Hyphosphere microbiome of arbuscular mycorrhizal fungi, a realm of unknowns

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Abstract

The extraradical hyphal-associated microbiome of arbuscular mycorrhizal fungi (AMF), the "hyphosphere microbiome," harbors a diverse reservoir of microbes. The biological interactions in the AMF hyphosphere have major implications for soil carbon and nutrient cycling, soil food web dynamics, and plant nutrition and health. Hyphosphere microbial communities are thought to assist AMF in accessing organic nutrients by degrading complex organic compounds that AMF are unable to do by themselves. The AMF, in return, provide an energy-rich microhabitat supplied with hyphal exudates that facilitates microbial growth and mobility in the hyphosphere. However, our current knowledge of hyphosphere entities, their trophic interactions and functional roles, and the underlying mechanisms facilitating microbial co-occurrence and co-operation, are largely incomplete. Here, we review the current state of knowledge on the identity and putative roles of AMF hyphae-associated microbes, with a specific focus on prokaryotes, and potential drivers of such microbial communities in the hyphosphere. Moreover, we discuss the knowledge gaps and open challenges that should be addressed and prioritized in future studies on the AMF microbiome. We also provide an appraisal of available and emerging tools and technologies, and highlight the need for innovative approaches to disentangle hyphosphere processes and answer the many unresolved questions.

Keywords: AM fungi, biological interaction, bacterial and archaeal communities, protists, nutrient and carbon cycling, competition and cooperation

Introduction

Arbuscular mycorrhizal fungi (AMF), from the subphylum Glomeromycotina, are the most widespread plant root symbiotic partners in a wide range of terrestrial ecosystems, interconnecting the plant root system to the soil environment (Brundrett & Tedersoo 2018; Spatafora et al. 2016). The mutualistic symbiotic association between AMF and plants was established more than 400 million years ago and currently exists in more than 70% of all vascular plant species (Brundrett & Tedersoo 2018; Remy et al. 1994; van der Heijden et al. 2015). The fungal partner contributes to plant mineral nutrient uptake, and resistance against multiple biotic (e.g., pathogens) and abiotic (e.g., salinity, drought, heavy metals) stresses (Faghihinia et al. 2020; Gao et al. 2020; Kikuchi et al. 2016; Smith & Smith 2011; Zai et al. 2021). As obligate plant symbionts, AMF fully depend upon photosynthate carbon (C) supply from their host plants to thrive and propagate (Smith & Read 2008). In consequence, AMF also contribute to soil C fluxes and stabilization in terrestrial ecosystems by facilitating the transfer of atmospheric C fixed by the plants into the soil (Jansa & Treseder 2017; van der Heijden et al. 2015).

Of the total AMF biomass, extraradical fungal hyphae represent a significant component, and these mycorrhizal hyphal networks are largely responsible for the lateral C fluxes in soil (Godbold et al. 2006; Kaiser et al. 2015; Talbot et al. 2008; Treseder & Cross 2006). Extraradical AMF hyphae have enormous potential to exploit soil micropores beyond the rhizosphere zone to access macronutrients, particularly phosphorus (P) and nitrogen (N), and micronutrients such as zinc, potassium and copper (Adeyemi et al. 2021; Bukovska et al. 2018; Gao et al. 2021; Jiang et al. 2021; Tamayo et al. 2014; Thirkell et al. 2016), and possibly also facilitate soil-plant water fluxes (Kikuchi et al. 2016; Püschel et al. 2021). However, AMF are unable to efficiently cleave and utilize complex organic compounds due to their limited exo-enzymatic repertoire (but see Koide and Kabir (2000)), suggesting that AMF organic nutrient acquisition is facilitated by other soil microorganisms (Rozmoš et al. 2021; Tisserant et al. 2013). This could explain the existence of the microbial communities associated with the surface of extraradical fungal hyphae, termed the "hyphosphere microbiome" (Artursson et al. 2006; Bonfante & Anca 2009; Jansa & Hodge 2021). Indeed, AMF affect the soil immediately surrounding their hyphae, the hyphosphere, through the exudation of a range of compounds and signaling molecules by the extraradical hyphae (Jiang et al. 2021; Zhang et al. 2018a) and recruit specific microbiota through a mechanism called the "hyphosphere effect" (in parallel to the “rhizosphere effect”) (Bakker et al. 2013).

To date, only limited research efforts have been dedicated to characterizing the hyphosphere communities associated with different AMF species, using compartmentalized microcosms with separate root and hyphal compartments or ingrown mesh cores, and/or manipulating soil conditions (Emmett et al. 2021; Wang et al. 2016; Zhou et al. 2020) (See section 6, Table 1 and Table S1). In addition, the relationships between AMF and the hyphosphere microbiome members, and the outcome of their interactions on organic nutrient utilization and nutrient cycling, were only investigated in a few studies so far using *in vitro* experimental setups and single or multiple bacterial genotypes (Bukovska et al. 2018; Jiang et al. 2021) (See section 6, Table 2). Thus, our current knowledge of the functioning of the hyphospheric microbiome and the underlying mechanisms of interactions between AMF and their associated microbiome is still largely incomplete (Jansa & Hodge 2021; Zhang et al. 2022).

The concept of the AMF microbiome offers a promising perspective to improve our understanding of microbe-AMF interactions, which subsequently influence plant nutrition and health (Artursson et al. 2006; Pivato et al. 2009; Zhang et al. 2022). Moreover, uncovering the link between AMF and hyphosphere microorganisms has significant implications for our understanding of soil nutrient and C cycling, and soil food webs.

This review scrutinizes and critically appraises the current state of knowledge on AMF hyphosphere and provide directions for future research efforts. It also highlights the methodological approaches and emerging tools and techniques that have great potential to contribute to a better understanding of the hyphosphere microbiome functioning. Contrary to the review of the AMF microbiome by Zhang et al. (2022), here the emphasis is on the functional role of microbes in the hyphosphere from a system-level perspective as well as providing a summary of important considerations for experimental design/approaches when studying the AMF hyphosphere microbiome. Particularly, we pay attention to a whole range of multitrophic interactions within the hyphosphere microbiome, not only positive, but also negative (such as competition for ammonium between AM hyphae and nitrification bacteria); in addition to focusing on P and N, we further include processes within the S and Si cycling, and the role of common mycorrhizal networks in soil-plant nutrient cycling, something overlooked in the previous review paper.

Hyphosphere and its functional role

The term "mycorrhizal hyphosphere" has been defined for several decades as the zone of interface between extraradical hyphae of mycorrhizal fungi and the adjacent soil (Andrade et al. 1997; Linderman 1991; Marschner 1995). This zone can further be separated into endo-hyphosphere (inner hyphosphere, i.e. inside the hyphae) and exo-hyphosphere (outer hyphosphere or the hyphosphere corresponding to the definition above). The exo-hyphosphere can further be operationally divided into hyphoplane (hyphal surface) and ecto-hyphosphere (i.e., the hyphosphere soil), although it is difficult to differentiate between them since it is a continuum without a clear demarcation separating them (Fig 1).

The AMF endo-hyphosphere can be colonized by obligate bacterial endosymbionts which are nutritionally dependent on their fungal host, that could confer specific functions such as providing essential nutritional/metabolic factors, and have been reviewed elsewhere (Bonfante et al. 1994; Desiro et al. 2014; Mosse 1970). In this review, we focus on the zones at the surface or in the immediate vicinity of the AMF mycelium. These zones are characterized by a variety of interactions between hyphae, soil minerals, organic and inorganic nutrients, gases, soluble compounds and active microbial communities that participate in various soil biogeochemical cycles. The AMF extraradical hyphae release exudates containing a variety of compounds, including sugars and amino acids, that can be sensed and/or used by soil microbes, mainly bacteria or archaea, and stimulate them to move towards the hyphosphere (Jiang et al. 2021; Luthfiana et al. 2021). Indeed, due to their superior capability to grow into soil micropores and forage for spatially and temporarily heterogeneous nutrient resources, while exuding a variety of

compounds along the way, extraradical hyphae provide a high-energy microhabitat for microbes and can facilitate their dispersal throughout the soil (Jansa & Hodge 2021; Jiang et al. 2021).

