Chapter 8

Arbuscular Mycorrhizal Fungi and their Value for Ecosystem Management

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1. Introduction

Arbuscular Mycorrhizal Fungi (AMF) are a group of obligate biotrophs, to the extent that they must develop a close symbiotic association with the roots of a living host plant in order to grow and complete their life cycle [1]. The term "mycorrhiza" literally derives from the Greek *mykes* and *rhiza*, meaning fungus and root, respectively. AMF can symbiotically interact with almost all the plants that live on the Earth. They are found in the roots of about 80-90% of plant species (mainly grasses, agricultural crops and herbs) and exchange benefits with their partners, as is typical of all mutual symbiotic relationships [2]. They represent an interface between plants and soil, growing their mycelia both inside and outside the plant roots. AMF provide the plant with water, soil mineral nutrients (mainly phosphorus and nitrogen) and pathogen protection. In exchange, photosynthetic compounds are transferred to the fungus [3].

Taxonomically, all AMF have been affiliated to a monophyletic group of fungi, i.e. the Glomeromycota phylum [4]. They are considered to be living fossils since there is evidence that their presence on our planet dates back to the Ordovician Period, over 460 million years ago [5]. Investigations on AMF taxonomy began in the nineteenth century with the first description of two species belonging to the genus *Glomus* [6]. Since that date, many Glomeromycotan species, genus and families have been discovered and characterized by means of traditional approaches based on the phenotypic characteristics (mainly spore morphology). Molecular DNA sequencing-based analyses have recently contributed to a great extent by shedding light on a previously unseen and profound diversity within this phylum [7].

Nevertheless, an open debate on the phylogeny of AMF, and in particular concerning some taxonomical groups, is still puzzling scientists [8–10] (Figure 1). Besides a general disagreement about the number of families and genera (Figure 1), what emerges from reference [8] is that Gigasporales are considered to be a separate order from Diversisporales. This is different from what has been reported in the tree on the right side of Figure 1, which was presented in reference [9], and supported by the recent reference [10].

Functionally, AMF form the so-called arbuscular mycorrhizae with plant roots. The most typical AMF structure, which also gives the name to this group of fungi, is the arbuscule (Figure 2). This structure, whose shape recalls that of a small shrub, forms inside the root cortical cells by branching in several very thin hyphae. In this way, the surface area, where the nutritional exchanges between the plant and fungus take place, is maximized. Fungal hyphae that grow between root cortical cells are able to produce other AMF structures, such as intercellular hyphae and vesicles (Figure 2). All these structures that grow inside the plant roots represent the intraradical phase of the fungus. Hyphae also grow outside the plant roots, and generate a network that extends over long distances and explores the soil beyond the nutrient depletion zone that normally characterizes the area surrounding the roots. At the end of the AMF life cycle, or in response to particular environmental conditions, spores (Figure 2) of variable size (up to 400 μm), depending on the species, are produced in the roots and/or in the soil. These, along with external explorative and running hyphae, represent the extraradical phase of the fungus. The synergic action of the intra-and extraradical phases is responsible for the ecological significance of the AMF, a soil-root-living key group of organisms [3].

1.1. The ecological roles of AMF

Arbuscular mycorrhizal fungi have a high relevance in many ecosystem processes. Since they can be found in many different plant species, they can provide their favorable services to almost all terrestrial ecosystems, from grasslands to forests, deserts and agroecosystems [11]. AMF can play several roles in such environments. The most agriculturally significant and frequently investigated one, from both the ecological and physiological points of view [12], is their positive effect on plant nutrition and, consequently, on plant fitness. In particular, they play a pivotal role in helping the plant uptake phosphorus from the soil [13]. Without AMF, it is rather difficult for the plant to absorb this macroelement from the soil, since it is mainly available in its insoluble organic or inorganic form. Besides phosphorus, AMF can also translocate water and other mineral nutrients (in particular nitrogen) from the soil to the plant. These nutritional exchanges are bidirectional. As a consequence, particularly efficient symbiotic associations have been demonstrated to stabilize through unknown mechanisms, with the plant selecting the most cooperative fungal partners and vice versa [14]. The AMF-inducible recovery of plant nutritional deficiency can inevitably lead to an improvement in plant growth, with a potential positive impact on productivity. Needless to say, AMF have attracted a great deal of interest from the agricultural world over the years [15].

AMF are also responsible for other services that favour the plants they colonize: (a) they positively affect plant tolerance towards both biotic (e.g., pathogens) and abiotic stresses (i.e., drought and soil salinity) by acting on several physiological processes, such as the production

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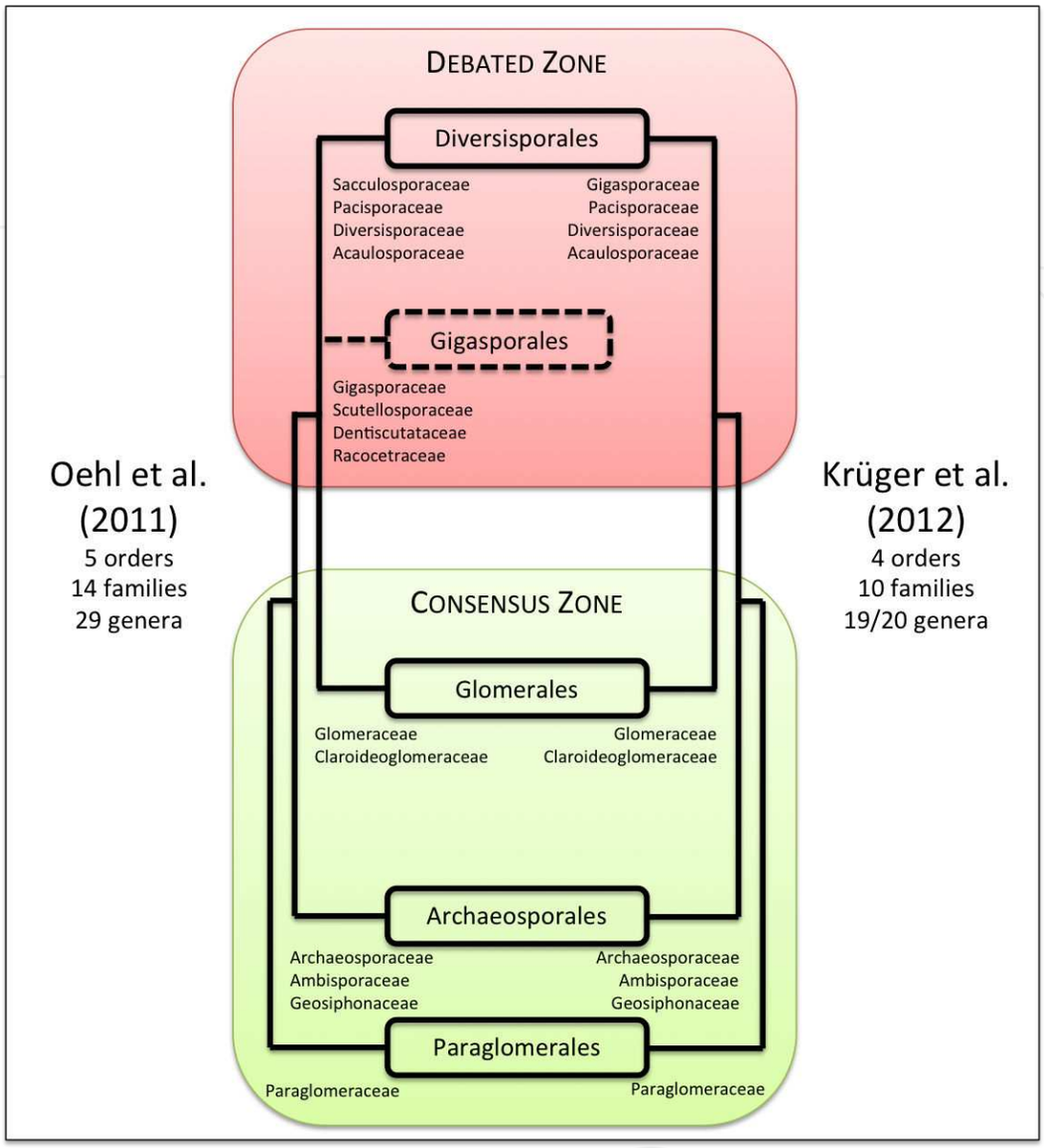

