The future has roots in the past: the ideas and scientists that shaped mycorrhizal research

Tansley 2018

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Summary

Our knowledge of mycorrhizas dates back to at least 150 years ago, when the plant pathologists A.B. Frank and G. Gibelli described the surprisingly morphology of forest tree roots surrounded by a fungal mantle. Compared with this history, our molecular study of mycorrhizas remains a young science. To trace the history of mycorrhizal research, from its roots in the distant past, to the present and the future, this review outlines a few topics that were already central in the nineteenth century and were seminal in revealing the biological meaning of mycorrhizal associations. These include investigations of nutrient exchange between partners, plant responses to mycorrhizal fungi, and the identity and evolution of mycorrhizal symbionts, as just a few examples of how the most recent molecular studies of mycorrhizal biology sprouted from the roots of past research. In addition to clarifying the ecological role of mycorrhizas, some of the recent results have changed the perception of the relevance of mycorrhizas in the scientific community, and in the whole society. Looking to past knowledge while foreseeing strategies for the next steps can help us catch a glimpse of the future of mycorrhizal research.

Introduction

There are many ways to speak about the past. In this review, rather than presenting a detailed analysis of the minutiae of ever-changing scientific results, I chose to speak at a more general level, to address mycorrhizal research from a historical and conceptual perspective, based on my experience in the field.

Starting at the end of the nineteenth century, researchers in the traditional fields of mycology and botany became more and more aware of the relevance of plant–fungal symbioses. One hundred and fifty years later, researchers still discuss issues in plant–fungal interactions and pose substantial questions in ecology, evolution, microbiology, plant pathology, agronomy and forestry sciences, as well as in applied economy and bioinformatics, just to name some of the relevant fields.

This interdisciplinary interest, fed by different experimental approaches, mirrors the awareness that the umbrella term 'mycorrhiza' covers a huge number of biological systems that include most plant species. According to Brundrett and Tedersoo (2018), 72% of vascular plants are arbuscular mycorrhizal (where Glomeromycotina fungi form inter-intracellular hyphal networks within the roots), 2.0% are ectomycorrhizal (where fungi of the Ascomycota or Basidiomycota produce a mantle surrounding the root tip as well as an intercellular hyphal network between the root epidermal and cortical cells), 1.5% are ericoid mycorrhizal (where mostly Ascomycota form coils inside the epidermal cells of the thin roots of Ericales) and 10% are orchid mycorrhizal (where mostly Basidiomycota colonize the cortical cells of orchid protocorms and roots). Just 8% of plants are completely nonmycorrhizal, and 7% have inconsistent nonmycorrhizal-arbuscular mycorrhizal associations. The "State of the World's Plants" report (2017, https://www.kew.org/science/who-we-are-and-what-we-do/strategic-outputs-2020/state-of-the-worlds-plants) lists about 391,000 species of vascular plants currently known to science; therefore, we can conclude that the number of mycorrhizal plant species ranges from 320,000 to 340,000, also taking into account that many non-vascular plants, like liverworts, interact with mycorrhizal fungi. All these plants associate with more than 50,000 fungal species (van der Heijden et al, 2015) and appear equally successful in colonizing different environments, from alpine and boreal zones to tropical forests and grasslands.
The impressive biodiversity revealed by these figures makes mycorrhizal association one of the most relevant biological processes of our planet, opening the question of how to understand and explore the complexities of fungal–plant interactions. Traditionally, mycorrhizas have been investigated following two main trajectories (O. Ferlian et al., unpublished): first, the ecological

104 one, which has developed crucial concepts, *i.e.* the demonstration that mycorrhizal symbiosis is a
105 determinant of plant biodiversity, and ecosystem variability (van der Heijden et al, 1998), controls
106 plant productivity, nitrogen, and phosphorus cycles, as well as soil aggregation and seedling
107 survival (van der Heijden et al, 2015). These concepts have produced potential applications such as
108 using mycorrhizas as powerful tools for sustainable agriculture focused on lower chemical inputs
109 and improved food security (Rodriguez and Sanders, 2015), as well as for preserving forest
110 ecosystems (Courty et al., 2010). In a second trajectory, another enormous wealth of data has been
111 developed under laboratory conditions trying to understand the mechanisms behind the complexity
112 of in-field associations. According to the classical biological reductionistic methods, cellular,
113 molecular, and physiological approaches have been applied to simpler biological systems, usually
114 represented by one plant species colonized by one fungal species.
115 The aim of this review is to draw up a map of the ideas (and to highlight the scientists) that changed
116 mycorrhizal research, searching for topics that were central in the nineteenth century, became
117 seminal in revealing the biological meaning of mycorrhizal associations along the decades, and are
118 still crucial today in the 'omics' era. The selection of these issues is the fruit of a personal
119 perspective, which mostly mirrors my main scientific interests.

120

121 **The portraits of our ancestors: a gallery of ideas from more than one hundred years of**
122 **mycorrhizal research**

123 A journey to the past of mycorrhizal research must start with the scientists who produced the basis
124 of our current knowledge; as in all other scientific fields, the most recent molecular studies of
125 mycorrhizal biology were developed on the shoulders of work by researchers in the past. In this
126 context, Albert Bernard Frank (1839–1900) is probably the most famous: he was the first to
127 recognize the widespread nature of the associations between plant roots and ectomycorrhizal fungi
128 (Trappe, 2005 for original quotations), even if other scientists provided contributions on other
129 plant- fungal associations in the same period. Franz Kamienski (1851-1912) showed that
130 *Monotropa hypopitys* was nourished by fungi associated with the roots of neighboring trees (Berch
131 et al., 2005), while Noël Bernard (1874-1911) established that *Neottia nidus-avis* needs for a
132 fungus, which is also forming mycorrhizae in adults, for seed germination (Selosse, 2017). Surely
133 Giuseppe Gibelli (1831–1898), professor of Botany at the University of Modena and then of
134 Torino, is worthy of being acknowledged: he provided beautiful drawings of chestnuts roots (Fig.