Scanning electron microscope images and earlier experimental efforts show that some bacterial groups are able to attach firmly to the surface of the AMF hyphae (Artursson & Jansson 2003; Bianciotto et al. 2001; Bianciotto et al. 1996; Jansa & Hodge 2021; Toljander et al. 2006) and there is also direct observation that some groups of bacteria are able to migrate along the water film-coated hyphae (Jiang et al. 2021). The movement of bacteria along such a "fungal highway" is essential for their ecological competence (i.e., the capacity to fulfill a specific ecosystem function) and competitive success due to many factors that limit their mobility and dispersal in the soil (Jiang et al. 2021; Junier et al. 2021; Otto et al. 2017). The role of fungal highways on bacterial dispersion in air-filled soil micropores have previously been highlighted for other groups of fungi (Deveau et al. 2018; Kohlmeier et al. 2005; Nazir et al. 2014; Otto et al. 2017; Wick et al. 2007). The fungal highway also facilitates contact between prey (e.g., bacteria) and their predators (e.g., protists), shaping the microbial communities and thus also soil food webs (Junier et al. 2021; Otto et al. 2017). These observations were made in some groups of fungi using novel tools such as 3D-printed devices or controlled microcosm systems, but not yet in systems that include mycorrhizal fungi (Abeysinghe et al. 2020; Aleklett et al. 2018; Mafla-Endara et al. 2021).

AMF hyphae-associated microbes have been shown to enable or at least facilitate production of extracellular lytic enzymes (which could be regarded as “public goods”) that degrade soil organic matter to liberate nutrients that the AMF can then obtain to meet their nutritional needs (Rozmoš et al. 2021; Zhang et al. 2018a). Under *in vitro* culture conditions, Zhang et al. (2018a) observed significantly greater phosphatase excretion by hyphosphere bacteria. Furthermore, there was greater expression of the mycorrhizal phosphate transporter gene GintPT and polyP synthesis gene Vtc4p in *Rhizophagus irregularis* in the presence of *Rahnella aquatilis* (phosphate solubilizing bacterium, PSB) compared to *R. irregularis* not in association with *R. aquatilis*. In a series of well-designed experiment, Jiang et al. (2021) observed a significant role of *R. aquatilis* in organic P utilization by the AMF, and that *R. aquatilis* dispersion along the hyphal highway and enhanced metabolism is likely due, at least in part, to AMF hyphal exudates. Wang et al. (2016) found that combined inoculation with PSB (*Pseudomonas alcaligenes M20, Bacillus megaterium C4,* or *Rahnella aquatilis HX2*) and *R. irregularis* resulted in higher phytate-P mineralization and microbial P biomass in the AMF hyphosphere compared to the treatments with *R. irregularis* or with the PSB alone. These observations suggest that AMF rely on their associated bacterial communities for the acquisition of P from organic sources in the soil.

There is also evidence for the potential role of some hyphosphere microbes in facilitating N acquisition from organic sources by the AMF hyphae, in addition to the effects of some soil bacteria on AMF germination and hyphal growth (Gryndler et al. 2000; Hildebrandt et al. 2002; Xavier & Germida 2003). For example, using quantitative real-time PCR, a significant positive correlation was observed between the hyphal proliferation of two AMF species, *R. irregularis* and *Claroideoglomus claroideum*, and the abundance of ammonia-oxidizing bacteria (including *Nitrosospira* sp.) in soil patches containing organic N (Bukovska et al. 2016). In contrast, however, a follow-up study with non-mycorrhizal controls showed that AMF hyphae actually suppressed the abundance of many soil microbes, including nitrification bacteria in a root-free soil that was supplied with organic nutrients or not (Bukovska et al. 2018). More

recently, it has been demonstrated that a substantial amount of (otherwise unavailable) N supplied as chitin could be used up by the AMF hyphae in the presence of *Paenibacillus* sp. in root-free organic N patches (Rozmoš et al. 2021). These findings suggest that, in some cases, the AMF specifically recruit beneficial bacteria for their own nutritional needs, likely providing them with C resources in return, as well as providing them with a microhabitat for convenient movement throughout the soil. The apparent contradictions in results presented here are likely linked to a still far too superficial understanding on AMF-bacteria relationships and interactions, and specifically what cues might be used by the AMF to recruit beneficial bacteria (and/or archaea). Nonetheless, evidence does suggest that AMF can alter the microbe's physicochemical environment via efficient acquisition and exporting of nutrients such as N and/or P from enriched patches and importing fresh C into the microbe's microhabitat (Bukovska et al. 2018; Jiang et al. 2021; Nuccio et al. 2013; Wang et al. 2019; Zhang et al. 2014).

Overall, the AMF hyphosphere provides an energy-rich habitat for microbes, facilitates the dispersal of microbes through soil matrix via water film-coated hyphae, facilitates contact between prey and predators, selectively recruits beneficial bacteria (and/or archaea), and can stimulate bacterial metabolic activities to degrade soil organic matter to liberate P and N that the AMF hyphae can then utilize. The hyphosphere and its microbial community would therefore be playing a greater role in soil processes than its physical size would indicate.

Hyphosphere residents

The hyphosphere microbiome differs from that of the mycorrhizosphere (soil zone under the influence of both roots and fungal components), the rhizosphere (soil zone under the influence of plant root components), and the bulk soil (traditionally defined as root-free soil) (Gahan & Schmalenberger 2015; Veresoglou et al. 2019; Zhang et al. 2018b; Zhou et al. 2020). These observations were made mainly by comparing soil microbial communities in root and hyphal compartments and also extracted AMF hyphae using amplicon sequencing (Emmett et al. 2021; Nuccio et al. 2013; Wang et al. 2016; Zhang et al. 2020; Zhou et al. 2020). To date, only a few studies have attempted to specifically characterize the hyphosphere microbial community (Tables 1 and S1). Based on that research, it has been proposed that "core AMF hyphosphere microbiome" does exist (in spite of lacking consensus as to its exact definition) and that it is AMF species- and soil conditions-independent (Emmett et al. 2021). In addition, microbial community analyses reported so far have provided relative abundances of microbes in destructively obtained samples, which does not necessarily address the absolute abundances of the different microbes and the spatial and temporal complexity of such communities in the hyphosphere unless combined with complementary techniques such as qPCR (Table 3) (Alteio et al. 2021). When relying on amplicon sequencing, inclusion of spike-in standards has the potential to enable absolute quantification (Tourlousse et al. 2017).

Recent compelling evidence suggests that bacteria are the dominant life form in the AMF hyphosphere microbiomes in terms of individuals and biomass (Bukovska et al. 2021). The results also indicate that despite possible differences in the relative abundance of bacteria at lower taxonomic levels

(e.g., species), the composition of the bacterial community may not differ substantially with regard to the dominant phyla detected in the AMF hyphosphere. Despite differences in the relative abundances of the different species present, it appears that the microbiome of the hyphosphere is dominated mainly by four bacterial phyla: Pseudomonadota, Actinomycetota, Gemmatimonadota and Bacteroidota (Tables 1 and S1). The substantial variation in taxonomic composition obvious at lower taxonomic ranks (genus and species levels) may be related to the context-dependence of or functional redundancy within the AMF-microbe interactions.

Although several high-throughput sequencing techniques have been used to identify the prokaryotic microbiota, mainly bacteria, in the AMF hyphosphere, we are not aware of any studies that similarly characterized the community of eukaryotes in the same zone, apart from indirect research comparing mycorrhizal and nonmycorrhizal pots (e.g., Gryndler et al. (2018)). Importantly, the significant role of bacterivores such as protists in the dynamics of bacterial populations should not be overlooked (Bukovska et al. 2018; Koller et al. 2013a; Mafla-Endara et al. 2021; Rozmoš et al. 2021). And of course, other organisms such as animals (e.g., collembolan and nematodes) or fungi (filamentous or not), may fulfil specific functions in the AMF hyphosphere, too (Poveda et al. 2019; Purin & Rillig 2008).

