Arbuscular Mycorrhizal Fungi and Associated Microbiota as Plant Biostimulants: Research Strategies for the Selection of the Best Performing Inocula

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Abstract: Arbuscular mycorrhizal fungi (AMF) are beneficial soil microorganisms establishing mutualistic symbioses with the roots of the most important food crops and playing key roles in the maintenance of long-term soil fertility and health. The great inter- and intra-specific AMF diversity can be fully exploited by selecting AMF inocula on the basis of their colonization ability and efficiency, which are affected by fungal and plant genotypes and diverse environmental variables. The multiple services provided by AMF are the result of the synergistic activities of the bacterial communities living in the mycorrhizosphere, encompassing nitrogen fixation, P solubilization, and the production of phytohormones, siderophores, and antibiotics. The tripartite association among host plants, mycorrhizal symbionts, and associated bacteria show beneficial emerging properties which could be efficiently exploited in sustainable agriculture. Further in-depth studies, both in microcosms and in the field, performed on different AMF species and isolates, should evaluate their colonization ability, efficiency, and resilience. Transcriptomic studies can reveal the expression levels of nutrient transporter genes in fungal absorbing hyphae in the presence of selected bacterial strains. Eventually, newly designed multifunctional microbial consortia can be utilized as biofertilizers and biostimulants in sustainable and innovative production systems.

Keywords: arbuscular mycorrhizal symbiosis; mycorrhizosphere; AMF associated bacteria; plant growth-promoting bacteria; biofertilizers; phosphate-solubilizing bacteria; siderophore production

1. Introduction

In the next decades, the major challenge for agriculture will be the adoption of a new paradigm, sustainable intensification, to meet human needs for the production of enough food at a global scale while maintaining environmental quality and reducing the input of chemical fertilizers and pesticides [1]. These objectives may be pursued by giving more attention to beneficial soil microorganisms that play key roles in the maintenance of long-term soil fertility and health, the reduction of chemical inputs in agriculture, the promotion of plant nutrition, and the production of safe and high-quality food [2]. Among them, arbuscular mycorrhizal (AM) fungi (AMF) represent a key functional group, positively affecting plant growth, nutrition, and health. AMF are obligately biotrophic organisms that establish

mutualistic symbioses with the roots of all major land plant taxa, including the most important food crops such as cereals, pulses, fruit trees, vegetables, medicinal plants, and other economically relevant species such as sunflower, cotton, sugarcane, tobacco, coffee, tea, cocoa, rubber, and cassava [3]. Within food crops, the only exceptions are represented by genera and species belonging to Brassicaceae and Chenopodiaceae, which are non-mycorrhizal plants.