1), where the features of the mycorrhizal structures are finely described. In Torino some years later, Oreste Mattirolo (1856-1947) hypothesized that truffles were ectomycorrhizal fungi, as witnessed by a painting in the Botanical Garden of Torino (Fig. 1). Many years passed before truffles – so loved in the cuisines of Mediterranean countries were acknowledged as true symbionts (Fontana and Palenzona, 1969; Martin et al 2010).

Inspired by the forests of larches and birches in the north of Europe, Elias Melin (1889–1979) laid the foundation for study of the physiology of ectomycorrhizas in trees: his observations on drained peat bogs led to the conclusion that coniferous and dicot seedlings require ectomycorrhizal associations for normal growth and development. After this discovery, Melin devoted his life to studying the structure, function, and importance of the ectomycorrhizal fungi (Lindeberg, 1989 and references quoted therein), moving from a description of the process in nature to experimental validation (Melin and Nilsson, 1950). Thanks to a protocol that allowed him to produce ectomycorrhizal symbioses under sterile conditions, he examined the competition for available nitrogen between soil microorganisms and plant roots, and suggested that ectomycorrhizal fungi primarily facilitate nitrogen uptake.

Under the direction of Professor Melin, Uppsala became an attractive centre for many students and researchers. Among them, Erik Bjorkman (1912-1973) developed his carbohydrate theory: under controlled conditions, he found a positive correlation between carbohydrate concentration in the roots and frequency of mycorrhizas in conifers. Increased illumination, combined with moderate deficiencies in nitrogen and phosphorus, resulted in increased carbohydrate concentrations in the pine roots and increased mycorrhizal infection. Bjorkman's theory, although criticised, inspired many other researchers (Lindeberg, 1989), such as Edward Hacskaylo (1925–2018) and Sagar Krupa. Indeed, the source–sink relationships in mycorrhizas represent an issue that has been often investigated (Allen et al, 1981; Reid et al, 1983), but photosynthesis in mycorrhizal plants remains to be fully clarified using advanced molecular techniques or computational models (Norby et al, 2016).

In the fifties, a new star was rising in the world of mycorrhizas: John Laker Harley (1911–1990), Professor of Forest Science at the University of Oxford from 1969 to 1979, and Fellow of St. John's College, Oxford. He was the giant who developed the biology of mycorrhizas, wrote the first book devoted to mycorrhiza, *The Biology of Mycorrhiza* (1959), and who, as the editor of *New Phytologist*, launched the journal as *the* place for publication of work on mycorrhizas. Thanks to his unforgettable personality and his productive school (just to drop a couple of names, his students included Sir David Read and Prof. Sally Smith, editors of the reference book Smith and Read,

168 1997), mycorrhizas were acknowledged all over the world, and interest in these plant–fungus
169 interactions no longer remained restricted to a limited community. The transfer of phosphate from
170 ectomycorrhizal fungi to their tree host was one of his main interests and many publications bear
171 witness to his experimental vision, which has provided strong experimental support to the concept
172 of nutrient exchange between mycorrhizal partners (Harley and Mccready 1952; Harley and
173 Brierley 1955). Indeed, with today's eyes, it seems that David Read transferred the wealth of
174 experimental data of his laboratory into an ecological vision, where different plant communities
175 have a dominant mycorrhizal type, depending on the physiological capacity of the symbiotic fungi
176 (Read 1991).
177 At the end of the 19th century, the roots of forest trees were not the only ones to be dug, studied,
178 and characterised. Many herbs and flowering plants were investigated, as detailed by Koide and
179 Mosse in their excellent "A history of research on arbuscular mycorrhiza" (Koide and Mosse,
180 2004). Pioneering observations on the so-called endotrophic mycorrhizas were conducted, initially
181 by P.A. Dangeard (1862-1947), J.M. Janse (1860-1938), L.Petri (1875-1946) and J. Gallaud (1904)
182 (Fig. 2), and later continued by MH Rayner (1890-1948). The terminology we still use today comes
183 from those observations: for example, J. Gallaud was the first to distinguish between Arum and
184 Paris types of arbuscules (Smith and Smith 2007, Dickson 2004). Beniamino Peyronel (1890-1975)
185 also played a crucial role, as he was one of the first to detect the so-called double infection, the
186 presence of the endomycorrhizal fungus coupled with other endophytic fungi. I refer the interested
187 reader to Koide and Mosse (2004), which contains a full reference list and extensive original
188 quotations.
189 Irrespective of the huge number of descriptions, many years were required before researchers
190 produced an experimental demonstration of the role played by these endophytic fungi we now
191 identify as arbuscular mycorrhizal fungi. Barbara Mosse (1962) was one of the first to reach this
192 objective; she used sporocarps of the fungus that now bears her name (*Funneliformis mosseae*) to
193 inoculate plants growing in autoclaved soil, and demonstrated a growth effect. Similarly,
194 Gerdemann (1964) grew plants in steamed soil, inoculated them with sporocarps (and treated the
195 control plants with sporocarp washings), setting up a protocol that became the standard for
196 mycorrhization experiments. It was becoming clear that arbuscular mycorrhizal fungi were not
197 parasites, as previously suggested (see Koide and Mosse, 2004).
198 A meeting organized in Leeds in 1974 (Fig. 3) by Sanders, Mosse, and Tinker (1975) brought
199 together, for the first time, scientists who were active in research on arbuscular mycorrhizal fungi,
200 as well as in ericoid and orchid mycorrhizas. Looking at the titles of the presentations, we

immediately perceive that the main issues under debate today (evolution and classification of the endophytes, nutrient exchange, systemic effects, multiple interactions) were already a major focus. The transfer of phosphate from the fungus to the plant was one of the first topics; more than one hundred pages of the book are devoted to this matter, covering theoretical aspects related to membrane structure, the accumulation of phosphate at the tips of mycorrhizal clover, as well as the quantification of phosphate flow in the extraradical hyphae. Many of these reports had their foundation in a very important paper published by Sander and Tinker in *Nature* (1973), where the authors calculated the phosphate inflow in mycorrhizal *vs.* control onion roots.