Figure 1. A schematic representation of two recently published and partly controversial phylogenetic trees of the Glomeromycota phylum (reference [8] for the tree on the left side and [9] for the tree on the right side). The one published in reference [8] was based on molecular (SSU, ITS, partial LSU rDNA, and partial β-tubuline gene) and morphological analyses (spore wall structures, structures of the spore bases and subtending hyphae, germination, and germination shield structures). The tree published in reference [9] was based on concatenated SSU rDNA consensus sequences (ca 1.8 kb).

of antioxidants, the increment of osmolyte production or the improvement of abscisic acid regulation [16,17], and the enhancement of plant tolerance to heavy metals [18]; (b) they help plants become established in harsh/degraded ecosystems, such as desert areas and mine spoils [19]; (c) they increase the power of phytoremediation (the removal of pollutants from the soil by plants) by allowing their host to explore and depollute a larger volume of soil [20,21]. Another crucial ecological role played by AMF is their capacity to directly influence the diversity and composition of the aboveground plant community. Several studies have

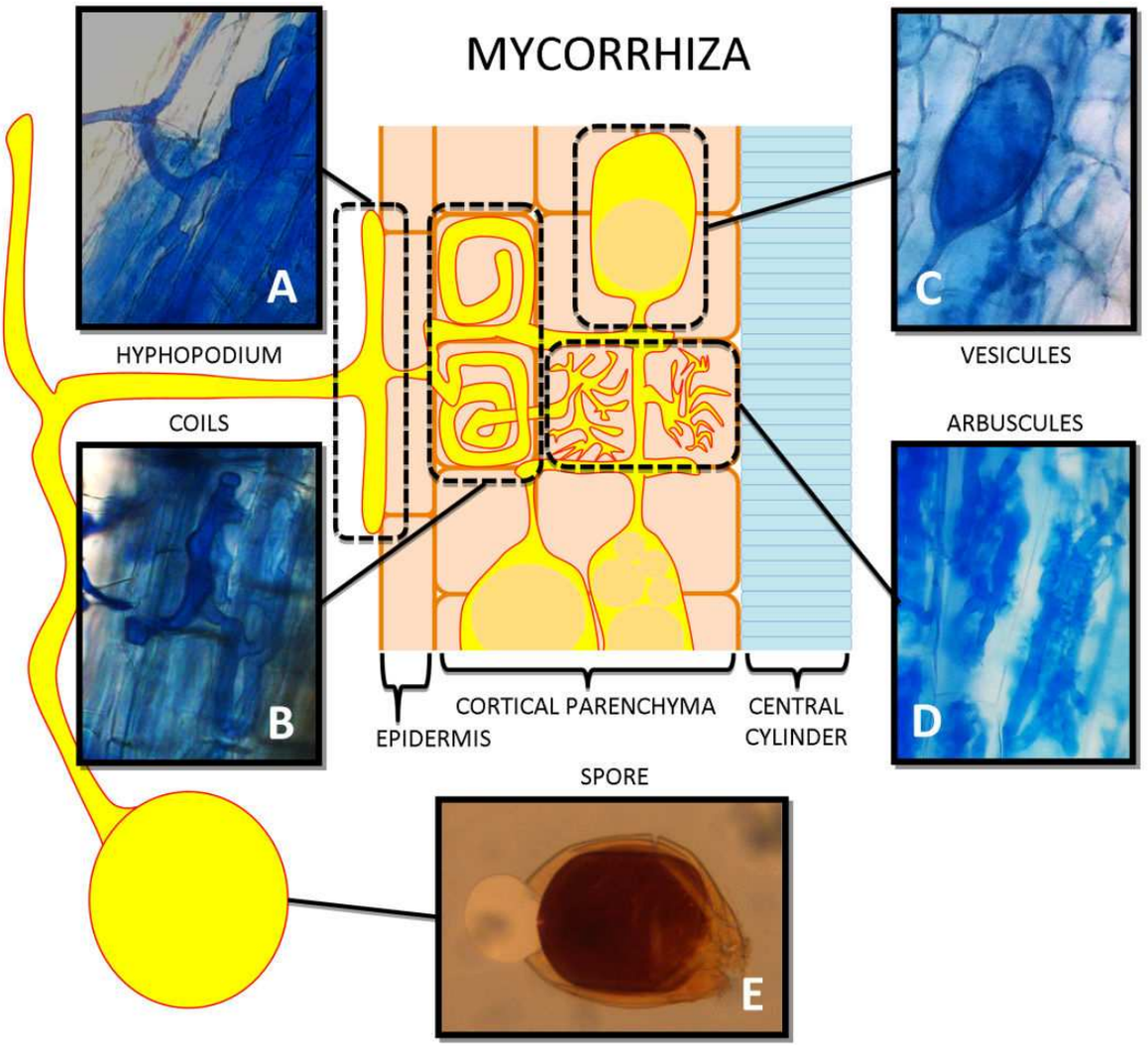

Figure 2. Extraradical and intraradical phases of AMF growth. The spore (Figure E, a *Scutellospora sp.* spore stained with Melzer's reagent and squeezed) germinates in the bulk soil and approaches the root of a host plant. The fungus penetrates through a hyphopodium (Figure A, stained with 0.1% cotton blue encountered in *Camellia japonica* L. roots) and develops intracellular coils, extracellular vesicles and intracellular arbuscules (Figures B, C, D) within cortical parenchyma, without entering the central cylinder where the vessels are.