In exchange for plant photosynthates, AMF facilitate the uptake and transfer of mineral nutrients, such as phosphorus (P), nitrogen (N), sulfur, potassium, calcium, copper and zinc, from the soil to their host plants by means of the extraradical mycelium (ERM) extending from colonized roots into the soil [3]. Such a fungal structure represents one of the critical elements of the AM symbiosis, as the flow of nutrients translocated to the root cells of host plants is highly dependent on its structure, extent, and interconnectedness. ERM functions as an efficient absorbing system, given the high surface-to-volume ratio of the mycelium, which is able to uptake soil nutrients beyond the depletion zone around roots, and the presence of nutrient transporter genes in the hyphae [4]. Besides plant nutrition improvement, AMF facilitate the completion of biogeochemical cycles, increase plant tolerance to biotic and abiotic stresses, carbon sequestration and soil aggregation [5], and the content of health-promoting phytochemicals [6,7] (Figure 1).
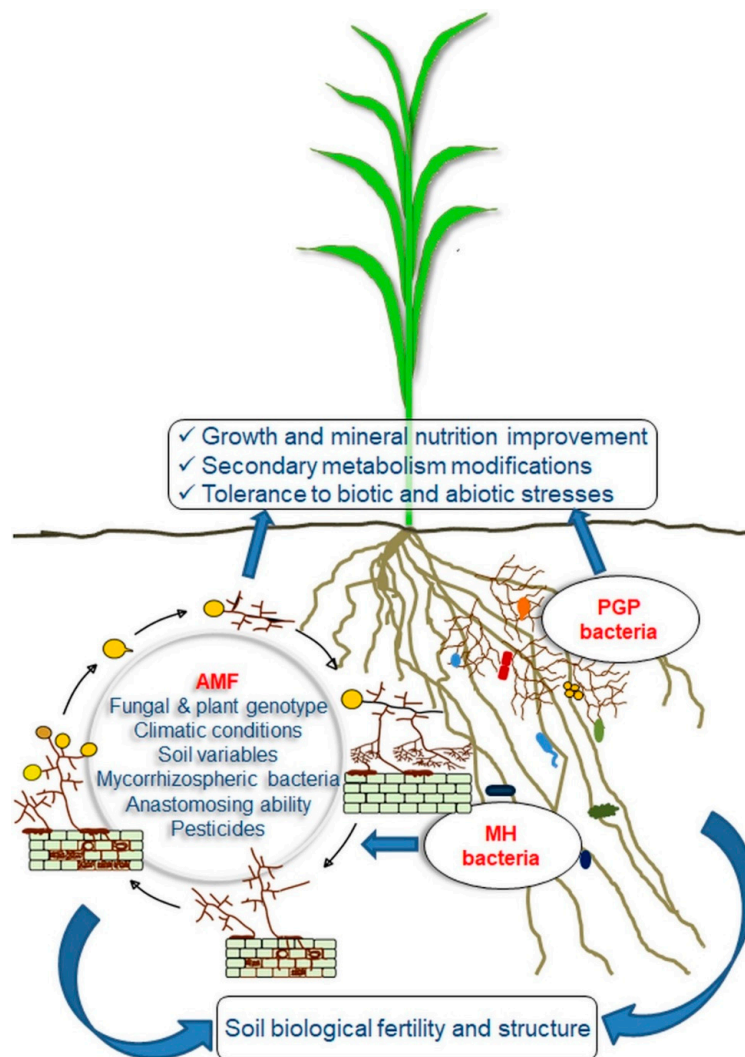

Figure 1. Schematic drawing representing the impacts of arbuscular mycorrhizal fungi (AMF) and beneficial bacteria on plant performance and soil fertility. On the left: a visual representation of the AMF life cycle and factors affecting the different AMF developmental stages; on the right: mycorrhizal helper (MH) and plant growth promoting (PGP) bacteria synergistically interacting with AMF.

Several studies showed that the multiple services provided by AMF are the result of the synergistic activity of diverse bacterial communities living in the mycorrhizosphere, strictly associated with their spores and extraradical mycelium and playing diverse plant growth-promoting (PGP) roles, from nitrogen fixation and P solubilization and mineralization to the production of indole acetic acid (IAA), siderophores, and antibiotics [8,9]. Such microbiota was identified not only by culture-independent methods but also by culture-dependent approaches, which allowed their functional characterization, aimed at detecting the best performing bacterial strains, to be used in combination with selected AMF as biofertilizers and biostimulants in innovative and sustainable food production systems [10].

The aim of this review is to provide an overview of the recent developments which contributed to disclose the biostimulant properties of AMF and their associated bacteria and to propose the best research strategies for the selection of functional isolates and consortia to be utilized as high-quality inocula in sustainable agriculture.