Twenty years had to pass before another seminal step occurred: the identification of the phosphate transporter in *Glomus versiforme* by Maria Harrison and Marianne van Buuren (1995). This study revealed the molecular basis of phosphate uptake by the fungus and described the first step of phosphate's journey from the soil to the fungus to the plant. The further physiological and molecular characterization of the arbuscular mycorrhizal-inducible plant phosphate transporters represented another milestone; thanks to many groups (among them, those led by Marcel Bucher, Sally Smith, Iver Jakobsen, and again Maria Harrison), we learnt about the symbiotic phosphate pathway (Harrison et al, 2002; Bucher 2007, Smith et al, 2011). This road is still open: phosphate is a hub where symbiotic fungal needs (Ezawa and Saito, 2018), mycorrhization, plant development, regulation of transporters, and hormones are interconnected, and such networks have yet to be completely deciphered.

In another important meeting held in Quebec some years later (1980) and organized by a scientist with a large vision of biology, J. Andre Fortin, in the context of the North American Conference on Mycorrhizas, an accurate mycorrhizal atlas was already available. The main mycorrhizal typologies were associated to specific pairs of partners with a strong, increasing interest in the ecological aspects of mycorrhizal symbiosis.

Even if largely incomplete, this gallery of scientists reflects the evolution of some ideas in mycorrhizal research: in less than one hundred years, times were already mature for discussing not only the descriptive aspects, but also for trying to understand the mechanisms operating at the ecological and molecular levels. A good example of the ideas stemmed from the knowledge already available in the eighties is provided by the powerful concept of interaction networks (Toju et al 2014, van der Heijden et al., 2015): plants associate with their dominant mycorrhizal fungi forming separate underground networks. However, borders are not so clear-cut: in temperate forests, ectomycorrhizal trees often harbour an understory of shrubs (e.g. *Vaccinium*) that bear ericoid mycorrhizal fungi. In these forests, fungi may form both ectomycorrhizal and ericoid mycorrhizal

associations, meaning that there might be linkages between the different fungal networks (van der Heijden et al., 2015). Ecology has put the taxonomic, anatomical, and physiological data into a novel, broader context.

Mycorrhizal fungi in the 'omics' era: first puzzle, how to name mycorrhizal fungi

Giving a name to the object of your biological investigation is a substantial starting point. For many years, ectomycorrhizal fungi were identified on the basis of the fruiting bodies from which they had been isolated, while collecting mycorrhizas in nature often led to a situation resembling the famous Latin expression "*mater semper certa est, pater numquam*" (the mother is always certain, but not the father). Indeed, there were no doubts on the name of the plant, while the name of the associated ectomycorrhizal fungus was always a difficult gamble. There were exceptions: *Cenococcum geophilum* with its black mycorrhizal mantle was very easy, even for mycologists with little experience, even if Douhan and colleagues (2007) later revealed that *C. geophilum* is indeed a species complex.

Applying molecular techniques, which were first focused on RNA ribosomal genes, allowed researchers to identify fungal symbionts with greater confidence (White et al, 1989; Gardes and Bruns, 1993), quickly moving from identification and diagnostic aims to more general issues of biodiversity and molecular ecology. A further crucial step was the genome sequencing of mycorrhizal fungi. The *Laccaria bicolor* genome (Martin et al, 2008) opened a window to an unknown world: the 65-megabase genome revealed unexpected features, most notably a battery of effector-type small secreted proteins, several of which are only expressed in symbiotic tissues. Another noteworthy finding was the lack of carbohydrate-active enzymes involved in degradation of plant cell walls. The capacity to degrade non-plant cell wall polysaccharides suggested a dual saprotrophic and biotrophic lifestyle of the mycorrhizal fungus, enabling it to grow in the soil and in living plant roots. The availability of a fungal genome provided tools to understand for the first time the processes by which symbionts interact with plants within their ecosystem.

On the wave of success of the *Laccaria* and truffle (Martin et al, 2010) genomes, Francis Martin promoted the Joint Genome Institute Mycorrhizal Genomics Initiative, which aimed to sequence phylogenetically and ecologically diverse mycorrhizal fungi (Basidiomycota and Ascomycota), including the major clades of symbiotic species associating with trees and woody shrubs (https://genome.jgi.doe.gov/Mycorrhizal_fungi/Mycorrhizal_fungi.info.html). Thanks to this huge wealth of data, many mechanistic answers were offered to explain the strategies of ectomycorrhizal

fungi, *i.e*. the loss of genes involved in plant cell wall degradation, and the acquisition of many small secreted proteins, as a tool to interact with the host (Martin et al., 2016).