confirmed that plant species richness can be altered not only by climatic and edaphic factors, but also by soil microbial assemblages [22–24]. The underlying mechanism is not completely understood, but could be related to the promotion of seedling establishment of secondary plant species [25]. Nevertheless, on some occasions, AMF can also negatively affect the diversity and growth of plants, which is particularly significant for the management of weeds [26]. Last but not least, AMF play a critical role in soil aggregation, thanks to their thick extraradical hyphal network, which envelops and keeps the soil particles compact. It has been suggested that glycoproteins (glomalin and glomalin related proteins) secreted by AMF into the soil could exert a key role in this process [27,28]. These proteins are exuded in great quantities into the soil, and could have implications on carbon sequestration. This potential capability of AMF is likely to contribute to a great extent to the soil ecosystem carbon dioxide (CO_2) sequestration

process. This aspect has led to the recognition of the importance of this group of organisms in processes related to climate change mitigation [29].

All the services offered by arbuscular mycorrhizal fungi confirm the need to study and describe all their features, including their biology, ecology, taxonomy, phylogeny and biodiversity. Over the years, several techniques have been developed to reach this goal: a brief history is reported in section 1.2.

1.2. Methods used in the study of AMF

This group of organisms has a constraining characteristic that makes their study very complex: as obligate symbionts, they cannot be cultivated in vitro, away from their host plant. The development of an artificial system that is capable of going beyond this barrier dates back to the 1980s, when in vitro transformed carrot roots were successfully colonized by AMF species [30]. Thanks to this method, the study of arbuscular mycorrhizae became easier and many researches on both physiology and genetics became possible [31,32]. Over the last two decades, many molecular and physiological mechanisms involved in the symbiotic process between plants and AMF have been discovered and described, thanks to the increasing innovations and opportunities offered by molecular biology. For example, it is now known how the infectious process of AMF arises, and many of the involved genes have been identified successfully [33].

Molecular biology has also revolutionized the analysis of the biodiversity of AMF, making it easier and more accurate to characterize the AMF community composition of large quantities of samples from many different ecosystems, from prairies to savannas, and from grasslands to forests (Table 1). The first studies on the diversity and distribution of AMF were mainly focused on the identification of the species that colonize the roots of a given plant in a given environment [34]. This was mainly due to the deficiency in the available investigation techniques, as they were primarily based on spore surveys and intraradical fungal structure morphological identification by means of microscopy. Such morphological identification surveys were time consuming and often lacked accuracy, since many species were easily confused with others. The situation changed radically when the use of DNA-based techniques became common, and the extraction of DNA from plant tissue was reduced to a few relatively easy steps that could be reproduced in any laboratory [35,36]. The load bearing principle is simple: by sequencing a specific DNA region, it is possible to univocally identify the corresponding AMF [37]. So far, the most used DNA target regions for AMF identification are located on the ribosomal genes (Small and Large ribosomal Subunits – SSU and LSU, respectively – and the Internal Transcribed Spacers – ITS1 and ITS2), as they show a rate of variability that is sufficient to discriminate between AMF species/isolates [9]. All this has led to the current era of molecular identification of AMF species [10]. Next-Generation Sequencing (NGS) tools represent a further step forward for biodiversity surveys of all organisms [38], including AMF. Over the last few years, the number of NGS-based AMF biodiversity studies has increased, while the spectrum of the target environments has broadened [39]. Furthermore, new primer pair sets for the specific amplification of AMF DNA sequences, capable of providing higher accuracy and a comprehensive coverage of the whole Glomeromycota phylum, have been

developed [40]. Nowadays, AMF assemblages are no longer studied only in plant roots, but also in the bulk soil [41–43]. The main result obtained from the application of NGS to the study of AMF biodiversity has been the discovery of an unpredictable diversity within the Glomeromycota phylum [39]. However, this series of innovative molecular tools has introduced a new issue, that is, the continuously increasing number of unidentified AMF DNA sequences from environmental samples with no correspondence whatsoever to sequences of known species [44]. This has naturally made scientists aware of the fact that the number of AMF species could be larger than expected. However, it is not reliable to have new species described on just the basis of short DNA sequences obtained by means of NGS tools. Instead, for each new suggested taxon, a series of steps needs to be followed to characterize the morphotype, the functional traits, and the ecological role offered when present in combination with other organisms in a given environment. Therefore, NGS tools cannot be considered as complete replacements of the traditional methods of identification and description of new species. The combined approach is still necessary to shed light on such a key group of organisms and to make them available for agricultural application and, more in general, for other practices useful for the wellbeing of humankind [45].

1. Reference	2. Year	3. Method	4. Target region	5. Studied compartment	6.Ecosystem	7. AMF sequences	8. OTUs
[39]	2013	Clon-seq/NGS	SSU	Plant root	Tropical, subtropical, temperate and boreal forests, subtropical and temperate grasslands, tropical and subtropical deserts and shrublands, and polar tundras (Africa, Asia, Oceania, Europe, North and South America)	2353/22391	204
[46]	2013	NGS	SSU	Soil	Prairie (Cananda)	1335521	120
[47]	2013	NGS	SSU	Plant root and Soil	Temperate forest (Estonia)	35738	76
[48]	2013	Clon-seq	SSU	Plant root	Mediterranean semi-arid soils (Spain)	467	30
[49]	2013	Clon-seq	SSU	Soil and plant root	Prairie (USA)	232	13
[43]	2012	NGS	SSU	Soil	Forest (Estonia)	13320	37
[50]	2012	NGS	SSU	Soil	Arable field (China)	59611	70

1. Reference	2. Year	3. Method	4. Target region	5. Studied compartment	6.Ecosystem	7. AMF sequences	8. OTUs
[51]	2012	NGS	SSU	Soil	Prairie - Chernozem (Cananda)	7086	33
[52]	2012	NGS	LSU	Plant root	Grassland (Denmark)	82511	32
[42]	2012	Clon-seq	SSU/LSU	Soil and plant root	Arable field (Italy)	427/364	20/23[a]
[53]	2012	Clon-seq	SSU	Plant root	Alpine meadow ecosystem (China)	4452	38
[54]	2011	NGS	SSU	Plant root	Broadleaf, mixed broadleaf and coniferous forests, botanical gardens, greenhouse	65001	73
[55]	2011	NGS	SSU	Plant root	Grassland, wood and heath (UK)	108245	70
[56]	2011	Clon-seq	SSU	Plant root	Hardwood forest (USA)	1598	17
[41]	2010	NGS	SSU	Soil	Mediterranean soils (Italy)	2815	19/80[a]
[57]	2010	Clon-seq	SSU	Soil and plant root	Vineyard (Italy)	681	37
[58]	2009	Clon-seq	SSU	Plant root	Woodland (UK)	617	33/37[b]
[59]	2009	Clon-seq	SSU	Plant root	Mediterranean semi-arid soils (Spain)	1443	21
[60]	2009	NGS	SSU	Plant root	Boreal forest (Estonia)	111580	47
[61]	2008	Clon-seq	LSU	Soil and plant root	Arable field (Italy)	183	8
[62]	2008	Clon-seq	SSU	Plant root	Boreal forest (Estonia)	911	26/27[c]
[63]	2008	Clon-seq	SSU	Plant root	Arable field (Mexico)	213	16
[64]	2008	Clon-seq	SSU	Plant root	Serpentine soils (USA)	1249	19
[65]	2008	Clon-seq	SSU	Plant root	Arable field (Sweden)	115	8
[66]	2007	Clon-seq	ITS	Soil, plant root and spores	Meadow (Germany)	180	>18
[67]	2007	Clon-seq	SSU	Rhizoids	Liverworts (World-wide)	150	10