2. Arbuscular Mycorrhizal Fungi

AMF belong to the phylum Glomeromycota, encompassing ten out of eleven families: Acaulosporaceae, Ambisporaceae, Archaesporacea, Claroidoglomeraceae, Diversisporaceae, Gigasporaceae, Glomeraceae, Pacisporaceae, Paraglomeraceae, and Sacculosporaceae (http://www.amf-phylogeny.com/, accessed on 7 January 2020). Given their status of obligate biotrophs, the AMF life cycle cannot be completed in the absence of host plants. It starts with an asymbiotic phase, during which spores germinate in response to physical factors such as moisture, temperature and pH, producing hyphae with a limited lifespan [11]. In the presence of root exudates from host plants, a differential hyphal morphogenesis occurs, with germling hyphae reorienting the direction of elongation and initiating a differential branching pattern [12–14]: this pre-symbiotic phase is followed by physical contact between AMF hyphae and host roots, with the differentiation of appressoria, which give rise to hyphae growing intercellularly within the root cortex, eventually penetrating in root cells and producing highly branched hyphal tree-like structures similar to haustoria, the arbuscules. Arbuscules are the key structures of mycorrhizal symbioses, as at their level nutrient exchanges between the two partners take place: AMF obtain carbon (up to 20% of plant photosynthates) and lipids from the host plant and release mineral nutrients absorbed and translocated by ERM [15–17]. Two types of root colonization have been detected: *Arum*-type and *Paris*-type [3]. In the *Arum*-type, the AM symbiont spreads intercellularly between cortical root cells, forming terminal arbuscules on intracellular hyphal branches [18]. In the *Paris*-type, the fungus grows directly from cell to cell within the cortex and forms intracellular hyphal coils and intercalary arbuscules along the coils. However, most of the data available on AMF derive from studies carried out on the *Arum*-type mycorrhizal symbioses, which are widely distributed in natural and agricultural ecosystems. Beyond arbuscules, several AMF species produce intraradical vesicles, which are spore-like storage structures containing lipids. After receiving host carbon, the fungal symbiont is able to grow extraradically, colonize the surrounding soil, absorb mineral nutrients to be transferred to the host plant, interact with rhizosphere and soil microorganisms, colonize the roots of other plant living nearby (even belonging to species, genera and families different from their host), and also translocate mineral nutrients from one host to another [19,20]. The life cycle is closed by the formation of asexual spores by ERM, functional to the maintenance of a high mycorrhizal potential of the soil and, consequently, of soil biological fertility (Figure 1).

3. AMF Functional Diversity: Colonization Ability and Efficiency

So far, 323 AMF species have been described (http://www.amf-phylogeny.com/amphylo_species.html, accessed 3 December 2019), though only a few species have been investigated for their functional diversity, in order to detect and select the best isolates to be used in agriculture. As a consequence, most of the available commercial inocula are prepared with *Rhizoglomus irregulare* (syn. *Rhizophagus irregularis*, formerly *Glomus intraradices*) and *Funneliformis mosseae* (formerly *Glomus mosseae*), that are generalist symbionts, widespread all over the world in almost all soils and climatic zones [3]. In order

to exploit the great inter- and intra-specific diversity, the general criteria to be applied when selecting the most efficient AMF isolates are outlined here.

The two fundamental fungal characteristics to be taken into account are colonization ability, which refers to fungal capacity of a rapid and extensive root colonization, and efficiency, represented by fungal symbiotic performance, in terms of plant growth and nutrition.

3.1. Colonization Ability

A high root colonization ability is the essential prerequisite for any AMF isolate to be designed for agricultural utilization, as it should be able to compete with highly competitive native AMF. AMF colonization ability does not depend only on fungal genotype, but also on soil characteristics and plant genotype, which may influence the different steps of mycorrhizal establishment, from spore germination to appressorium formation and intraradical growth.

The first variable affecting the competitive ability of an AMF strain is represented by spore dormancy, which may be relieved by storage at 5–10 °C for 5–6 weeks; nevertheless, it is extremely important to know which AMF isolates produce dormant spores when selecting strains for inoculation. As an example, different species of the genera *Glomus*, *Funneliformis*, and *Acaulospora* show spore dormancy, while species such as *Gigaspora gigantea* and *Gigaspora margarita* are able to germinate as early as one day after incubation [21]. It is unfortunate that only a few works have investigated this critical element, which should be further studied not only at the species but, most importantly, at the isolate level, as the producers of commercial inocula often reproduce their own strains.