Phylogenomics was also used to reconstruct the evolution of plant-interacting fungal groups and to trace their common origins. For example, Kohler et al. (2015) demonstrated how ectomycorrhizal fungi evolved to depend on their green hosts, but in order to exploit the protective plant niche, they had to lose the potential to degrade lignocellulose compounds. Interestingly, these ectomycorrhizal features emerged with similar genetic adaptations across multiple clades, including symbiotic fungi with fully diverse strategies, as those colonizing heathers and orchids. So, thanks to this balance between losses and gains, they acquired evolutionary stability. Parallel to the evolution of ideas, the Linnean names of many sequenced fungi changed; for example, *Pezizella ericae*, which I investigated 40 years ago together with Vivienne Gianinazzi Pearson (1979), is now called *Rhizoscyphus ericae*.

Giving a name to arbuscular mycorrhizal fungi has been an endless tale that has stretched over many years. As Koide and Mosse (2004) summarize, these symbiotic fungi were first called 'phycomycetoid fungi' to distinguish them from those living inside the Ericaceae or Orchidaceae. The name was related to the morphology of the fungi, which are aseptate and coenocytic. Peyronel (1923, 1924) was one of the first to assign arbuscular mycorrhizal fungi to a defined taxon. He was successful in tracing the extraradical hyphae from mycorrhizal roots to spores of *Endogone fuegiana*, *E. vesiculifera*, and other *Endogone* species. As a consequence, the hypothesis was that phycomycetoid fungi were Zygomycetes-related, since on the basis of their zygospores and chlamydospores, Endogonaceae were placed inside the Mucorales (Koide and Mosse, 2004). The name was successful; for example, in the Leeds meeting many years later, arbuscular mycorrhizal fungi were listed as *Endogone*-related (Sanders et al., 1975). Gerdeman and Trappe (1974) provided a very detailed list of the more common arbuscular mycorrhizal fungal genera and wisely commented that their revision of Endogonaceae (Endogonales, Zygomycetes) offered a temporary solution to a difficult taxonomic problem.

Many new genera and families were proposed over the years, but only the advent of molecular tools enabled the development of new ideas. Schußler et al. (2001) established the relationships among arbuscular mycorrhizal fungi and between them and the other fungi: arbuscular mycorrhizal fungi were identified as a phylum (Glomeromycota), which was a sister group to Ascomycota and Basidiomycota. Even if we have used these concepts for years, many aspects were not fully clear: for example, *Endogone* did not group with the Glomeromycota, but *Geosyphon pyriforme*, a very peculiar fungus, was added to the Glomeromycota, even if it does not produce arbuscular

mycorrhizal symbioses. Lastly, Zygomycetes turned out to be a polyphyletic group, without a defined taxonomic meaning.

As for the ectomycorrhizal fungi, the sequenced genomes gave the green light to clear analysis of phylogenetic relationships. As already suggested by the mitochondrial genomes (Lee and Young, 2009; Pelin et al 2012), examination of the genome of *Rhizophagus irregularis* (Lin et al 2014; Tisserant et al, 2013) revealed closer relationships with Mucoromycota than with the Dikarya. The decisive word arrived with a phylogenomics analysis performed by Spatafora and colleagues (2016). Thanks to the sequencing of new genomes, their study clearly indicated that Mucoromycota constitutes a phylum with three subphyla, Mucoromycotina, Mortierellomycotina, and Glomeromycotina: this study unambiguously defines the phylogenetic position of arbuscular mycorrhizal fungi. It is true that our arbuscular mycorrhizal fungi are now relegated from phylum status to a lower subphylum level, but many shared phenotypic features among the three subphyla, for example the presence of endobacteria (Bonfante and Desiro 2017), have provided a good rationale for explaining similarities (hyphal morphology) and dissimilarities (nutritional styles) among these enigmatic fungi.

We can conclude this endless tale by claiming that one hundred years ago (1923) Beniamino Peyronel, who looked at the coenocitic hyphae running between mycorrhizal roots and the spores of *Endogone*-like fungi, was not so far from our current views.

Signalling: a central question of our time?

One of the major questions of the community studying plant–microbe interactions is the nature of the signals exchanged between the partners and how they are perceived. Oliveira Chagas and colleagues (2018) compiled an exhaustive list of the molecules so far identified as involved in plant–microbe interactions. However, and probably differently from our expectations, the scientists previously quoted in the portrait gallery were already well aware of the crucial role played by the early events where unknown molecules act as the driving factors. For example, one of Elias Melin's interests focussed on the root exudates that stimulate the growth of ectomycorrhizal fungi, *i.e.* the so called 'M-factor' (Melin, 1954). The effect of this factor was demonstrated in experiments where excised pine roots, cultivated in tissue culture, were placed on the surface of nutrient agar that contained suspensions of finely divided mycelia of different ectomycorrhizal fungi. The stimulating effect of the root exudate on the fungi was very evident. Several attempts were made to purify and characterize the active principle but, much to Melin's disappointment, these attempts produced no definitive results (Lindeberg 1989).

With a very similar approach, Hepper and Mosse (1974) studied the interaction between their arbuscular mycorrhizal fungi (*F. mosseae*, at that time called *Endogone*) and root organ cultures of *Trifolium pratense*. The pre-germinated spores were stimulated by exudates diffusing from the growing roots in the absence of any physical contact (Fig. 4) The stimulation of hyphal branching was impressive. While similar observations were nicely confirmed by the group of Manuela Giovannetti in Pisa (Giovannetti et al, 1993), only many years later did other studies identify the plant bioactive molecules that stimulate the branching and metabolism of pre-symbiotic hyphae in arbuscular mycorrhizal fungi as strigolactones (Akiyama et al., 2005; Besserer et al., 2006).