Factors shaping the hyphosphere microbiome

Our current knowledge suggests that interactions in the hyphosphere are regulated by a number of factors, including fungal identity (Agnolucci et al. 2015; Bukovska et al. 2016; Emmett et al. 2021; Zhou et al. 2020), quality of soil organic matter, particularly organic P and soil P level (Gao et al. 2020; Jiang et al. 2021; Wang et al. 2019; Zhang et al. 2014; Zhang et al. 2018b), organic N quality and mineral N availability (Bukovska et al. 2018; Nuccio et al. 2013; Veresoglou et al. 2019), as well as soil physico-chemical properties (Emmett et al. 2021; Svenningsen et al. 2018).

By combining ^{13}C-DNA stable isotope probing (^{13}C-DNA-SIP) with MiSeq sequencing in compartmented mesocosms with split-root systems, Zhou et al. (2020) found distinct active microbial communities associated with different AMF species, *Funneliformis mosseae*, *Gigaspora margarita* and *R. intraradices,* that had simultaneously colonized single cotton plant roots. Greater hyphal density, ^{13}C abundance and bacterial OTUs richness were observed in the hyphal compartments with *F. mosseae* or *R. intraradices* than in those with *Gi. margarita* (Zhou et al. 2020). The authors also found greater relative abundance of *Streptomyces* and *Bacillus* in the hyphosphere of *R. intraradices* and *F. mosseae*, and the greatest abundance of *Pseudomonas* in the hyphosphere of *Gi. margarita* (Zhou et al. 2020). Accordingly, Emmett et al. (2021), using 16S rRNA gene sequence analysis, compared the AMF hyphae-associated microbiome of two AMF species, *R. irregularis* and *Glomus versiforme*, and observed greater enrichment of Gammaproteobacteria and Alphaproteobacteria on extraradical mycelium of *R. irregularis* compared to those associated with *Glomus versiforme*. The evidence that AMF species can, to some extent, determine the composition of microbial communities in their hyphosphere is thus robust, albeit for a still relatively

small number of examples. Furthermore, absolute quantification of microbial taxa within the communities (which are likely very relevant to ecosystem functions they confer) is still largely missing.

Identification of distinct microbial communities associated with hyphae of different AMF species might have been caused by differences in the composition of their hyphal exudates and/or by their different developmental and metabolic traits (Luthfiana et al. 2021). Notably, composition of hyphal exudates can change in response to nutrient availability in the vicinity of the hyphae, which likely influences recruitment of bacteria. Luthfiana et al. (2021) showed that the concentrations of 18 metabolites containing sugars, amino acids, and organic acids were significantly higher in hyphal exudates of *R. clarus* at low P than at high P supply. Conversely, the concentrations of 10 compounds in the hyphal exudates of *R. irregularis* were significantly lower under low P than under high P conditions (Luthfiana et al. 2021). Jiang et al. (2021) found a significant increase in bacterial (PSB) abundance and AMF (*R. irregularis*) hyphal biomass in the presence of organic P compared with treatments without organic P under in vitro culture condition. Thus, it appears plausible that organic P triggers changes in composition of hyphal exudates, hyphal growth/branching, and consequently the composition of the hyphosphere microbiome. The details behind such a process have yet to be revealed.

Further, Nuccio et al. (2013) observed the influence of *G. hoi* on relative abundance of nearly 10% of bacterial taxa inhabiting decomposing litter, suggesting that N acquisition by AMF is one possible mechanism by which AMF alter bacterial populations in an organic patch (i.e., experimentally created organic-rich soil microsite) (Bunn et al. 2019). In a pot experiment system consisting of spatially discrete organic patches containing different organic N forms and *Andropogon gerardii* as host plant, Bukovska et al. (2018) firmly established (using various qPCR assays), a significant suppression of the microbial abundances, particularly ammonia oxidizing bacteria, in the presence of *R. irregularis* hyphae networks. This suppression was attributed to the competition that occurs between AMF and microbes for free ammonium ions. Interestingly, Wang et al. (2019) reported significant changes in the alkaline phosphatase-harbouring bacterial community associated with the *F. mosseae* hyphosphere of the leek root system in response to different forms of P (KH_2PO_4 or phytin) with a higher relative abundance of *Pseudomonas* in phytin treatments compared to KH_2PO_4 and the control treatments. It is worth noting that alkaline phosphatase activity has been shown to be positively associated with the relative abundance of rare microbial taxa (Liu et al. 2021; Wei et al. 2019). Overall, there is good evidence that both synergistic and antagonistic interactions between AMF and microbes are expected to be regulated to some extent by nutrient availability in the hyphosphere or by nutrient status of the hyphae and/or the host plants.

The information discussed above would point to the existence of a degree of specificity between AMF and certain soil bacteria that is context-dependent (Artursson et al. 2005; Cruz-Paredes et al. 2021; de Boer 2017; Scheublin et al. 2010). A number of mutually interacting factors would therefore shape the hyphosphere microbiome and its functional role in the plant-AMF-soil continuum, particularly with respect to the C and nutrient cycling. Direct experimental evidence for this happening across environmental and temporal gradients at different scales, however, is still largely lacking.

Knowns and unknowns

To stimulate beneficial bacteria or other microbes, AMF are assumed to exude various compounds into the hyphosphere to provide C resources that facilitate microbial growth and their metabolic activities (Jiang et al. 2021). Some microbes may intimately associate with hyphae by colonizing the AMF hyphal surface (Scheublin et al. 2010; Toljander et al. 2006), which could give them a competitive advantage for acquiring hyphal exudates. AMF hyphal colonization allows bacteria to take advantage of living in a nutrient- and water-rich environment, which clearly could facilitate their growth, proliferation, and metabolic activities, as well as mobility throughout the soil. Given the availability of resources, a high degree of competition among microorganisms for the available resources in this microhabitat is likely. Considering that AMF influence the hyphosphere microbial colonization particularly through modulating the composition of their hyphal exudates (Luthfiana et al. 2021), a degree of specialization would be expected to exist in the AMF microbiome. AMF may need to recruit different functional groups of microbes to exploit a wide range of organic compounds in the soil and this may have consequences in conferring specific functional services to the plants and other components of ecosystems. However, there is a scarcity of information about functional traits of different microbial taxa in the AMF hyphosphere and the processes that govern their interactions with the AMF. In other words, the fundamental question of "who does what?" remains largely unanswered.

On one hand, there appears to be a spectrum of associations between hyphae and bacteria ranging from tightly attached to non-attached bacteria (Toljander et al. 2006) and the tightness of association and the ability of attachment is different among various bacterial groups (Bonfante & Anca 2009; Scheublin et al. 2010). Further, it remains unclear as to which taxa are just casual opportunists and which are mutualistically dependent on the specific niche provided by the AMF hyphae. On the other hand, at least some of the AMF hyphae-associated microbes have been demonstrated to provide specific services to the AMF, including enhancement of organic nutrient uptake (Rozmoš et al. 2021; Zhang et al. 2016). However, the underlying mechanisms by which AMF select their symbionts and balance their own need for essential nutrients such as N and P with those of their bacterial companions are not well understood. It also remains unclear whether and to what extent bacteria (or other hyphae-associated microbes) provide other benefits to the AMF such as boosting AMF defence mechanisms or inhibiting AMF pathogens.

Using a bacterium without flagella, *Micrococcus luteus*, Jiang et al. (2021) recently claimed that non-motile bacteria were unable (unlike the flagellated *Rahnella aquatilis*) to reach a distant organic P patch even in the presence of AMF hyphae, suggesting that bacterial motility (and particularly the presence of flagella) was required for their migration along the hyphae. However, scanning electron microscopy of AMF hyphae from an unsterile pot experiment revealed a plethora of microbes on the surface of the hyphae coated with liquid water film or mucilaginous substances (Bukovska et al. 2018; Holátko et al. 2021; Jansa & Hodge 2021). There appear to be some groups of bacteria that are immobile on the bumpy, rough surface of the mycorrhizal hyphae. It also is possible that the rough surface of the hyphae could provide various microhabitats that can be inhabited by functionally and/or structurally diverse microbes (Jansa & Hodge 2021). However, it has not yet been possible to unequivocally show that

colonization of hyphal surfaces requires motility traits since bacteria are capable of various means (swimming, gliding, swarming) by which to move along a hydrated surface.