1. Reference	2. Year	3. Method	4. Target region	5. Studied compartment	6.Ecosystem	7. AMF sequences	8. OTUs
[68]	2007	Clon-seq	LSU	Soil and plant root	Arable field (France)	246	12
[69]	2007	Clon-seq	SSU	Plant root	Grassland (Sweden)	185	19
[70]	2007	Clon-seq	ITS	Plant root	Volcanic desert (Japan)	205	11
[71]	2006	Clon-seq	SSU	Plant root	Polluted soils (Italy)	115	12
[72]	2005	Clon-seq	SSU	Plant root	Warm-temperate deciduous forest (Japan)	394	5
[73]	2004	Clon-seq	SSU	Plant root	Wetland (Germany)	546	35
[74]	2004	Clon-seq	LSU	Plant root	Grassland (Denmark)	158	11
[75]	2004	Clon-seq	ITS	Plant root	Pasture (UK)	30	10
[76]	2004	Clon-seq	SSU	Plant root	Grassland (Japan)	200	8
[77]	2004	Clon-seq	SSU	Plant root	Grassland (UK)	606	9
[78]	2003	Clon-seq	ITS	Plant root	Afromontane forests (Ethiopia)	92	20
[79]	2003	Clon-seq	SSU	Plant root	Boreal forest (Estonia)	16	6
[80]	2002	Clon-seq	SSU	Plant root	Seminatural grassland (UK)	88	24
[81]	2002	Clon-seq	SSU	Plant root	Woodland (UK)	232	13
[82]	2002	Clon-seq	SSU	Plant root	Tropical forest (Republic of Panama)	1536	18/23[d]
[83]	2002	Clon-seq	SSU	Plant root	Tropical forest (Republic of Panama)	558	18
[84]	2001	Clon-seq	SSU	Plant root	Arable field (UK)	303	8
[36]	1999	Clon-seq	SSU	Plant root	Seminatural woodland (UK)	141	6/8[e]
[85]	1998	Clon-seq	SSU	Plant root	Woodland (UK)	253	6/10[b]

a-taxa obtained with different primer sets; b: taxa obtained at different study sites; c-taxa obtained from forest ecosystems of different ages and management intensities; d-taxa obtained from roots of different plant species; e-taxa obtained at different sampling times.

Table 1. The table shows an overview of DNA-based studies on the diversity of Arbuscular Micorrhizal (AM) fungal communities. For each study, the following are reported in sequence: 1. Reference, 2. Year of publication, 3. Used method (Clon-seq=cloning and sequencing; NGS=next generation sequencing), 4. Studied DNA region (SSU=Small Subunit; LSU=Large Subunit, ITS=Internal Transcribed Spacer), 5. Compartment from which the DNA was analyzed, 6.

Ecosystem from which the samples were collected, 7. Number of DNA sequences and 8. OTUs (Operational Taxonomic Units) from arbuscular mycorrhizal fungi.

2. The impact of humans on AMF biodiversity

Most human activities have an arguable impact on the physical and biological aspects of soil. As mentioned before, AMF are among the most widespread soil microorganisms, and each human activity that has an impact on soil, such as agricultural practices, therefore has a side effect on them. These practices, alone or in combination, exert an enormous selective pressure on AMF that shapes their community structure and evolution by modifying several of their biological features, such as sporulation strategy, resource allocation and spatial distribution [86]. As in natural ecosystems, AMF are also present and active in agricultural ecosystems, where they colonize several major arable crops (sorghum, maize, wheat and rice). Many studies have indicated that AMF diversity, effectiveness, abundance and biodiversity decline in agroecosystems subjected to high input practices [41,42]. Modern intensive farming practices that implement deep and frequent tillage, high input inorganic fertilization and pesticide use are evidently a particular threat to AMF. This is surely a drawback for agriculture, since the more AMF biodiversity losses, the fewer AMF functional traits the host plant can benefit from. On the other hand, the activity and diversity of AMF, following conversion from conventional to organic farming, have not yet been investigated thoroughly. However, the available data seem to indicate that AMF respond positively to the transition to organic farming through a progressive enhancement of their activity [87]. Even though it is difficult to discriminate between the effects that different agricultural treatments exert on AMF communities, they are here considered separately, and their role in shaping AMF communities will be analyzed.

2.1. Tillage: A conventional practice detrimental to AMF

One of the most ancient and representative agricultural techniques is tillage. Tillage has played a crucial role in the evolution and technological development of agriculture, particularly for food production. The benefits produced by tillage include a better conservation of water and soil fertility, the abatement of weeds and the preparation of a suitable seedbed. To fulfill these tasks, the undisturbed soil is mechanically manipulated in an effort to modify the physical characteristic of the soil and eliminate weeds. The physical, chemical and biological effects of tillage on the soil can be both beneficial and negative, depending on the methods that are used. The inappropriate use of tillage techniques can therefore have a dramatic impact on the soil structure and on soil microorganism community assemblage. It is possible to identify different tilling levels, ranging from a very low impact, “No-tillage”, to a high impact, “conventional tillage”. A continuum of intermediate conditions lies in between these two extreme situations, e.g. varying frequency and intensity of the plowing.

The mechanical soil disturbance experienced by AMF in tilled agricultural soils has no equivalent in natural ecosystems. This is why tillage has been widely recognized to be one of the principal causes of the modification of the AMF communities that colonize plant roots in

agricultural fields [88]. Mycorrhizal diversity, at a family level [88], and the timing of root colonization [89] can be affected negatively. As a consequence, the effectiveness of AMF [90] is likely to be reduced. Periodically repeated mechanical soil disturbance destroys the extra-radical mycelial network formed by AMF. This very complex underground structure can reach lengths of up to some tens of meters in one gram of soil [91], and represents a soil "highway" for nutrient transport. For this reason, it is often claimed to be closely correlated to biodiversity, biomass production and the functioning of plant communities [22,25,92].