A key fungal characteristic directly linked to AMF establishment and persistence in the field is represented by spore germination, which is affected by different factors such as soil pH and nutrient content, temperature, soil bacteria, and pesticides. Poor information is available on soil variables, suggesting that the different AMF strains show optimum germination when cultivated in environments with characteristics similar to those from which they were originally isolated. Thus, for example, *Acaulospora laevis*, predominant in low pH soils, germinates well at pH 4–5, while *Dentiscutata heterogama* (formerly *Gigaspora heterogama*), isolated from warm climates, germinates best at 34 °C [21], although nine AMF, isolated and maintained in tropical areas, showed very different germination rates, ranging from 8% to 78%, when cultured in the same environmental conditions [22]. It has long been known that spore germination can be stimulated by soil microorganisms, from Actinobacteria to Pseudomonads, although the most relevant role is played by bacteria living in intimate association with AMF, often located on and within spore wall layers (mycorrhizospheric bacteria) [9]. Actually, many bacterial taxa able to degrade biopolymers were recently detected in spore homogenates by culture-independent methods, suggesting a possible chitinolytic activity on chitin of spore walls that could enhance spore germination [23,24]. It is interesting to note that a recent molecular work reported the ability of six AMF isolates to recruit different bacterial communities on their spores, belonging to Actinomycetales, Bacillales, Burkholderiales, Pseudomonadales, and Rhizobiales, possibly exerting an activity on spore germination [25]. As to pesticides, their effects on spore germination are different depending on the target organisms. Several fungicides, like copper hydroxide and mancozeb, were able to inhibit spore germination of *F. mosseae* in vivo, while flutolanil, azoxystrobin, fenpropimorph, and fenhexamid inhibited germination of *R. irregulare* spores in vitro [26,27]. On the other hand, other fungicides, such as fosetil Al, metalaxyl and different herbicides, seem to exert no activity on spore germination even if the results obtained on the same substance in different investigations were often contradictory [28].

After germination, another important variable affecting the competitive ability of AMF towards native fungi is represented by the ability of germlings to produce an extensive and interconnected hyphal network, which is essential for increasing the chance of coming into contact with a host root. Germling growth may be affected by the same environmental variables quoted above, but depends largely on fungal genotype as it can range from 0.25 up to 104 and 544 mm of hyphal length per germling in the same experimental in vitro conditions [11]. It is important to underline that the possibility to contact host roots and to establish the symbiosis is greatly extended by the ability of germling

hyphae to become interconnected through hyphal fusions (anastomoses): this capacity represents a fundamental survival strategy for AMF germlings, which can plug into compatible extraradical networks, gaining immediate access to plant-derived carbon [29]. Anastomosis formation is highly related to the fungal genotype, as species belonging to the families of Glomeraceae and Acaulosporaceae show a high frequency of hyphal fusions, while members of the family Gigasporaceae do not form fusions interconnecting different hyphae [30,31]. The length, viability, and interconnectedness of germling hyphae are affected by various pesticides: for example, fungicides containing benomyl and fenhexamid, even at doses below the recommended field rate, inhibited hyphal growth of *F. mosseae*, affected mycelial viability, and induced abnormal hyphal branching, while the herbicide glufosinate ammonium decreased mycelial growth and viability, and also the anastomosis rate [32,33].

When AMF germlings come into contact with a host root, a differential hyphal morphogenesis is induced, characterized by an increase in hyphal branching, functional to the production of appressoria on the root surface [12,34]. Appressoria are swollen, multinucleate structures formed as early as 36 h after the contact between germlings and roots [35], and represent the signs of fungal recognition of the host plant. A prompt production of a large number of appressoria, which is requisite for a rapid root colonization, characterizes the most infective AMF, as it makes them highly competitive with native symbionts. Several works investigated this AMF functional trait: an old, but not obsolete work, reported that the number of appressoria may range from 2.6 to 21.1 and from 4.6 to 10.7 per mm of root length in field-grown strawberry and apple, respectively [36], while more recent works found 10.2–80.5 appressoria per plant in parsley and aubergine inoculated in microcosms with *F. mosseae* [37,38]. The same fungus showed variable results depending on host plants: for example, it produced 3.6 appressoria per mm of root in *Medicago truncatula*, 9.7 in *Prunus cerasifera*, and 1.26 in *Trifolium pratense* [39–41]. On the other hand, *G. margarita* produced only 0.01 appressoria per mm of root when inoculated on *Allium cepa* [42]. The dynamics of appressoria formation was monitored in a time-course experiment, showing that the first structures were produced after 36, 48, and 60 h, depending on the fungal genotype [35].