Strigolactones derive from carotenoid metabolism (Al-Babili and Bouwmeester, 2015) and were first studied as root-exuded molecules that elicit the germination of parasitic plants (Cook et al., 1966). Later, strigolactones emerged as key plant hormones that control several aspects of plant biology and physiology, such as the repression of shoot branching (Waters et al. 2017). Strigolactone production is conserved from Charales to Embryophytes (Delaux et al., 2012), suggesting that their function in the rhizosphere probably came about as a secondary feature of their active release from the roots into the soil (Kretzschmar et al., 2012, Bonfante and Genre, 2015).

Despite the emerging understanding of the role of strigolactones, the molecular mechanisms underlying the hyphal branching of arbuscular mycorrhizal fungi, first observed by Hepper and Mosse (1974), remain poorly known (Lanfranco et al., 2018b). Data from RNA sequencing of germinated spores of *Gigaspora margarita* after a treatment with the synthetic strigolactone analogue GR24 confirmed the findings of Besserer and colleagues (2006, 2008), revealing the up-upregulated expression of mitochondrial genes (Salvioli et al., 2016) as well as of some genes related to cell wall components (encoding chitin deacetylase, chitin synthase). The data suggest that not only the mitochondrion, but also other fungal compartments are sensitive to strigolactones. However, despite the availability of sequenced genomes of arbuscular mycorrhizal fungi, the fungal strigolactone receptor has not yet been identified.

Along the same line, after many years, the M factor acting on ectomycorrhizal fungi is still waiting to be identified, even if some flavonoids, such as rutin and quercetin, have been hypothesized to be sensed by ectomycorrhizal fungi (Lagrange et al, 2001). By contrast, many molecules of fungal origin that are perceived by the host plant have been investigated in both ectomycorrhizal and arbuscular mycorrhizal fungi. Ditengou and colleagues (2015) demonstrated that *Laccaria bicolor* emits volatiles, identified as sesquiterpenes, during the interaction with host and non-host plants (poplar and Arabidopsis, respectively). The main effect of the volatiles, together with fungal auxin, is to induce lateral root formation, which is the first developmental cue to induce mycorrhization.

As reviewed by Martin et al (2016), other active components of the molecular dialogue in ectomycorrhizal symbioses are the fungal mycorrhiza-induced small secreted proteins, which interact with plant hormone receptors, thus altering root development.

The molecules that are released by arbuscular mycorrhizal fungi and interact with the plant host during the presymbiotic phase are mainly chitin-related. Chitin is a crucial cell wall component of arbuscular mycorrhizal fungi, and changes its structural organization during the fungal life cycle (Bonfante-Fasolo 1988). In most spores of arbuscular mycorrhizal fungi, chitin is laid down in fibrils that are spatially organized with a particular helicoidal form (Fig 5), but this organization is lost during germination, when chitin morphology changes together with alterations of the cell wall. Moving from the extra to the intraradical phase, the wall becomes progressively thinner and thinner, and at the end in the arbuscular branches, the wall is barely present, with an amorphous structure, and chitin fibrils are no longer detectable, even if chitooligomers are still present (Bonfante et al 1990 a).

To explain these observations, I hypothesized, with the self-confidence of a young researcher (Bonfante-Fasolo 1988), that i) the fungal wall might release chitooligomers that act as signals for the plant during the intraradical phase, and ii) that the fungal wall was thinning due to the activity of a plant chitinase. The second hypothesis was surely wrong, but thanks to the collaboration of Thomas Boller in Basel, an outstanding expert in chitinases, and of Pietro Spanu, at that time an undergraduate student in Torino, we performed a detailed study of plant chitinase expression, revealing that it was limited to the early moments of the interaction (Spanu et al, 1989). This observation has been largely confirmed by the detection of many chitinases and pathogenesis-related proteins in transcriptomic studies (Giovannetti et al, 2015, Fiorilli et al., 2015) and has provided the basis of novel ideas, *i.e.* that the fungus induces priming in the host plant, thus activating a range of molecules related to innate immunity (including chitinases), thereby raising the basal level of defences in the plant (Pozo and Azcón-Aguilar, 2007; Martinez et al, 2017; Chialva et al, 2018).

Currently, we know that lipochitooligosaccharides and chitooligomers act as signalling molecules in the pre-symbiotic phase, eliciting calcium spiking, a key part of the symbiotic pathway involved in the initial stages of root colonization (Maillet et al 2011, Genre et al., 2013; Sun et al., 2015). The discovery that GR24 treatment led to an increase in the release of chitin oligomers (Genre et al., 2013) by arbuscular mycorrhizal fungi and, subsequently, to amplification of the calcium spiking response, offered the first experimental evidence of the interaction between the signalling molecules released by the fungal and plant partners (Bonfante and Genre, 2015). However, the origin of the

fungal bioactive chitooligomers and lipochitooligomers is largely unknown. Are they the degradation product of chitin thanks to fungal/plant chitinases, or are they the product of specific catabolic pathways that lead to short oligosaccharides?

Chitin is a double-faced molecule: on the one hand it may act as signal for symbiosis, but on the other, pathogenic fungi also release chitooligomers (Sánchez-Vallet et al 2015). The way in which plants manage to understand the origin and the length of the chitin fragments thanks to a plethora of chitin receptors is the focus of many recent reviews (Barker et al 2017, Zipfel and Oldroyd 2017). In the context of arbuscular mycorrhizal fungi signalling, Miyata et al., 2014 Zhang et al., 2015 Carotenuto et al (2017) revealed that the rice lysin-motif receptor-like kinase OsCERK1 plays a central role in perceiving the short-chain chitooligomer signals and activating the downstream conserved symbiotic signal transduction pathway. Could chitooligomers have a role in modulating plant responses also during the intracellular colonization? At the moment, we lack experimental evidence. Small secreted proteins are the likely candidates for the signals that modulate plant responses to arbuscular mycorrhizas and ectomycorrhizas (Kloppholz et al, 2011, Kamel et al, 2017; Plett et al 2011), but this does not exclude a role for other bioactive molecules.