Water film thickness on the surface of the AMF hyphae may be a critically important factor contributing to bacterial movement along the hyphae (Jiang et al. 2021). Thus, the microbe migration and community composition along the hyphae could be influenced by the thickness and temporal dynamics of liquid water films. For example, it is plausible that the tortuosity of fungal highway increases dramatically in dry or water-unsaturated soils. The conditions in which bacteria are able to travel along the hyphae thus need to be explored from both AMF and bacterial perspectives.

In parallel to the interactions between AMF and bacteria, there are also interactions among bacteria themselves and with other soil microbes such as protists and/or saprotrophic fungi in the hyphosphere that may induce positive or negative feedbacks on the AMF microbiome. Zhang et al. (2014) indicated that organic P acquisition by AMF hyphae was influenced by the interactions among phosphate solubilizing bacteria. In addition, free-living bacterial grazers such as protists could alter bacterial community structure via selective feeding on bacteria or serving as their intermittent host and retaining them inside the protist transiently (Amaro & Martín-González 2021; Amaro et al. 2015; Simek et al. 1997). Soil protists may also return some of the N they ingest (~30%) as free ammonium ions back to the soil (Bonkowski 2004), which can be taken up by other soil microorganisms, including AMF, and passed eventually onto plants (Bukovska et al. 2018; Koller et al. 2013b). Rozmoš et al. (2021) showed that the addition of an amoeboid protist, *Polysphondylium pallidum*, to AMF hyphosphere in the presence of *Paenibacillus* sp. significantly enhanced N uptake by AMF and their associated plants from an organic (chitin) source. These findings suggest that the interactions between AMF and their associated bacteria should not be addressed without considering the critical role of bacterivores involved in the complex soil food webs. It is also likely that hyphosphere interactions occur synergistically, where the effect of a hyphosphere microorganism on AMF is enhanced by the presence of another organism from a different functional group (guild). Furthermore, it is possible that bacterial movement along hyphae could be aided by eukaryotes such as protists (Rubinstein et al. 2015), but this still remains to be directly demonstrated (Jansa & Hodge 2021). Uncovering the functional overlaps (redundancy) in the AMF microbiome by assembling communities based on their function (observed growth promotion, enhanced nutrient acquisition, etc.) also deserves further investigation. We have summarized known and hypothesized interactions between AMF hyphae and other microorganisms in the hyphosphere in Fig 2.

Directions for future research

Recent experiments in characterizing the AMF microbiome using high-throughput sequencing techniques has led to the identification of a diversity of bacteria in the hyphosphere (Table 1 and S1). These studies have begun to shed light on the interactions between individuals or communities of AMF and various bacterial and archaeal taxa and have paved the way for more detailed functional studies of various combinations of AMF and microbes (Table 2).

To date, AMF microbiome research has focused almost exclusively on specific AMF-bacterial interactions, and less effort has been made to understand AMF microbiome functioning at the community level. Although focusing on bipartite interactions within a single functional type (e.g., AMF-PSB) could provide insights into the level of mutualism within such combination of microbes, the importance of mutualism at the community level is underestimated because synergism or complementarity usually occurs among functionally distinct partners. Moreover, a network of trophic interactions binds microorganisms together and cross-feeding cannot simply be ignored. Viewing the AMF microbiome from a systems-level perspective requires that we examine and interpret interactions not only between AMF and microbes, but also between different phylogenetic groups and/or functional guilds of microbes (e.g., bacteria, protists, archaea, and fungi). With the exception of the recent study by Rozmoš et al. (2021) on the interactions between bacteria and protists, we are not aware of any research that addresses specifically the interactions between different life forms in the AMF microbiome.

It is also important to acknowledge that capturing the whole system, with its dynamics and stability, resistance and resilience to environmental perturbations, is experimentally challenging mainly due to a plethora of simultaneous interactions. While expecting the occurrence of such interactions in the AMF hyphosphere, it still remains challenging to identify key species or genes and their metabolic interactions due to spatial and temporal (micro)scales and due to significant diversity of the relevant microbiomes. To decipher the complexity of players and interactions, novel *in vitro* and mesocosm experimental setups using state-of-the-art approaches are required to adjust and alter specific microbial strain/guild-related factors such as absence/presence/abundance, and environmental factors including pH and/or nutrient availability.

Despite widely criticized artificiality of the approach, several *in vitro* culture systems have been used in a few recent studies. For example, Jiang et al. (2021) designed a two-compartment Petri plate system to test whether the PSB bacterium, *R. aquatilis* HX2, enhanced the uptake of organic P by *R. irregularis* by migrating along the hyphae to obtain nutrients. In an innovative way, they created an air gap by cutting the solid medium to halt the unspecific migration of bacteria through the medium (Fig. 3-a). The same principle was used to design a three-compartment Petri dish system to test the effects of different levels of organic P on bacterial movement along the hyphae (see Jiang et al. (2021)). However, it is still extraordinarily challenging to identify bacteria that actually use the fungal highway to reach nutrient-rich microsites in natural soil.

Rozmoš et al. (2021) investigated the recycling of organic N via a microbial loop by applying a synthetic approach using ^{15}N-labelled chitin as an organic N source in a compartmented *in vitro* culture system (Fig. 3-b). This study is unique in that it is the first attempt to unravel the biological interactions between AMF, bacteria and protists under controlled settings, as this previously was assumed to have significant implications in nutrient cycling in soil (Koller et al. 2013a). Although these kinds of simplified and artificial experimental setups with low complexity may overlook the heterogeneity of soil microbes, such studies are of particular interest as they contribute to a better understanding of the biological interaction occurring in soil, especially when combined with isotope labelling approaches. In addition, a major strength of the above study is the possibility to establish bacteria-free controls. It is worth mentioning that each inoculum and each open pot culture, including the non-mycorrhizal (mock)

inoculants, contains specific microbiomes that cannot be precisely reconstructed with soil washes (Gryndler et al. 2018). This means that full replicability of microbiome manipulation experiments under open pot settings may be difficult to achieve, unless novel revolutionary methods such as molecular editing tools are applied to genetically manipulate complex microbiomes (Rubin et al. 2022; Tringe 2022).

In spite of that, there have been also some extraordinarily well-designed mesocosm experiments using root-free compartments, delimited from the root-zones by meshes of various sizes (from 25 µm to 50 µm), aiming to characterize the AMF hyphosphere associated microbiome and the factors that shape such a community. For instance, in a series of mesocosm experiments, Emmett et al. (2021) examined the effects of different soils, AMF species, and nutrient levels on the bacterial community in the AMF hyphosphere. The novelty of their experimental setting was that they exposed root-associated AMF networks grown from microbiome-free (or nearly-free) inoculum to a complex bacterial community in nonsterile field soils (Fig 3-c). By doing so they gave the AMF hyphae access to a natural pool of different bacterial species/genotypes and allowed the AMF to preferentially recruit the bacteria from such a complex pool. Thus, using field soil as a microbial starter for the AMF hyphae may reflect the complexity of biotic interactions that take place in natural environments. It would also allow us to ensure that all mycorrhizal and non-mycorrhizal treatments are exposed to an identical and relevant microbial input (rather than sieve-washing and filtrating unsterilized soil inoculum which lead to significant losses and could potentially narrow down the diversity of the microbiome (Ehlers et al. 2008)). In fact, inoculation with open pot-produced AMF inoculant will introduce a lot of microbes with the inoculant itself (e.g., Zhou et al. (2020)) and cannot really be corrected by applying soil washes to the non-mycorrhizal pots (Gryndler et al. 2018).

