complexity, while their endobacteria operate as multipliers of fungal genetic variability, providing diversity for natural selection.

Mycobiota in the future: discoveries and agricultural applications

Mycobiota and fungal-associated bacterial communities may have applications in agriculture as part of the microbial revolution, the widespread use of beneficial microbes to improve crop yields and prevent diseases. Fungi and their associated bacteria may be used to produce inocula to coat seeds [60], but the development of synthetic microbial consortia represents a new target for high quality crops [61]. Many AMF have already been used in field experiments, leading to remarkable successes [62]. Like some soil bacteria [63], AMF with their endobacteria may represent a source of novel natural products, such as antibiotics and other pharmaceutical compounds. However, new approaches like microfluidics [64] have to be developed to set up the experimental conditions where fungi and bacteria interact leading to the synthesis of these potential new molecules. As is done for the human microbiota [65], it will be crucial to use multiple culture conditions for the plant microbiota, and to push a rebirth of classical microbiological techniques to grow currently uncultivable microbes. Like opening Russian nesting dolls, exploring the complex interactions of plants, their mycobiota, and the microbiota of the mycobiota is vastly expanding our understanding of what it means to be a holobiont.

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