Looking at the observations produced by Melin and Mosse, we can conclude that their inspiring experiments with plant exudates and their observation of the fungal phenotype have opened one of the crucial chapters of plant–fungal interactions in our time. The issue is still open: Bonfante and Genre (2015) commented that the molecules involved in interkingdom symbiotic signalling, such as strigolactones, and chitin-related molecules, also have key roles in plant and fungal development, originally unrelated to symbiosis. Therefore, the symbiotic role of these molecules relies on the co-evolved capacity of the arbuscular mycorrhizal partners to perceive them as symbiotic signals.

Despite the striking effects of strigolactones, it is hard to believe that plants seeking arbuscular mycorrhizal symbiosis rely exclusively on released strigolactones to capture the fungi that are present in the soil. The discovery of a plant transporter of N-acetylglucosamine in maize and rice that is also required during the pre-contact phase (Nadal et al., 2017) suggests that other signalling molecules could be important during the pre-contact phase. Lastly and surprisingly, ectomycorrhizal plants have been poorly investigated in this context. Looking at the fungal factors, chitin-related molecules seem to be shared by pathogenic and arbuscular mycorrhizal fungi, opening the question of whether they could also function in signalling in ectomycorrhizal symbioses.

The colonization process: how cellular studies predicted future ‘omics' data

If we stop and look at the old drawings by G. Gibelli, J. Gallaud, or B. Peyronel, and compare them with the beautiful schemes of ectomycorrhizas in the publications by Martin and colleagues (2016), or with the iconic arbuscules shown in the Brundrett et al (1984) or by Maria Harrison's group (Bravo et al 2017), we will have no doubt of the beauty and richness of the details in the recent publications. However, many basic points of information, *i.e.* fungal structure, host anatomy, and plant cell specificity, were already correctly identified at the dawn of research on mycorrhizas. The finding that fossils of the Rhynie chert have root associations similar to modern arbuscular mycorrhizal fungi reflects the excellent knowledge of our colleagues of the past (Kidston & Lang 1921).

Moving from morphology to physiology, the functional ideas that are at the basis of our current concepts mostly stemmed from transmission electron microscopy observations. Transmission electron microscopes became operative at the end of the fifties. In Torino, Arturo Ceruti (1911–2000) funded one of the first Centres of Electron Microscopy, providing an important tool to look at mycorrhiza with a new technique. One of the major and longstanding concepts that developed at that time was the existence and importance of the interface, *i.e.* the area of physical contact between the plant cell and the mycorrhizal fungus (Fig. 6). Silvano Scannerini (1940–2005) was one of the first to adopt the terminology proposed for plant–pathogen interactions (Bracker and Littlefield, 1973). At the 1974 Leeds meeting, the different types of interfaces originating during the interaction between *Ornithogalum umbellatum* and its endogenous arbuscular mycorrhizal fungi were carefully described and listed. Moreover, they were assigned different numbers: the plant cell wall–fungal wall contact was named IT8, and at the moment of fungal penetration and plant membrane invagination, the interface was named IT24 (live-walled hosts and endophyte with matrix in the middle). Finally, when the fungus was collapsing, the interface was identified as IT25 (Scannerini et al, 1974). Luckily, no one today speaks about the interface numbers, but indeed this catalogue provided the basis for understanding the structure and the nature of the material laid down between the fungus and the invaginated surrounding membrane.

Cytochemical approaches allowed us to detect polysaccharides and proteins, revealing that the composition of the matrix is related to the plant cell wall (Scannerini and Bonfante-Fasolo, 1979). Indeed, years later work using more sophisticated *in situ* techniques detected many plant cell wall components, *i.e.* pectins, cellulose, hemicellulose, and the hydroxyproline-rich protein extensin (Bonfante et al 1990a, b; Balestrini et al, 1996 a,b; Balestrini and Bonfante 2014 for a review) in the interface compartment, observations that are still valid today. The activation of genes involved in cell wall synthesis was nicely confirmed by many transcriptomic studies in both arbuscular