Appressoria produce intraradical hyphae able to establish the mycorrhizal symbiosis by rapidly spreading in the apoplastic space between root cortical cells, although the levels of root colonization greatly vary among AMF and plant genotypes. While such variability among different AMF species have been assessed in countless experiments aimed at evaluating fungal performance in terms of plant growth, the susceptibility of different plant genotypes to mycorrhizal colonization has been investigated only in recent works, reporting large differences among 11 sunflower cultivars (range 8.6–78.7%) and 108 durum wheat varieties (range 10–44%) [43,44].

3.2. Efficiency

The efficiency of the different AM fungal isolates is generally interpreted as their ability to increase plant growth and nutrient uptake, and evaluated by considering the relevant fungal variables such as ERM development, extent, interconnectedness, viability, and rate of nutrient uptake and translocation, that are directly linked to the occurrence of fungal transporter genes in the absorptive extraradical hyphae [4].

ERM length density, assessed after destructive extraction from the soil, showed a large variability among AMF species, ranging from 1.1–6.9 to 3–5 and 10 m/g soil in *Acaulospora laevis*, *F. caledonius* (formerly *Glomus caledonium*), and *Scutellospora calospora*, respectively [11]. Recent works have reported higher hyphal lengths (up to 22 m/g soil) produced by *R. irregulare* isolate BEG 87 [45]. It is worth mentioning the ERM growth rate, which was 738–1067 and 3.1–3.8 mm/day in bidimensional and tri-dimensional experimental systems, respectively [20,46].

ERM structure and interconnectedness have been investigated by nondestructive tests, which provided both qualitative [47,48] and quantitative data. For example, ERM produced by members of the family Glomeraceae, widely distributed in agricultural soils, is highly interconnected by means of anastomoses between contacting hyphae (67–77% in *F. mosseae*), reaching the value of

100–410 anastomoses per gram of soil [20,31,49]. On the contrary, hyphae of members of the families Gigasporaceae, Ambisporaceae, and Paraglomeraceae are not able to fuse after contact, in vivo [50]. Nevertheless, within the Glomeraceae family, self-incompatible interactions between contacting hyphae may occur, with frequencies ranging from 5% to 32% [29,50,51]. Further extensive studies addressed such a clue, revealing major differences among three glomalean AMF: in particular, when grown in symbiosis with five different plant species, *F. mosseae* and *R. irregulare* ERM showed anastomosis frequency of 26–48% and 36–54%, respectively, while *F. coronatus* never exceeded 7.7% [52]; length and density affect AMF symbiotic performance, positively correlating with plant growth responses and nutrient levels [53]. Specifically, AMF isolates showing a high anastomosing ability are able to tolerate soil disturbance, such as tillage, by producing large mycorrhizal networks capable of re-establish interconnections after disruption [54–57]. ERM length and structure may be affected by pesticides, as reported by a recent work performed using a whole-plant experimental system, i.e., in *F. mosseae*, ERM length and density decreased in the presence of the herbicides dicamba and glufosinolate and the fungicides benomyl and fenhexamid, while ERM length and density increased in the presence of two mycorrhizospheric bacteria, *Ensifer meliloti* (formerly *Sinorhizobium meliloti*) and *Enterobacter ludwigii* [58]. Such recent novel data stress the need for further studies to evaluate the impact of agrochemicals and biocontrol agents on ERM structure and activity in a large number of AMF taxa in order to detect the most resilient isolates able to maintain a high mycorrhizal inoculum potential in soil.

Beyond the mentioned phenotypic parameters, viability, which is the most important factor affecting ERM functionality in soil, has been poorly investigated. A few studies reported that metabolic activity occurred in 63–96%, 96–100%, and 100% of extraradical hyphae in *R. irregulare*, *F. mosseae*, and *Rhizoglomus clarum* (formerly *Glomus clarum*), respectively [46,59,60]. A recent study posed the interesting question of whether ERM could survive and maintain colonization ability after plant harvest, thus representing a source of inoculum for the successive crops. The authors, utilizing an in vivo whole-plant experimental system and two worldwide distributed glomalean AMF, *F. mosseae* and *R. irregulare*, revealed that ERM viability and functionality are uncoupled from the host plant lifespan, as, after shoot removal, its growth from detached roots was comparable with that from intact plants and continuous for at least 150 days [61]. Accordingly, ERM represents a long-term survival structure able to maintain mycorrhizal potential and biological fertility in agricultural soils.