An ecological shift in AMF communities is particularly noticeable when frequently and infrequently tilled agroecosystems are compared [42,63,88,93]. This is probably due to the different tolerance to hyphal disruption among the different AMF species [94,95]. Although AMF species can colonize plants from spores, this process often requires a certain amount of time. Faster root colonization can be reached in the presence of a viable and well-structured underground mycelial network that facilitates AMF proliferation and speeds up plant root penetration [96]. On the other hand, AMF species differ greatly in their capacity to restart colonization from fragmented mycelium or root fragments [97]. Intense tillage could be a factor that favors those AMF species that are more able to proliferate from fragmented hyphae or root fragment [98], and could therefore determine a shift in AMF community assemblages. A clear example of this is the large presence of Glomeraceae species found in tilled soil all over the world [99]. AMF species belonging to this group are able to randomly connect hyphae in close proximity after disruption, a condition that can easily be found in disturbed soil. This allows these species to proliferate more easily and to rapidly become dominant over slow-growing AMF. The members of the Gigasporaceae family, for example, use spores as the main source of root colonization, but do not regrow from hyphal fragments [97].

2.2. Fertilization

Another agricultural practice that has major ecological fall-outs is chemical fertilization. This practice is often claimed to be fundamental in improving the growth performance of plants, but it is sometimes abused. In addition to the environmental drift and the possible pollution of underground water reservoirs, the presence in the soil of high levels of fertilizer dramatically alters the interaction between plants and microbial communities. The central role of arbuscular mycorrhizae in plant nutrition makes them very susceptible to changes in soil nutrient availability. Generally, in a nutrient-rich environment, a plant can directly uptake enough nutrient from the soil, without the "catering" service provided by the AMF partners. As a result, the dependency of plants on their AMF partners gradually diminishes, and AMF community richness and diversity decline [42,53,100,101]. It is thought that fertilization can alter the performance of this symbiosis, making microbial partners costly, and even parasitic [102]. It has been hypothesized that the enrichment of soil resources, due to high input fertilization, could lead to a reduction in plant allocation to roots and mycorrhizas [103], and an accumulation of nutrient resources in epigeous plant sinks [104]. A reduction in host plant resource allocation to the fungal partners can therefore result in a decrease in AMF root colonization [105], and an increase in fungal competition for limited C resources. Moreover, this reduction in host nutrient availability is thought to shift the competitive balance between

microbes, favoring more aggressive, antagonistic microbial genotypes [106–108]. This change in competitive balance can alter the evolution of the functional traits of AMF by reprogramming AMF to reduce their allocation to structures devoted to nutrient exchange (arbuscules and coils), and increase their allocation to internal storage and growth structures (vesicles and intraradical hyphae) [103,109,110]. This is likely to result in an incremented presence of highly competitive AMF which, on the other hand, will be less beneficial to the host crop [111].

Particular AMF taxa have been found to be more sensitive than others to specific fertilization conditions [42,50,53,65,93,112]. This is probably due to the different taxon-related ability of the AMF taxa to manage nutrient absorption. For instance, *Acaulospora* species have been demonstrated to be very effective in P uptake, and in the transfer to the host plant, compared to Glomeraceae species [113]. In line with these findings, Acaulosporaceae species have been considered to decrease to a great extent under high input P fertilization [50]. The same thing has been observed for Gigasporaceae in N-enriched soils [50,103]. On the other hand, Glomeraceae species, such as *Rhizophagus intraradices*, are able to cope well with nutrient rich environments [50,53].

2.3. Crop rotation

The choice of crop and rotation made by the farmer has a crucial impact on AMF communities. Even though AMF are commonly recognized as generalist symbionts that show the ability to interact with different plant species, some plant-fungus combinations can perform better than others. The choice of the partner is not univocal, but is believed to be driven by a reciprocal reward mechanism between the two symbionts involved [14]. This means that both the plant and the AMF communities can exert an important role in modifying the community composition of the partner [22,23]. Thus, different cultivation practices that involve a variation in plant diversity, such as monoculture, fallow and crop rotation, could show different and profound effects on AMF community assemblages.

Monoculture can be highly deleterious for AMF communities, and result in a significant reduction in mycorrhizal root colonization [114] and mycorrhizal diversity [115,116]. The effect of continuous monocropping, especially when crops that are not highly dependent on AMF-mediated nutrition (e.g. wheat) are used, favors the selection and proliferation of less cooperative and more aggressive fungal symbionts. These are likely to enact similar behavior to parasitism [102,106]. In addition, intensive tillage treatments, which are necessary in the case of monoculture practices, can overly disperse fungal propagules, thus allowing fewer AMF isolates to dominate the community profile. The dominion of AMF species with a poor mutualistic attitude could be toned down by alternating the cultivation of plant species that are less dependent on AMF with 'break crops', such as *Brassica* [117] or legumes [118]. The former is a non-mycorrhizal crop that can therefore act as an inhibitor of the dominant AMF species proliferation. The latter represent the opposite approach, since legumes are AMF-dependent crops that favor the overall propagation of AMF communities. This is the fundamental principle of crop rotation, a practice that can exert a control function that prevents particular AMF from dominating the soil matrix. Hence, crop rotation has the potential of driving AMF communities to be less parasitic [86]. It has been experimentally demonstrated

that crop rotation promotes higher AMF diversity [115,119], and can reshape AMF communities derived from agricultural fields to be more diverse and similar to the ones detected in natural ecosystems [87].

3. AMF biodiversity restoration

Agricultural fields, degraded lands and the so-called "third landscapes" are all soil environments in which humans have had an impact on the ecological balances, by unchaining a series of inevitable ecosystem alterations. Therefore, the restoration of such balances should be a necessity. Owing to their role in the promotion of plant health, soil nutrition improvement and soil aggregate stability, AMF are primary biotic soil components that, when missing or impoverished, can lead to a less efficient ecosystem functioning. The presence of a high degree of AMF biodiversity is in fact typical of natural ecosystems and indicates good soil quality [120]. Consequently, a process that aims at the re-establishment of the natural level of AMF richness is a pivotal step towards the restoration of the ecological balances. As previously mentioned, the cultivation practices adopted for major crops include anthropic inputs that can impact AMF occurrence and/or diversity. Of these, the use of fertilizers and pesticides also has an adverse impact on production costs, and should be reconsidered due to the heightened social concern about the corresponding environmental drift [121]. As a consequence, the need to benefit from AMF as a biofertilizer, with a view to sustainable agriculture, is becoming increasingly urgent. An appropriate management of these symbiotic fungi would lead to a great reduction in chemical fertilizer and pesticide inputs, a key target for growers facing a crisis, and having to deal with a more environmentally aware clientele. Two main strategies are possible to achieve this goal: the direct re-introduction of an AMF pool (referred to as "inoculum") into the target soil, or the selective management of the target ecosystem. These strategies can be selectively adopted when a population of AMF propagules of low effectivity is present, or when the indigenous AMF are absent or very low. This means that the AMF restoration process is suitable for different purposes, e.g. greenhouse and open-field cultivation, and even in helping the rehabilitation of degraded lands.