Another advantage of the study by Emmett et al. (2021) is that not only mycorrhizal and nonmycorrhizal root-free soils were collected and analyzed, but also the AMF hyphal samples. In fact, many studies (Nuccio et al. 2013; Wang et al. 2016; Zhou et al. 2020) to date have characterized the microbial community of the soil sampled from the hyphal compartment but not from the hyphae itself (see Table S1). The community in the soil of the hyphal compartment may not well reflect the community of bacteria that are tightly attached to the hyphal surface. The reason is that the hyphal exudates spread out only a few micrometers, so that the soil from the hyphal compartment as a whole is only slightly affected by the hyphae. However, collecting undisturbed hyphal samples is challenging, and we are not yet fully aware of the effects of washing, agitation and centrifugation on the recovery and intactness of the hyphosphere microbial community. Although it may introduce some confounding effects and may not fully mimic natural soil, a possible and practical approach is to mix the soil with autoclaved sand and/or glass beads (Emmett et al. 2021; Wang et al. 2019; Zhang et al. 2018b; Zhou et al. 2020) to better be able to collect the fragile and highly dispersed AMF hyphae.

Another innovative mesocosm experiment conducted previously by Wang et al. (2016) consisted of hyphal compartments inoculated with various PSB strains (Fig 3-d, Table S1). The originality of that study was based on the combination of complementary techniques including 16S rRNA gene amplicon sequencing, terminal restriction fragment length polymorphism (T-RFLP) analysis and $^{13}CO_2$ pulse labeling. T-RFLP fingerprinting and DNA fractionation into different buoyant density fractions led to recognition of the hyphal associated PSB that were actively involved in translocation/utilization of recent (^{13}C-labeled)

photosynthates to soil. Indeed, stable-isotope probing (SIP) approaches combined with high-throughput sequencing and/or multiple omics approaches would be a powerful means to identify microbes that are metabolically associated with/dependent on the living AMF hyphae, and not blur the observation with microbes that consume dead hyphal walls/biomass (Dumont & Hernández García 2019; Radajewski et al. 2000) (Table S3). The SIP approaches, which are independent of cultivation, have been extensively applied in various systems, including diverse microbial communities in marine environments (Mayali & Weber 2018), soil (Liu et al. 2018; Schwarz et al. 2018) and rhizosphere (Pett-Ridge & Firestone 2017). New developments and refinements in these techniques could be applied to the AMF microbiome to characterize hyphosphere communities, identify isotopically (^{13}C, ^{15}N, or ^{18}O) labeled, partially labeled or unlabeled microbial guilds, estimate their population sizes, analyze their gene expression and metabolic networks, decipher their functional roles and their positions in food webs, besides providing insights into element fluxes and in microbial cross-feeding in the hyphosphere. We have summarized some of the potential applications of SIP-omics approaches in AMF microbiome research in Table S3. We have also provided a summary of possible experimental design considerations in future AMF microbiome research in Table 3.

The microbes on the surface of the AMF hyphae can also be screened by nanometer-scale secondary ion mass spectrometry (NanoSIMS) to trace fluxes of stable (C, N, or O) isotopes at the microbial cell level. This technology has been applied to visualize and quantify ^{15}N and ^{13}C in AMF hyphae (Nuccio et al. 2013) and to visualize C and N utilization on the hyphal surfaces of ectomycorrhizal fungi (Gorka et al. 2019; Kaiser et al. 2015; Mayerhofer et al. 2021). Application of NanoSIMS coupled with SIP-Raman microspectroscopy in AMF microbiome research could also help link microbial species identity with function in the hyphosphere at the single-cell level. Moreover, a combination of NanoSIMS with fluorescent *in situ* hybridization (FISH) approaches (NanoSIMS-FISH) or with gold nanoparticle DNA-hybridization (Kubota et al. 2014) could also provide as yet unexplored options to link identities of hyphosphere microbes with their functions (Musat et al. 2016).

In addition, novel microtechnologies such as microfluidic soil chips mimicking soil environment combined with advanced microspectroscopy techniques such as vibrational-infrared (IR) absorption, Raman scattering and synchrotron radiation based X-ray microspectroscopy offer opportunities to overcome common obstacles in the study of soil microbiomes in physically and chemically controlled microenvironments in real time (Aleklett et al. 2018; Arellano-Caicedo et al. 2021; Mafla-Endara et al. 2021; Pucetaite et al. 2021). These technologies have been recently applied for the studies of microbial interactions in the rhizosphere (Massalha et al. 2017; Noirot-Gros et al. 2020), formation of soil biogeochemical interfaces (Huang et al. 2017), soil microbial dispersal and interactions (Mafla-Endara et al. 2021), and fungal growth and foraging behaviour at the single hyphal scale (Aleklett et al. 2021). In AMF microbiome research, the dynamics of *in situ* hyphosphere-bacteria interactions at the cellular and subcellular resolution could be visualized and monitored by designing a microfluidic imaging platform where microbe(s) are introduced to the hyphosphere and the microbe's behaviour or chemical responses to the defined environmental condition (e.g., nutrient supply, signalling gradients) monitored with minimal system disturbance.

Finally, a key element to consider in future research is how the presence of specific AMF-bacteria associations in the hyphosphere might drive fitness outcomes in AMF and plant species. Deciphering these complex interactions could eventually lead to the development of novel strategies and technologies to harness the full advantage of beneficial bacteria or specific inter-organismal interactions. Such knowledge could be utilized to develop more profitable microbial inoculants or combination of microbes for maintaining plant and soil health, improving agricultural sustainability and increasing crop yield particularly in low-input systems (Bonfante et al. 2019; Jiang et al. 2020; Messa & Savioli 2021; Ray et al. 2020). Enhancing phytoremediation of polluted or contaminated soils could also be envisaged as an additional benefit of improved knowledge of AMF hyphosphere communities. Yet, more experimental studies are needed to validate the interactions which so far have been postulated only theoretically or based on extremely simplified models. Besides, it needs a dedicated research to specifically address the importance of these fine-scale interactions at a full plant or plant community levels, taking into account plant C expenditure to AMF (and its associated microbes) and redistribution of symbiotic benefits and costs among plant individuals involved in common mycorrhizal networks (Walder et al. 2012; Weremijewicz & Janos 2013). It also needs more attention to broaden the scope to other symbiotic fungi, namely the fine root endophytes recruiting from Mucoromycotina, which occupy a very similar ecological niche as the Glomeromycotina (Orchard et al. 2017; Sinanaj et al. 2021).

Concluding remarks

The AMF provide both nutritional and non-nutritional benefits to various microorganisms in their hyphosphere, as they represent a microhabitat coated with a water film, rich in carbon/energy sources to facilitate bacterial growth and translocation over long distances. In return, bacteria enhance the nutrient uptake capacity of AMF by breaking down organic nutrients, which AMF only have a limited capability to do.

The AMF and microbes could engage in both synergistic and antagonistic interactions. Synergistic interactions may include nutrient resource interdependence, facilitation of movement along hyphae, production of molecules that support biofilm formation, and possibly production of volatile organic compounds that enhance microbial coexistence. Antagonistic interactions may include competition for available resources and/or production of suppressive (biocidal) compounds. Synergistic and antagonistic interactions may change with AMF identity and substrate (soil) properties such as nutrient availability.

The results of next generation sequencing of 16S rRNA gene of AMF hyphae extracted from pots have suggested that the hyphosphere microbiome is possibly structured by a selection at higher taxonomic ranks. However, the relative abundance of various microbes at different taxonomic levels may simply reflect differences in their environment and fungal host. Thus, hyphosphere microbial communities should be studied not only at a broad phylum resolution, but more revealingly also at lower taxonomic levels where the full suite of ecological functions should become more apparent. This may eventually reveal that

the current notion of high-rank selection of bacteria within AMF microbiome is merely based on our limited understanding of functional diversity within those high-ranks.