AMF efficiency is highly correlated with the rate of P translocation to the host plant: alas, only scanty information is available, showing that in *F. mosseae*, P fluxes in hyphae were $3.4 \times 10^{-8} \, \mathrm{mol \, cm^{-2} \, s^{-1}}$ [62]. However, as the transfer of nutrients flowing in the extraradical hyphae can occur exclusively through appressoria, which are the unique structures connecting soil-based to root-based mycelium, a high number of appressoria produced on the root surface is a key factor affecting not only AMF colonization ability but also their efficiency.

Studies on the occurrence of nutrient transporter genes in AMF extraradical hyphae have mostly been performed in vitro, using transgenic root organ cultures and few species, i.e., *R. irregulare* and *R. intraradices*. The results showed that a number of nutrient transporter genes (ammonium, phosphorus, zinc) are differentially regulated, depending on the availability of various mineral or organic compounds [4,63]. However, as transformed roots show an altered hormonal balance and sugar acquisition, possibly affecting the physiology of the mycorrhizal symbiosis, diverse whole-plant experimental systems were devised, encompassing other AMF species, *F. mosseae*, *F. coronatus*, and *G. margarita* [64]. Further extensive investigations focusing on nutrient transporters gene expression in extraradical mycorrhizal mycelium produced by a large number of AMF isolates are needed in order to achieve a deeper knowledge of differences in AMF efficiency and to select the best performing symbionts to be used as inocula, if also meeting the other quality characteristics concerning colonization ability and efficiency.

4. AMF Efficiency in the Enhancement of Plant Health-Promoting Compounds

In the light of the new findings on plant secondary metabolism being modulated by AMF, the concept of efficiency should be expanded to take into consideration the production of health-promoting compounds, a theme of the highest concern not only to scientists but also to consumers and producers as phytochemicals may reduce oxidative damages, prevent chronic and heart diseases, and decrease the risk of mortality from cancer [65–67]. The levels of such compounds, mainly represented by carotenoids, glucosinolates, polyphenols, including flavonoids, isoflavones and anthocyanins, are affected by different variables such as plant genotype, agronomic techniques, soil characteristics, and also by mycorrhizal symbioses [6].

For example, sweet basil (*Ocimum basilicum*) inoculated with *Glomus* spp. increased the production of rosmarinic and caffeic acids, and of essential oils [68,69], while *R. intraradices* affected the gene expression of key enzymes involved in basil rosmarinic acid biosynthetic pathway [70]. *Echinacea purpurea* inoculated with *R. irregulare* and *G. margarita* showed higher concentrations of caffeic acid derivatives, alkylamides, and terpenes [71], while *R. irregulare* inoculated on *Stevia rebaudiana* enhanced its content of the health-promoting compound steviol glycoside [72]. Interestingly, diverse AMF isolates differentially affected the production of specific phytochemicals; for example, the levels of thymol derivatives in the roots of *Inula ensifolia* were more enhanced by *R. clarus* than by *R. irregulare* [73], while in basil leaves the production of camphor and alfa-terpineol were enhanced by *Gigaspora rosea* but not by *G. margarita,* which decreased the total content of essential oils, in particular that of eucalyptol, linalool, and eugenol [68].