3.1. AMF inoculation and the role of enterprises

The re-introduction of AMF into soils that are impoverished in belowground biodiversity is a complex strategy, but it can be very rewarding. Unfortunately, the production of AMF inoculum on a large-scale is very difficult using the techniques currently available. The main obstacle to the production of an AMF inoculum lies in their peculiar symbiotic behaviour, the AMF compulsorily requiring a host plant for growth. This means that AMF are propagated through cultivation with the host plant, and this usually requires time-demanding protocols and cumbersome infrastructures. The maintenance of AMF reference collections requires methodologies that are rather different from those used for other microbial collections and inoculum production. Unlike non-obligate symbionts, the production of AMF inoculum requires the control and optimization of both host growth and fungal development. Thus, these propagation techniques involve high costs that are not apparently competitive with fertiliza-

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tion-related costs. The impossibility of rapidly assessing AMF colonization on the host plant, together with the complexity of AMF species identification, also contribute to the pitfalls of inoculum agricultural usability. Moreover, the management of the high amount of inoculum necessary for extensive use is very challenging. It has been suggested that AMF is more suitable for plant production systems that involve a transplant stage, as inoculation is carried out more easily, and smaller quantities of inoculum are needed. At a first glance, establishing an open-field, large-scale inoculation treatment would seem technically impractical and economically prohibitive. However, once AMF biodiversity has been restored, AMF-friendly practices, such as fall cover cropping [122], can be put in place in order to help the AMF persist. If no detrimental agricultural practices are carried out, the biodiverse mycelial network will remain unaltered and infective in the future. For example, in revegetation schemes, it would be totally impractical to restore an entire degraded land, which often appears as a highly extended surface, through inoculation. A particular approach must be considered when it is necessary to face these situations. First, the ability of specific cover crop mixtures and even target indigenous plant species to elevate the native AMF inoculum has to be taken into account as a potentially successful selective management tool to aid the recovery of desertified ecosystems [123]. However, since ecosystem functioning is supported by a close liaison between the aboveground plant diversity and belowground AMF diversity [22], the excessive loss of AMF propagules in degraded ecosystems could, in some cases, preclude either natural or artificial revegetation. For this reason, an inoculation step may also be needed. Although it would be too laborious and expensive to re-introduce AMF and cover plants into entire lands, a smaller-scale approach should be adopted. Taking inspiration from the idea of creating the so-called "fertility islands" [124], only small patches of cover plants could be inoculated with AMF. This could lead, in time, but with reduced costs, to the re-establishment of a mycelial network that would also be able to allow native plant species to quickly recover the nutrient impoverished land.

Hence, AMF restoration would only represent an initial cost and, if soil AMF persistence is favoured, this cost could be subjected to amortization over the years. This makes the application of AMF particularly attractive since, as already demonstrated [125,126], it could provide considerable savings for growers and for degraded land recovery projects, in comparison to conventional fertilization. It is important that the end-users cultivate a portion of their crop without inoculum in order to assess the cost-effectiveness and the beneficial effects on plant fitness due to AMF inoculation [127]. Growers are starting to understand the significance of sustainable agricultural systems, and of reducing phosphorus inputs using AMF inocula, especially in the case of high value crops, such as potted ornamental plants. These crops can easily be regarded as the result of organic crop farming, and be sold at a premium price to an eco-friendly orientated consumer class. However, the absence of solid inoculation practices still represents a problem, and applied research should therefore be focused on defining the best inoculum formulation strategies [128] and imparting know-how to the growers.

Since large-scale AMF production is impractical for growers, the significance of AMF has not been ignored by the commercial sector, and many AMF-based inocula are nowadays available for sale. AMF inoculum production began in the 1980s and flourished in the 1990s. Nowadays,

several companies produce and sell AMF inocula. In recent years, these products have come under increasing scrutiny by scientists and end-users. Most manufacturers advertise their products by pointing out their suitability for a wide range of plants and environmental conditions. Unfortunately, their promises made about these products and the results seen are too often worlds apart. This has led to radical generalisations, both positive and negative, about the efficacy of the currently available products. The problem is that success, in terms of root colonization and plant response, is unpredictable since no plant does best with the same AMF mix [129]. In terms of fungal content, the manufacturer's tendency is to introduce a more or less biodiverse mix of AMF. Some companies have chosen the approach of single formulations, while others produce a range of differently shaped products for their target end-users. Glomeraceae species are usually used, but also Gigasporaceae, Scutellosporaceae and Acaulosporaceae families are gradually being introduced to commercial inoculum production. These few used species can be routinely propagated for spore applications, are found in association with a large variety of host plants and are geographically distributed all over the world.

Great problems arise in formulating the inoculum product in its most suitable state for the market. In the coming years, it is likely that greater regulation and controls will be introduced concerning the production and selling of AMF inocula. In Europe, the regulation of these products varies from country to country, with some having very strict regulations, while others are less demanding. In North America, Canada, for instance, considers AMF inocula to be only supplements and not fertilizers. In the USA, registration may fall either to the fertilizer or the pesticide sectors, depending on the supposed action of the formulated AMF inoculum. However, in most countries, AMF are no longer considered dangerous for human or animal health, and no infectivity or toxicity tests are therefore necessary. Normally, an application for registration has to be filled in and a series of meticulous information needs to be attached to the registration request. These data should also be reported on the inoculum label, and should include the list of all the ingredients and their concentrations, a detailed taxonomic description of the AMF, the isolate's history, the geographic origin and distribution, some literature on the beneficial effects of the isolate, a list of possible contaminants, an official safety data sheet, information about the producer, the number of viable AMF propagules or the percentage of colonization expected on reference plants after a known quantity is inoculated, the list of recommended plant hosts, the suggested soil conditions for inoculum effectiveness, the recommended application method/dosage, the suggested storage conditions, the expiration date and information on the manufacturing processes. Other information regarding previous tests performed with different soil, and which confirms the climatic conditions and the beneficial effect of the inoculum should also be added in order to highlight the reliability of the product and to help direct the consumer. Preventing over-regulation will be crucial in assisting the development of SMEs (Small and Medium Enterprises), and in helping refresh the market with this eco-friendly biotechnological tool.