The AMF microbiome research can be advanced through utilizing carefully-designed experimental setups, by collecting actively growing hyphae, providing identical microbial inputs to both mycorrhizal and non-mycorrhizal treatments, using only axenically-produced AMF inoculants, providing field soil as a microbial starter for the AMF hyphae, considering the temporal dynamic of hyphosphere microbial community by collecting hyphal samples at multiple time points and viewing the AMF microbiome from a systems-level perspective through studying both eukaryotes and prokaryotes in the hyphosphere. In addition, integrating a range of complementary approaches including omics-based technologies, SIP and isotope-enabled imaging tools, could provide exciting perspectives to elucidate the roles of individuals, populations, genes, proteins, and metabolites in the AMF microbiome. This would also advance our understanding of the fate of C in AMF hyphal exudates and other elemental fluxes in the hyphosphere.

A key element of future research in the AMF microbiome should be a specific focus on better understanding of the functional processes underlying microbial interactions in the hyphosphere in order to facilitate development of optimal combinations of microorganisms that could be used as efficient and competent soil inoculants in sustainable agriculture. The AMF-bacteria interactions appear to be of paramount importance in low-input agricultural systems where biological mechanisms rather than chemical fertilizers are sustaining soil quality and plant production. Thus, increasing our understanding of fundamental aspects of AMF hyphosphere ecology and underlying mechanisms appears critical to securing sustainable food production for the future.

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Author contribution:

Faghihinia and Jansa conceived and developed the study. Faghihinia drafted the paper. Faghihinia, Jansa, Halverson and Staddon contributed to revisions and all approved the final version.

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Table 1- Prokaryotic organisms detected in arbuscular mycorrhizal fungal (AMF) hyphosphere with either positive or negative response to the presence of AMF using next-generation sequencing tools, under various experimental setups and using diverse soils and biological materials, reported in previously published research (referred to with numbers). The number of reported significant positive and negative responses of the different taxa to the presence of AMF hyphae in the different studies is indicated by blue and red arrows, respectively. Positive and negative responses are when the relative abundance of bacteria increases and decreases, respectively, in response to the presence of AMF hyphae. Background color represents overall positive response in blue (blue arrows > red arrows), negative response in red (red arrows > blue arrows) and neutral response in purple (red arrows = blue arrows). White background and empty cells mean that no data is currently available for that particular taxonomic level of the relevant organism(s). The table shows the substantial variation in AMF-bacteria interactions particularly at lower taxonomic levels.

Phylum	Class	Order	Family	Genera
Pseudomonadota[1,2,3] ↑↑↑	Alphaproteobacteria[4,5] ↑↑↑↓	Rhizobiales	Rhizobiaceae	*Sinorhizobium*[3] ↑
			Bradyrhizobiaceae[6] ↑	*Bradyrhizobium*[3] ↓
		Hyphomicrobiales	Methylobacteriaceae[6] ↑	*Microvirga*[1] ↑↑
		Sphingomonadales[4] ↓		
	Betaproteobacteria[4,5] ↑↓	Burkholderiales	Comamonadaceae	*Ramlibacter*[1] ↑↑
				Variovorax[3] ↓
			Oxalobacteraceae[6] ↑	
	Gammaproteobacteria[4] ↑	Pseudomonadales	Pseudomonadaceae[6] ↑	*Pseudomonas*[1,2] ↑↑
		Enterobacterales	Enterobacteriaceae	*Citrobacter*[1] ↑
		Cellvibrionales	Cellvibrionaceae	*Cellvibrio*[2] ↓
	Deltaproteobacteria[5] ↑	Myxococcales[4] ↑		
Actinomycetota[1,5] ↑↑↑↓	Actinomycetes	Actinomycetales	Streptomycetaceae[6] ↑	*Streptomyces*[1,3] ↑↑↓
			Nocardiaceae	*Rhodococcus*[1] ↑↑
	Actinobacteria	Micromonosporales	Micromonosporaceae	*Micromonospora*[1] ↑
		Corynebacteriales	Corynebacteriaceae	*Corynebacterium*[1] ↑
		Micrococcales[4] ↓	Micrococcaceae[6] ↑	
	Rubrobacteria	Gaiellales[4] ↓		
Gemmatimonadota[1,5] ↑↑↑↑↓	Gemmatimonadetes	Gemmatimonadales[4] ↓	Unclassified-f-Gemmatimonadaceae[1] ↑↑	
Bacillota[2,3,6] ↑↓↓	Bacilli[5] ↑	Bacillales	Bacillaceae	*Bacillus*[1] ↑
			Paenibacillaceae	*Brevibacillus*[1] ↑
				Paenibacillus[1] ↑↑
	Clostridia[5] ↑	Clostridiales[2] ↓		
Bacteroidota[2,3,5] ↑↓↓	Cytophagia	Cytophagales[4] ↑		

	Chitinophagia	Chitinophagales[4]↓	Chitinophagaceae	*Flavisolibacter*[2]↑
Chloroflexota[3]↓				
Fibrobacterota	Fibrobacteria	Fibrobacterales[4]↑		
Verrucomicrobiota	Spartobacteria	Chthoniobacterales[4]↓		
Acidobacteriota[1,3,5]↑↑↓↓				
Planctomycetota[5]↑	Planctomycetacia	Planctomycetales	Planctomycetaceae	*Singulisphaera*[1]↑
Cyanobacteriota[1,2,5,6]↑↑↓↓↓	Cyanophyceae	Synechococcales	Leptolyngbyaceae	*Leptolyngbya*[2]↓
Fusobacteriota	Fusobacteria	Fusobacteriales	Fusobacteriaceae	*Cetobacterium*[1]↑

Note: The summaries of the data in this table are from six peer-reviewed publications based on three main criteria: 1) inclusion of the hyphal compartment in the experimental design 2) reporting of bacterial abundance both in the presence and absence of AMF 3) application of 16S rRNA gene sequencing to identify hyphosphere bacteria. References: 1: Zhou et al. (2020); 2: Wang et al. (2019); 3: Zhang et al. (2018b); 4: Emmett et al. (2021); 5: Nuccio et al. (2013); 6: Wang et al. (2016).

Table 2- Studies documenting possible ecosystem functions of selected AMF hyphosphere microbes

Ecosystem function	Bacteria	AMF species	Plant species	Substrate	Experimental set up	Result	Reference
• N cycling • Biodiversity • Trophic interactions	*Paenibacillus chitinolyticus* CCM 4527 *Paenibacillus ehimensis* CCM 4526	*R. irregularis* SYM5	*Cichorium intybus* L. (Ri T-DNA transformed chicory roots)	Modified MSR medium filled with ^{15}N labelled sources	Compartmented *in vitro* cultivation system using monoxenic culture of *Rhizophagus irregularis*	Release of N from chitin, enabling subsequent N acquisition by the AMF. Including a protist *Polysphondylium pallidum* further increased AMF N gain.	Rozmoš et al. (2021)
• P cycling • Trophic interactions	*R. aquatilis* HX2 (PSB)	*R.irregularis* MUCL 43194	*Daucus carota* L. (Ri T-DNA transformed carrot roots) and *Zea mays* L., cv Xianyu 335 (Maize)	Mesocosms soil (pH:6.4, mineral N:7.2 mg/kg, Olsen-P: 4.9/kg)	A series of experiments: two-compartmented petri plates, one *in vitro* culture and one mesocosm	Enhancement of organic P mineralization by the AMF both under in vitro culture and soil conditions	Jiang et al. (2021)
• P cycling • Trophic interactions	*R. aquatilis* HX2 (PSB)	*R. irregularis* MUCL 43194	*Daucus carota* L. (Ri T-DNA transformed carrot roots)	Modified MSR medium	*In vitro* culture in two-compartmented petri plates	Release of P and enhancement of P acquisition by AMF, which is further increased by fructose exuded by AMF.	Zhang et al. (2018a)
• N cycling • Microbial competitions	Bacteria (ammonia oxidizers), fungi, and protists (*Acanthamoeba* sp.)	*R. irregularis* BEG 158	*Andropogon gerardii* Witman	Alkaline soil contains sand and zeolite (pH: 7.88, total P: 797 mg/kg, total N: 0.13%)	Compartmented pot experiment with different spatially distributed organic patches	Suppression of microbial activity, particularly that of the nitrifiers, and of microbially-mediated soil N losses in presence of AMF hyphae	Bukovska et al. (2018) and (Bukovska et al. 2021)
• P cycling	*Pseudomonas alcaligenes* (PSB)	*R. irregularis* BEG 141	*Medicago sativa* L. cv. Aohan	A moderately acidic soil (pH:6.4, mineral N:7.2 mg/kg, Olsen-P: 4.9 mg/kg)	Compartmented pot experiment	Facilitation of soil phytate mineralization	Zhang et al. (2014)

Table 3- A summary of possible experimental design considerations in the study of the AMF hyphosphere microbiome.