Despite the good results obtained by utilizing medicinal plants and herbs, only a few food crops have been investigated for their levels of health-promoting compounds upon mycorrhizal inoculation, i.e., lettuce, onion, tomato, maize, artichoke, strawberry, pepper, and sweet potato [7]. Most experimental works utilized either AMF inocula composed of a mixture of species, obtained from commercial producers or single species inocula, often represented by *R. irregulare* or *F. mosseae*. Also, molecular studies focused on the assessment of the levels of transcripts encoding the enzymes of the pathways relevant to the production of health-promoting secondary metabolites mainly utilized the same two species [7]. This has impaired the evaluation of the efficiency of different AMF, aimed at selecting the best performing symbionts in the production of beneficial phytochemicals. Accordingly, in the years to come, in-depth investigations should fully exploit the wide physiological and genetic diversity of AMF, testing the highest possible range of diverse species, isolates, and lineages within isolates. In addition, transcriptomic studies would allow the identification of AMF strains differentially expressing genes relevant to the biosynthesis of nutraceutical compounds in food plants.

5. Mycorrhizospheric Bacteria and Their Functional Significance

It has long been known that AMF colonization ability and efficiency may be mediated by a third partner of the symbiosis, the diverse and abundant bacterial communities living in the mycorrhizosphere, i.e., associated with mycorrhizal roots, spores, sporocarps, and extraradical hyphae [74]. Later, by ultrastructural studies, bacteria were detected in spore wall layers, within the peridial hyphae surrounding spores [75,76], and inside the cytoplasm [77–80]. Culture-dependent approaches allowed the isolation of many different bacterial taxa from the mycorrhizosphere of *Glomus versiforme*, *R. clarus*, *G. margarita*, *F. mosseae*, and *R. irregulare* [81–84]. A recent work isolated from *Rhizoglomus irregulare* (formerly *R. intraradices*) spores as many as 374 bacterial strains [85]. Culture-independent methods provided an in-depth description of the different bacterial taxa associated with spores: for example, PCR denaturing gradient gel electrophoresis (PCR-DGGE) identified the bacterial communities associated with *F. geosporus*, *Septoglomus constrictum*, and *G. margarita* spores [23,24], and those strongly associated with the spores of six AMF isolates, three belonging to *F. mosseae*, one to *F. coronatus*, and two to *R. irregulare*—the 48 relevant sequences were affiliated with Actinomycetales, Bacillales, Burkholderiales, Pseudomonadales, Rhizobiales, and Mollicutes-related endobacteria [25].

The mycorrhizospheric microbiota showed different functional activities, ranging from the role of "mycorrhiza helper" (MH) [86] to that of "plant growth promoters" (PGP) (Figure 1). MH bacteria may increase spore germination and mycorrhizal symbiosis establishment: for example, *Streptomyces* spp., *Pseudomonas* sp., and *Corynebacterium* sp. improved the germination of *F. mosseae*, *G. versiforme*, and *G. margarita* spores [81,87–89]. The enhancement of spore germination was ascribed to Actinobacteria, a group of bacteria frequently associated with AMF spores, able to hydrolyze chitin, the main component of spore walls [23,25,76,90]. Other MH bacteria, such as *Klebsiella pneumoniae*, *Trichoderma* sp., and *Paenibacillus validus*, increased germlings hyphal growth [91–93], while one bacterial strain belonging to Oxalobacteriaceae enhanced not only spore germination and germling growth but also root colonization [94]. In addition, the development of AMF extraradical mycelium (ERM) may be promoted by strains of *Paenibacillus rhizosphaerae*, *Azospirillum* sp., *Rhizobium etli*, *Pseudomonas* spp., *Burkolderia cepacia*, and *E. meliloti* [45,95–98] (Figure 1).