In order to allow the AMF inoculum market to develop, scientists should define a series of 'best practices' that could be adopted by these SMEs to solve serious issues related to their product quality. One of these issues arises from the need to control the biological composition

of the product, especially for the possible presence of pathogens, but above all to assess its quality in terms of AMF composition. Being obligate symbionts, AMF are non-axenically culturable, while only a few can be monoxenically cultured. Therefore, an inoculum is produced above all using a containerized-culture, either in greenhouses, growth chambers, or in fields, and, as a result, cannot be completely free from external microorganisms. There is increasing awareness of the risk of pathogens, and many concerned producers are even making use of agrochemicals in an attempt to avoid contamination of their product. Others have instead decided not to include host root residues in their formulation, in order to avoid pathogen carry-over. Alternatively, surface sterilization of the incorporated colonized roots can be introduced without affecting the viability of the AMF propagules [130]. As far as quality control in terms of AMF composition is concerned, it is essential to verify whether the product effectively has the potential described on the label. With AMF, in order to confirm the fungal identity, such an assessment can be done through morphological identification of the spores [131,132]. Unfortunately, this technique requires a great deal of labor and there are very few experts in the world that are able to conduct a reliable identification solely on the basis of spore morphology [133]. Quick and user-friendly molecular techniques have been developed to detect AMF strains from complex matrices, such as soil [41,42] and AMF inocula [129,134]. The discrimination of AMF, on the basis of these techniques, relies almost completely on the sequencing of the ribosomal genes, the genetic region on which the AMF phylogenesis was constructed (4), and is still under debate [8–10]. Molecular techniques also allow the inoculated isolates to be reliably traced inside the host plant and their persistence in the soil to be established [135]. The use of Realtime qPCR and specific primers appears to be a very promising tool for the tracing of AMF isolates and their quantification in the host roots after application [136]. A recent study has even used laser microdissection to qualitatively monitor the arbuscule formation in *Camellia japonica* L., after inoculation with a highly biodiverse AMF inoculum [134]. Such a quality control is very important to exclude poor quality or defective AMF inocula from the market.

3.2. Key steps and current techniques for inoculum production

The actual inoculum propagation and formulation process entails a series of key steps that are crucial for the good quality of the final product. The most determining aspect of inoculum formulation is the choice of the AMF content. As mentioned before, the tendency is to introduce a mix of several AMF into commercial inocula. The most scientifically investigated AMF isolate, i.e. *Rhizophagus irregularis* DAOM197198 [137], is also one of the most frequently used for commercial inoculum formulation. This species is a very generalist symbiont that can colonize a large variety of host plants, survive long-term storage, is geographically distributed all over the world and, last but not least, adapts well to both in vivo and in vitro propagation. These characteristics make this isolate of *R. irregularis* suitable to be a premium component of commercial inocula. As previously mentioned, several other AMF that mainly belong to Glomeraceae species, but also to Gigasporaceae, Scutellosporaceae, and Acaulosporaceae families, are gradually being introduced into commercial inoculum production. It is important to notice that AMF are sometimes marketed as consortia that contain ectomycorrhizal fungi, saprophytic fungi and plant growth-promoting rhizobacteria (PGPR), in order to increase the

product potential for plant protection and production. The proper choice of the inoculum AMF content is unfortunately constrained by a lack of knowledge on the specificity of the relationships between a specific AMF strain and a particular crop, and on the compatibility and competition of the AMF strains for niches in the soil environment [128]. When AMF are examined as a community, there is abundant evidence that fungal growth rates can be host- and niche-specific. In reference [60], it has been suggested that partner specificity in AM symbiosis may occur at an ecological group level of both the plant and fungal partners. In [14], it has been demonstrated how reciprocal "rewards" stabilize cooperation between the host-plant and the fungus, thereby enforcing the best symbiotic combinations. Thus, the best way of finding the most cooperative and specific AMF isolates for the formulation of more targeted inocula is to directly screen what nature offers, by fathoming out the naturally occurring symbiotic combination set. For example, some AMF species are commonly recognized to be more stress tolerant than others, and are usually found in stressed and polluted soils [18,138]. Native AMF from areas affected by osmotic stresses can potentially cope with salt stress in a more efficient way than other fungi [139]. Thus, it is preferable to take this into account when "tuning" an inoculum to a particular kind of degraded/stressed soil and in order to avoid failure of the revegetation process [140,141]. Optimal benefits will only be obtained from inoculation after a careful selection of the favorable host/niche/fungus combinations. For this reason, natural or semi-natural ecosystems, in which the desired host plant is well established, represent a valid source of naturally selected AMF. However, this highly selective inoculum formulation requires time and hard work. An intriguing approach would be to formulate a series of highly biodiverse inocula, including several AMF species/strains of different geographical/environmental origin, which would be capable of offering benefits to multiple host plants under different environmental conditions, thus making researchers switch from looking for a superstrain to formulating a superinoculum.

AMF can use a number of different types of propagules to colonize new roots with different degrees of efficiency [142]. These are components of the extraradical and intraradical phase of AMF. The extraradical phase comprises spores and a mycelium that forms the hyphal network. Several fungal structures, inside both living and dead root fragments, can represent a source of inoculum [143]. Vesicles, in particular, have been shown to be very infective [97]. Considering that a number of different propagule types exist, it is of primary importance to determine the most eligible and user-friendly to be adopted as inoculum sources. Unfortunately, this is more complex than may be expected, since different AMF taxonomical ranks differ in their ability to propagate from a given propagule. As already mentioned, for instance, it seems that propagation through mycelial fragmentation may be more important for species of the Glomeraceae family, whereas spore germination may be the preferential type of propagation for species in other families (e.g. Gigasporaceae). In reference [144], the authors tested the establishment of a biodiverse community of AMF in a pot culture using different sources of inoculum from the field. They found that spores were successful in establishing most species of Acaulosporaceae, Gigasporaceae and Scutellosporaceae, whereas Glomeraceae species were only dominant when root fragments or soil cores were used. It is important to consider that these different propagation strategies can also reflect on the potential agricultural use of a particular AMF inoculum.

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Once the AMF content has been selected, pure monospecific cultures are normally obtained from a single spore, or a small piece of colonized root fragment, or mycelium collected directly from field plants, or obtained from AMF collection cultures. The AMF propagule spreads and colonizes the root apparatus of the host plant, and the subsequent pot-culture generations lead to the production of high quantities of AMF inoculum. Several organizations throughout the world have research culture collections (The International Culture Collection of VA Mycorrhizal Fungi, INVAM; The Banque Européenne des Glomales, BEG; The Canadian National Mycological Herbarium, DAOM; The Canadian Collection of Fungal Cultures, CCFC; The non-profit Biological Resource Center ATCC; The Glomeromycota In Vitro Collection, GINCO; NIAS, National Institute of Agribiological Science) and provide users with reliable AMF propagules to start propagation. Moreover, detailed information on species origin and distribution, spore morphology, and molecular biology and biochemistry are often provided by these organizations. The common purpose of these available AMF collections is to provide a stock source of pure and reliable material for fundamental and applied research use.