Considerations in future experimental design	Justification
Collecting only hyphal samples (e.g. Emmett et al. (2021) and Gahan and Schmalenberger (2015)) and NOT "only mycorrhizal and nonmycorrhizal root-free soils	The hyphal exudates reach only a few micrometers, so that the soil from the root-free compartment is only slightly affected by the hyphae.
Collecting only actively growing hyphae to identify the microbial hyphosphere community and NOT extracting hyphal samples including dead hyphal wall/biomass	To identify microbes that are metabolically associated with/dependent on the living hyphae, and not blur the vision with microbes that consume dead hyphal walls/biomass
Using only axenically produced AMF inoculants	Every inoculant, including the non-mycorrhizal (mock) inoculants, contains specific microbiomes which are impossible to be precisely reconstructed with soil washes or otherwise (Gryndler et al. 2018).
Ensuring identical microbial inputs into all treatments, e.g. mycorrhizal and nonmycorrhizal pots	To achieve comparability of treatments without confounding the effects of different microbial inputs (avoid comparing live and autoclaved inoculum).
Provide field soil as a microbial starter for the mycorrhizal hyphae to make a choice of the microbial companions (e.g. Emmett et al. (2021))	To create conditions under which the natural community of soil microbes is exposed to hyphae (and not the roots to prevent mycorrhizal formation in non-mycorrhizal treatments) and better reflect the complexity of biotic interactions in natural soil communities.
Consider the temporal dynamic of hyphosphere microbial community by collecting hyphal samples at multiple time points (e.g. Emmett et al. (2021))	To obtain more than a single snapshot of microbial community in the hyphosphere and also to understand whether the temporal changes in bacterial abundances are related to/caused by changes in the composition of hyphal exudates or whether they occur as a result/effect of increases in the abundance of their more vigorous competitors or bacterial grazers.
Viewing the AMF microbiome from a systems-level perspective that includes multifaceted interactions (metabolic/social networks) and characterizing the community of eukaryotes in the hyphosphere	Eukaryotes influence the abundance and dynamics of prokaryotes and thus nutrient availability in the hyphosphere (e.g., protists feed on bacteria and return some of the N they ingest back to the soil thus improving its availability for the AMF, e.g. Rozmoš et al. (2021))
Combining next generation sequencing technologies with complementary techniques such as quantitative PCR (or spiked-in DNA standards)	To address spatial and temporal complexity and abundance dynamics of microbial communities in the hyphosphere
Applying SIP-OMICs to relate element fluxes to microbial taxa (more details in Table S3)	To directly trace key players in C, O and N transformation/utilization in the hyphosphere

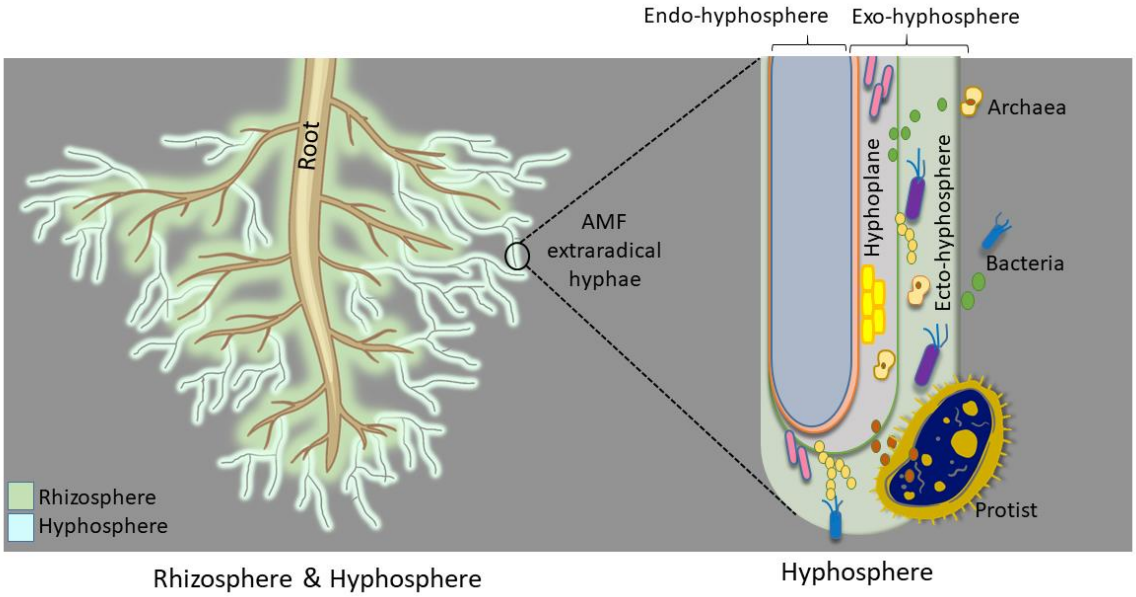

Fig 1. A schematic representation of AMF hyphosphere, which could operationally be separated into endo-hyphosphere (inner hyphosphere, intracellular space) and exo-hyphosphere (outer or extracellular hyphosphere). Exo-hyphosphere can further be divided into hyphoplane (hyphal surface) and ecto-hyphosphere (hyphosphere soil).

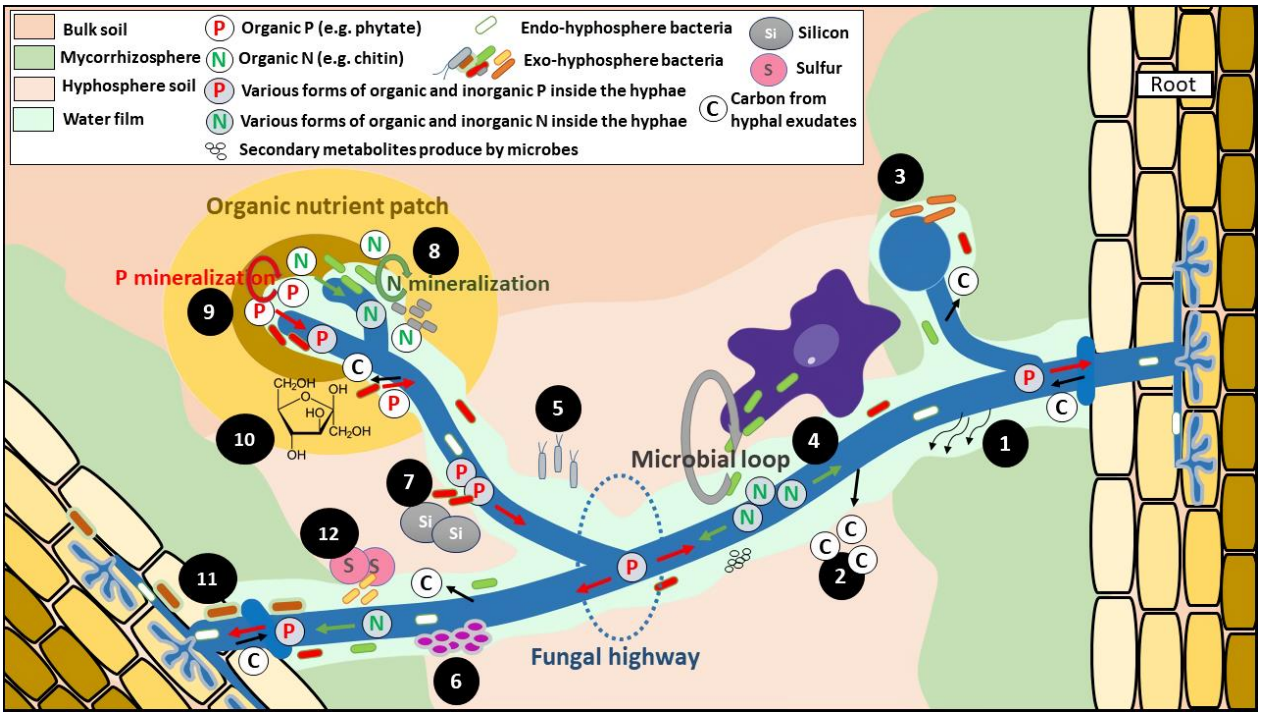

Fig 2. Known and hypothesized interactions between AMF hyphae and other microorganisms in their hyphosphere. Numbers refer to different processes taking place within AMF hyphosphere microbiome;