PGP bacteria show multifunctional activities, encompassing nitrogen fixation, P solubilization and mineralization, the production of indole acetic acid (IAA), siderophores, and antibiotics while supplying fundamental nutrients and growth factors [8,9]. Such activities represent key characteristics to be taken into account when selecting the best AMF and bacterial combinations for the production of inocula for agricultural use. For example, as P is rapidly immobilized in the soil, forming insoluble compounds with aluminium/iron and with calcium in acid and alkaline soil and thus becoming unavailable to plants, P-solubilizing bacteria may work in synergy with AMF to increase P availability and plant P uptake. Indeed, P-mobilizing bacteria, such as *Streptomyces* spp., *Leifsonia* sp., *Bacillus pumilus*, *Lisinobacillus fusiformis*, and *E. meliloti*, isolated from AMF spores of *R. irregulare*, showed synergistic action with AMF, promoting the mineralization of soil phytate and facilitating P uptake by mycorrhizal plants [45,99]. Similarly, the isolation from the mycorrhizosphere of bacterial strains possessing the *nifH* gene amplicon suggested a possible role in plant acquisition of nitrogen [85]. On the other hand, some PGP bacteria are able to produce IAA, a phytohormone of the auxin class, which plays a key role in the regulation of plant growth, increasing plant cell division and root formation, thus affecting water and nutrient uptake [100–102]. Accordingly, IAA producing bacteria isolated from *R. irregulare* and *F. mosseae*, such as *E. meliloti* and *Paenibacillus favisporus*, enhanced the growth of AMF extraradical hyphae, the fungal structure fundamental for absorbing and translocating P from the soil to plant roots [45,95]. An important role in the promotion of plant growth is played by mycorrhizospheric bacteria able to protect plants against soil-borne pathogens, either by directly producing antibiotics or indirectly producing siderophores, high-affinity iron-chelating compounds which mediate iron acquisition by pathogenic microorganisms [85,103–105]. Moreover, the facilitation of plant iron acquisition by siderophores-producing bacteria represents an additional benefit, as iron is an essential element in key biochemical processes like photosynthesis and respiration [106,107]. Interestingly, many of the bacteria isolated from AMF spores showed multiple PGP activities, i.e., 17 actinobacterial strains were able to produce siderophores and IAA to mineralize phytate and solubilize inorganic phosphate, and ten putative N-fixers to produce siderophore and solubilize P [85]. A recent work confirmed such data, reporting the occurrence of diverse bacterial functional taxa in a commercial AMF inoculum: 14 isolates showed the best combination of PGP traits, such as the production of IAA and siderophores, while 6 of them were also able to solubilize P, i.e., *Bacillus megaterium*, *Streptomyces* sp., and *Enterobacter* spp. [108]. These strains, both as single- and multi-strain inocula, deserve further in-depth studies in order to evaluate their efficiency as biofertilizers and biostimulants, able to boost plant growth, nutrition and health in sustainable food production systems (Figure 1). New remarkable findings showed that several members of the mycorrhizospheric microbiota may establish a more intimate relationship with their host plants as root endophytes [109,110]: considering that they can reach 10^5–10^7 CFU per g of root [111,112], their possible beneficial effects should be further investigated in the years to come.

6. Conclusions and Perspectives for Future Studies

The multiple beneficial activities of AMF and their associated bacteria discussed so far highlight the complex networks of interactions taking place in the mycorrhizosphere, functional to plant growth, nutrition, and health. The tripartite association among host plants, fungal symbionts and their associated bacteria shows beneficial emerging properties that could be efficiently exploited in sustainable food production. Although much is known on a very small number of AMF species, often studied singly in sterile conditions, very little is known about the high physiological and genetic inter- and intra-specific diversity of AMF and their associated microbiota. Further in-depth studies should be performed on different AMF species and isolates, and on their associated bacteria, both singly and in various combinations, in order to evaluate their colonization ability and efficiency when inoculated with a number of plant hosts. The studies carried out in microcosms should be followed by investigations in the field to assess the ability of the selected AMF and bacteria to compete with native microorganisms and to maintain their beneficial activities. Once detected as the best performing inocula, they could be differentiated by assessing their resilience against diverse environmental conditions, from soil types to drought, salt, biotic stresses, and pesticides. Transcriptomic studies could reveal the expression levels of nutrient transporter genes in fungal absorbing hyphae in the presence of selected efficient bacterial strains, possibly leading to the detection of the best synergistic combinations of AMF and associated bacterial communities, enhancing nutrient availability and plant performance. At the same time, transcriptomics could increase knowledge on the differential expression of genes encoding enzymes relevant to the biosynthesis of nutraceutical compounds in food plants. Eventually, newly designed multifunctional microbial consortia could be commercially reproduced and utilized as biofertilizers and biostimulants in sustainable and innovative production systems.

Conflicts of Interest: The authors declare no conflict of interest.

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