A pivotal step during AMF inoculum propagation is the choice of an adequate host plant. The criteria required for the host plant are its high mycorrhizal dependency and potential, i.e. its capacity of being highly colonized by a high number of AMF species, and its inclination to promote growth and sporulation, its suitability to grow under growth chamber or greenhouse conditions and its production of an extensive root system with a high number of fine feeder roots in a short time. A series of plants are commonly recognized as actual AMF "trap" plants, due to their mycorrhizal dependency and lack of specificity, and they are routinely used as host plants during propagation. These include clover (*Trifolium* spp.), plantains (*Plantago* spp.), ryegrass (*Lolium perenne* L.), the tobacco plant (*Nicotiana tabacum* L.), leek (*Allium porrum* L.), Sudan grass (*Sorghum bicolor* (L.) Moench), corn (*Zea mays* L.) and bahia grass (*Paspalum notatum* Flugge).

Pasteurization, steaming and/or irradiation are necessary to avoid contamination of the growing media. The use of a well-aerated substrate is also recommended. The manufacturer must provide the customer who intends to introduce the AMF inoculum to a target plant with basic information and assistance concerning its chemical and physical characteristics, such as nutrient content, pH and salinity. In particular, when elevated quantities of inoculum are used in agricultural fields, or in a pot-culture, controlling the nutrient content is of crucial importance, as it might lead growers to rethink their normally adopted fertilization practices. Conventionally, inoculum formulation processing consists of sieving the substrate and chopped roots of the trap plant in order to retrieve AMF propagules that can be included in the inoculum. This means that the carry-over of a certain amount of nutrients to the final product is unavoidable. Nevertheless, if trap plant pots are not over-fertilized, as it should be during inoculum formulation, the nutrient content will be negligible. A solution to the problem could be the laborious approach of completely separating the spores, mycelium and colonized trap plant root fragments from the used growing media. These substrate-free propagules could then be mixed with an inert-like carrier at a desired rate. The amendment of the inoculum should be compatible with the AMF, almost inert and only serve to support mycorrhizal development. Optimum P and N, but also other macroelement levels, have to be tuned to

specific plant–AMF combinations, as mentioned in the previous section, in order not to reduce AMF propagation and diminish plant dependency on mycorrhization after inoculation. Other edaphic factors, such as pH, salinity, soil temperature, moisture and soil aeration, should also be controlled to optimize AMF inoculation. Since the inadequacy of the nutrient composition dramatically affects AMF development, conventional soil analyses should be performed on the formulated inoculum, in independent official laboratories, as a quality control step. This way, the manufacturer will be provided with a certificate that guarantees the customers the validity of the data reported on the label and, therefore, enhances the quality of the inoculum. During experimental tests on the beneficial effects of inoculants, researchers often adopt an important practice in order to be able to differentiate between the effects of the inoculum carrier and the AMF portion, i.e. the use of a sterilized inoculum as a control, the so-called "mock" inoculum [145]. This practice of including a non-inoculated and a "mock" inoculated control should be considered by end-users who are willing to assess the eventual beneficial effect of AMF inoculation.

A few alternatives to the pot-culture method are available, regarding inoculum production and formulation. Other soilless culture systems, such as aeroponics and hydroponics, enable the production of pure clean spores and maximize growing conditions for the host plant [146]. Aeroponic inoculum production has long been scientifically validated [147,148], and could soon reach massive commercialization levels. Root-organ monoxenic culture is another method that allows the successful large-scale propagation of AMF which can be used directly as an inoculum. Unfortunately, the protocol for this method of propagation is not easily adjustable to all AMF strains. So far, several dozens of AMF species and strains have been propagated in vitro with the right synthetic growth medium and growth conditions. This type of culture consists of AMF inoculated excised roots (often *Daucus carota* L.) that have acquired the ability to uncontrollably proliferate, without the epigeous portion, after transformation with an *Agrobacterium rhizogenes* Conn. strain. This method of propagation does not require high specialization, and facilitates the control of AMF strain purity. As mentioned before, it is suitable for large-scale production, as a massive number of spores (several thousand), mycelium and colonized roots [149] can be obtained from one Petri dish in just 4 months, and from the consecutive subcultures [150]. AMF propagated with this technique have been shown to successfully re-colonize plant roots [151,152]. A possible further advantage of the AMF inoculum production process could be the use of bioreactors with liquid transformed root-organ cultures aimed at the large-scale propagation of AMF [153]. These tools may become suitable for commercialization in the near future and will lead to reduced labor and enhanced automation. However, as the AMF are produced in association with transformed roots, the product will only be intended for research use and may not be used for open-field inoculation.

The final product could become available on the market as a powder or granular substrate made from mixed inert-like materials, such as peat, compost, vermiculite, perlite, quartz sand, micronized zeolite and expanded clay, where colonized root fragments (1-5 mm long), spores and hyphal networks are uniformly distributed. Liquid inocula, dedicated to horticultural use, obtained from a hydroponic culture, or from a spore/mycelium suspension in a liquid carrier, represent a possible alternative final product [154]. As a final step before commercialization,

the AMF composition should be characterized in order to control inoculum purity and to trace the inoculated strains. This prevents poor quality inocula from being put on the market.

The storage methodologies should preserve a product's high and consistent quality, and be simple and inexpensive at the same time. AMF viability and efficiency can be maintained for several months at room temperature (20-25°C), but the inocula must be kept in their packaging and must be partially dried. The main inconvenience that could occur during the storage period is that spores can sometimes become dormant, thus decreasing germination rates drastically [155]. However, a cold-storage period could be used to break dormancy [156]. Longer-term storage of liquid or dry inocula could be conducted at 5°C for both in vivo and in vitro propagated AMF [127]. Research culture collections are often stored using more sophisticated and expensive preservation techniques. These include the maintenance of monospecific inocula on living host plants (with regular molecular checks regarding the AMF identity), or alginate bead mediated encapsulation-drying and cryopreservation [157,158].

4. Perspectives

Future research in this field will have to concern the formulation of AMF isolate collections, with comprehensive information on host-preference, edaphic and climatic adaptation, and stress and disturbance tolerance. This will help manufacturers address their product towards different uses, including agricultural use, as well as new fields of application, such as the green architecture of urban sites [159]. At the same time, farmers will have to begin asking for assistance from experts in the field when introducing AMF to their cropping systems. Scientists should also carry out large-scale multi-location field trials, and conduct cost-benefit analyses, in order to increase awareness among the end-users of AMF inocula.

By 2050, global agriculture will have the task of doubling food production in order to feed the world [160]. At the same time, dependence on inorganic fertilizers and pesticides must be reduced. For these reasons, significant advances in AMF research are needed to allow their stable use in agriculture. Their application and synergistic combination with other functionally efficient microbial consortia that include PGPR (Plant Growth Promoting Rhizobacteria), saprophytic fungi and other helper microorganisms [161], will help farmers develop a more sustainable cropping system.

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