(1) AMF hyphae are the main and rapid pathway for the transfer of plant C to soil microbes (Kaiser et al. 2015). AMF stimulate a range of beneficial soil microbiota (mainly bacteria) through their hyphal exudation of a variety of molecules such as fructose, glucose, trehalose, etc. (Jiang et al. 2021; Wang et al. 2016). Therefore, different fungal genotypes are thought to associate with different microbial communities due to inherent differences in hyphal exudation patterns.

(2) Plant-derived C released by AMF leads to selection of certain bacteria, e.g., *Bradyrhizobium*, *Pseudomonas*, and *Burkholderia* (Drigo et al. 2010). Transmission of organic C from the AMF to surrounding bacteria is likely to be selective (e.g., AMF-mediated C transfer preferentially to *Opitutus* spp. *Mucilanginibacter, Ohktaekwangia,* and *Massilia* spp (Hünninghaus et al. 2019)).

(3) Possible role of some bacteria in promoting AMF spore germination and mycelial growth, e.g., Rhizobiales, Bacillales and Pseudomonadales (Agnolucci et al. 2015).

(4) Protists (e.g., *Polysphondylium pallidum)* increase N availability in the hyphosphere by releasing N from consumed bacterial biomass (in a process termed soil microbial loop) (Henkes et al. 2018; Rozmoš et al. 2021), supporting the hypothesis that interactions between AMF and protists could further alter prokaryote community composition/activity/abundance/growth pattern in the hyphosphere. AMF hyphal networks could also provide a prey-rich microhabitat for the protists.

(5) Movement of motile bacteria toward the resource-rich patches along the fungal highway (Jiang et al. 2021).

(6) Positive responses of some bacteria to the presence of AMF hyphae, e.g., *Pseudomonas, Burkholderia, Streptomyces, Rhodococcus*, Myxococcales, etc. (See Table 1). These bacteria can bind tightly to hyphae or loosely swim in the water film surrounding the hyphosphere (Bonfante & Anca 2009; Jansa & Hodge 2021). They are likely to provide services to AMF such as enhancing nutrient availability or promoting AMF resistance to pathogens. (7)

Cooperative interactions between AMF and phosphate-solubilizing bacteria (PSB) in the presence of silicon increase P availability in the hyphosphere and enhance P uptake by AMF (Etesami et al. 2021).

(8) Organic N acquisition by AMF hyphae with microbial support (e.g., *Paenibacillus* sp.) (Nuccio et al. 2013; Rozmoš et al. 2021). Cooperative or antagonistic interactions between AMF and microbes can be determined based on nutrient patch quality (e.g., suppression of some ammonia oxidizing bacteria by AMF due to competition for free ammonia as a shared resource (Bukovska et al. 2018; Veresoglou et al. 2019)).

(9) Cooperative interactions between AMF and PSB (e.g., *Pseudomonas alcaligenes, Rahnella aquatilis)* in the hyphosphere and enrichment of some alkaline phosphatase-harboring bacteria that enhance the mineralization of organic P and microbial P biomass (Jiang et al. 2021; Wang et al. 2016; Zhang et al. 2014).

(10) Fructose exuded by the AMF, as a source of energy, stimulates P release by PSB and acts as a signal molecule to trigger P mineralization by bacteria (Gao et al. 2020; Zhang et al. 2018a).

(11) The AMF highway interconnecting different plants belonging to the same or different plant species (so called common mycorrhizal network) may facilitate the transfer of some bacteria into the plant (e.g., the migration of *Bradyrhizobium diazoefficiens,* a N-fixing rhizobial strain, into legumes via the rhizodermis cracks along the AMF hyphae (de Novais et al. 2020)).

(12) Mobilization of organically bound sulfur (S) can be facilitated by some hyphosphere bacteria such as Gammaproteobacteria (including *Pseudomonas* and *Stenotrophomonas*) and Actinobacteria, which enhances S uptake by AMF and plants (Gahan & Schmalenberger 2015).

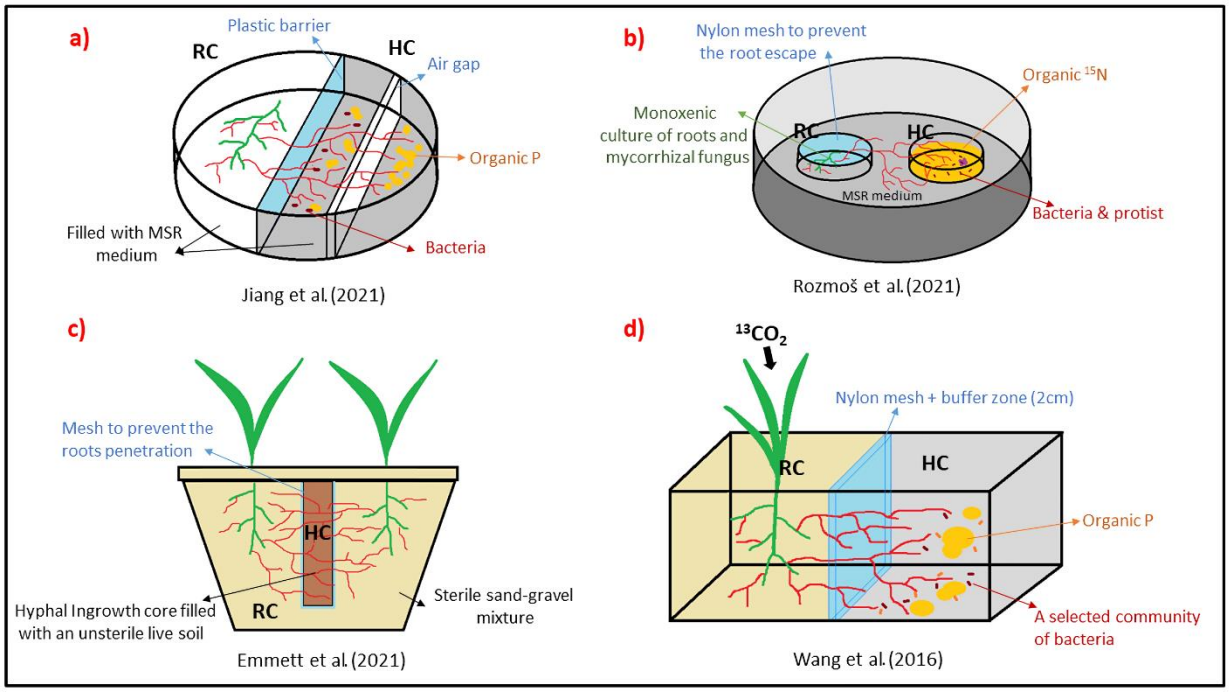

Fig 3. Four examples of well-designed *in vitro* and mesocosm experimental designs aimed at characterizing the microbial community and deciphering biological interactions in the AMF hyphosphere. RC and HC stand for plant root and hyphal compartments, respectively.