463 mycorrhizal and ectomycorrhizal symbioses, as well as all the events that lead to new synthesis of
464 plant membranes required for accommodation of the fungus (Balestrini et al., 2017, Guether et al.,
465 2009; Genre et al, 2005; Genre et al, 2012).
466 The eighties were also the years in which researchers compiled a detailed atlas of all mycorrhizal
467 associations. For example, Larry Peterson provided beautiful descriptions of ectomycorrhizas and
468 orchid associations (Peterson et al 2004) and, in the same period, together with Vivienne Gianinazzi
469 Pearson, we provided an accurate description of the morphology of ericoid mycorrhizas. The hair-
470 roots of Calluna showed a peculiar anatomy, consisting of a few epidermal cells filled up by the
471 coiled fungal symbiont (Bonfante- Fasolo and Gianinazzi-Pearson, 1979, 1982). Interestingly, the
472 ultrastructure of ericoid plant cells was very similar to that of arbuscular mycorrhizal fungi-
473 colonized cortical cells (Scannerini and Bonfante, 1983), Fig. 6. However, by using *in situ*
474 techniques and antibodies against pectin and polygalacturonase, we observed that the fungal cell
475 walls of the two endosymbiotic fungi had very diverse compositions and that the ericoid fungus
476 releases polygalacturonase enzymes mostly at the contact with the thin hair roots. We concluded
477 that, in contrast to arbuscular mycorrhizal fungi, ericoid fungi produce cell wall-degrading enzymes
478 and that this capacity was probably modulated during the interaction with host and non-host plants
479 (Perotto et al., 1995). Indeed, genomics and transcriptomics of arbuscular mycorrhizal fungi
480 (Tisserant et al, 2013; Chen et al 2018, Salvioli et al 2016) and of ericoid fungi (Kohler et al 2015,
481 Martino et al 2018, Perotto et al., 2018) have demonstrated that arbuscular mycorrhizal fungi do not
482 possess genes coding for cell-wall degrading enzymes, in contrast to ericoid fungi, largely
483 confirming the hypotheses advanced about 25 years before!
484 In the absence of molecular data, ultrastructural observations allowed researchers to look beyond
485 the hedge: the deep re-organization of the cortical cells following the fungal colonization suggested
486 a reprogramming of the molecular plant machinery which has been largely confirmed by RNA-
487 sequencing studies in all the mycorrhizal symbioses (Martino et al, 2018, Veneault-Fourrey et al,
488 2014; Peter et al., 2016; Fochi et al, 2017; Fiorilli et al, 2015; Sugimura and Saito, 2017). The
489 interface was identified as a constant feature present in all the mycorrhizal associations; this led to
490 the concept that the interface is a novel compartment, and a cellular marker of compatible
491 interactions (Bonfante 2001). Going further, the enlarged nucleus of the host arbusculated cells as
492 well as its loose chromatin organization (Fig. 7) suggested reduplication events, a hypothesis that
493 has already received partial experimental confirmation (Fusconi et al, 2005.; Genre et al., 2008).
494 Cellular studies between the seventies and the eighties gave therefore the foundation for the
495 functional concepts that are at the forefront of research today: mineral or organic nutrients have to

cross plant–fungal interfaces, and the nutrient transporters have to be located at the fungal–plant membranes. The functional characterization of such nutrient transporters (transporting molecules from minerals to organic compounds), as well as the events that allow new membrane biogenesis, and the regulatory machinery, already belongs to the molecular era of mycorrhizal research, as summarized in many recent reviews (Mclean et al, 2017; Pimpryka and Gutjahr 2018, Lanfranco et al., 2018a).

The genetics underlying colonization events

At the end of the eighties we already had a good deal of knowledge of mycorrhizal morphology, but a crucial bit was missing: the genetic control that plants exert on entry by the fungus. This important discovery was made in Dijon, when the plant geneticist Gerard Duc, collaborating with two "mycorrhizal" colleagues, Vivienne Gianinazzi-Pearson and Silvio Gianinazzi, discovered that mutant plants that were not successful in producing active nodules were also resistant to mycorrhization. This finding was validated for pea and fava bean (Duc et al., 1989) and represented a paradigm shift, since for the first time it demonstrated that mycorrhization is under the genetic control of the host plant.

The second conclusion we have come to with Duc and colleagues discovery, was that a common genetic basis governs root symbioses in legumes. Indeed, this has been one of the most deeply investigated issues in mycorrhizal research. During the years we have learned that a common genetic pathway operates for the establishment of root symbioses; work since 2000 has identified a common set of genes, found in studies conducted by many groups (among them, Martin Parniske in Germany, and J. Stougaard in Denmark; see Parniske, 2008 for a review). For example, these studies found that nuclear calcium spiking was one of the first detectable events in root symbioses (see Oldroyd, 2013). Very interestingly from an evolutionary point of view, these molecular determinants are also present in non-leguminous plants (Gutjahr et al 2008).

In the context of a mycorrhizal excursus, we have to note that the Dijon discovery by Gerard Duc and colleagues on the one hand reinforced the plant-centric view that has dominated mycorrhizal research for many years, and on the hand other introduced mutants as crucial tools to understand mycorrhizal functioning. Thanks to them, we have learned that many genes control the signalling/early phase, while others are directly related to mycorrhizal functioning. Interestingly, most of these mutants share a similar phenotype: the arbuscules are stunted and not fully developed, suggesting that plant genetic determinants control arbuscule morphology (Gutjahr and

528 Parniske,2013). An exhaustive list of the plant genes that play a crucial role in a functioning
529 mycorrhiza (from lipid transfer, to phosphate uptake and ATPase activity), as well as in
530 transcriptional regulation, can be found in Pimpryka and Gutjahr (2018).
531 These molecular data provide some explanations for the popular advertisement "mycorrhizal plants,
532 bigger plants", but indeed they do not offer convincing explanations on the mechanisms: how AM
533 fungi, which exclusively colonize hypogeous organs in vascular plants, may have an impact on
534 epigeous organs? Which is the basis of the systemic effects? Are microRNAs and hormonal balance
535 the main drivers?
536 What else is missing from our understanding? In the genomic era, we have learned how important
537 genetic variation in different lines of the same plant species can be. To better investigate the genes
538 that are responsible for efficient mycorrhization, natural variation studies have to be undertaken, as
539 has already been done in some pioneering work (Dreher et al., 2017). Natural variation is also
540 relevant for the fungal partners. We already have some diffuse information telling us that different
541 isolates of the same mycorrhizal species lead to different effects at the systemic level (Roger et al.,
542 2013). It will be crucial to identify the roles of such fungal factors, to have a more balanced view of
543 mycorrhizal interactions.
544 In the context of sustainable agricultural and forestry practices, in a moment in which some of the
545 innovations of the Green Revolution are seen as no longer affordable, we may need to come back to
546 plant varieties that were not selected as responding to fertilizers and to test whether they can
547 achieve high productivity under a lower-fertilization regimen, but with the help of an efficient
548 mycorrhizal fungus. Therefore, one of the next challenges will be to identify the plant and fungal
549 genes that lead to a highly compatible couple that produces the best yield for crop plants. This could
550 be a novel trait to be selected when using breeding approaches to generate new crop varieties
551 (Sawers et al, 2008). Lastly, it will be crucial to remember that arbuscular mycorrhizal fungi do not
552 work alone, but they belong to a complex microbiota (Chialva et al., 2018). Tailoring the
553 interactions of crop plants and their associated microbiota may provide a crucial advance for
554 sustainable agriculture.
555
556 **Concluding thoughts: chance and needs in mycorrhizal symbioses**
557 A walk through mycorrhizal research from the middle of 1800 to today reveals that many of the
558 crucial questions we are facing now were first asked many years ago. Interestingly, our colleagues
559 of the past provided many replies (exudates as sources of signalling molecules, transport of mineral
560 nutrients investigated at the whole organism level, attempts to give a name to the fungal symbionts)

and maybe (possibly by chance) many of these replies are still the right ones. Of course, the replies mirror the technical tools that were available at the moment. Moreover, not every question may have a reply, especially if it is not asked at the right time. For example, Barbara Mosse (1973) described the presence of bacteria-like organisms inside the spores of *Funneliformis mosseae*; some years later I observed similar organisms the first time that I looked at a mycorrhizal section under the electron microscope (Bonfante 2014), but the invention of PCR was needed for successful naming of these organisms (Bianciotto et al 1994, Bonfante and Desiro 2017).

This is a great time for the mycorrhizal scientific community: this is not only due to the powerful tools that are now available, but also to a crucial change in the perception of mycorrhizal symbiosis. Today mycorrhizas are perceived as relevant not only by researchers, but also by society. We can illustrate the societal impact of our research putting a strong emphasis on the role of our fungi as biofertilizers and bio-protectors. In a moment in which the environment is seen as a precious gift that has to be preserved, we can take many positive actions. It has been a wonderful opportunity to work on mycorrhizas. Over more than 40 years, I have seen that biology is a river in flood and that mycorrhizal research is a part of it. In looking to the future of mycorrhizal studies, we can learn much by examining their roots in the past—and I look forward to future developments in our understanding of these remarkable biological systems.

Acknowledgements

My sincere thanks to: Guillaume Becard and David Baker for the invitation to the IMMM in Toulouse (2017) stimulating me in thinking of the past in mycorrhizas; Dr Vivienne Gianinazzi-Pearson for her help in giving a name to some of the scientists attending the Leeds meeting in 1974 and shown in the congress picture (Fig 3); the team of Library of Department of Life Sciences and Systems Biology (Dr. Franco Estivi with Professor Guglielmo Pandolfo and Dr. Alessandra Fenoglio) for their competent and kind assistance in the search of the old volumes; Dr. Mara Novero for her continuous help, also in the plate preparation; and Luisa Lanfranco, Silvia Perotto, and Andrea Genre for reading the draft. A lot of thanks to Jennifer Mach for the generous text editing. Lastly, I would like to thanks the three reviewers, and apologize to all the colleagues whose work has not been quoted here for space reasons.

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Keywords: Mycorrhizas, History, "Omics" approaches, Plant Microbiota, Evolution, Mutants, Signalling molecules, Colonization Process

953 Legends

954 **Fig. 1 a.** The drawings from Giuseppe Gibelli (1883) illustrate chestnut roots colonized by an
955 unknown fungus leading to the typical rounded mycorrhizal tip.

956 **Fig. 1b.** Oreste Mattirolo had a painting made by an unknown artist on the occasion of an
957 international exhibition held in Torino in 1911. The painting illustrates how a pig and a dog are
958 looking for truffles under an oak. The root systems bear the typical ectomycorrhizal roots.
959 Reproduced with the permission of the Department of Life Science and Systems Biology- Library
960 of the Botanical Garden, University of Torino.

961 **Fig. 2**. Top line: In his 1905 reports, J. Gallaud provided beautiful illustrations of endomycorrhizas
962 depicting the different morphologies of arbuscules as reproduced here and showing details of the
963 root symbioses in *Sequoia* and *Arum*, respectively. The drawing on the left side illustrates an *Allium*
964 arbuscule where some branches are already collapsing; according to the terminology of the time
965 these fungal structures were defined as sporangioles. The same morphology can be appreciated in a
966 section of a root of *Lotus japonicus* colonized by *Gigaspora margarita* and stained with wheat germ
967 agglutinin conjugated with a fluorescent probe to reveal the fungal wall and seen under confocal
968 microscopy (Courtesy of Dr. Mara Novero)

969 **Fig. 3** The group picture illustrates the scientific community who convened in Leeds (1974) for the
970 first meeting on endomycorrhizas. In the first row: from right, third position: Silvano Scannerini,
971 Jack Harley, Mrs. Harley, Bernard Tinker, Vivienne Pearson (not yet Gianinazzi); from left, first
972 position: Francis Sanders; Geff Hadley; Barbara Mosse; Glynn Bowen, and after one person, C.
973 Hepper. David Read is in the last line, the third from the left. Paola Bonfante is in the second row.

974 **Fig. 4.** On the left side: Drawing by C. Hepper and B. Mosse (1975) showing the effect of *Trifolium*
975 exudates on the hyphal branching of *F. mosseae* (a) when compared to the untreated fungus(b). On
976 the right side, an experiment, similar to the one developed by Akyama *et al.* 30 years later (2005),
977 illustrates how the germinating spore of *Gigaspora margarita* is triggered to branch by GR24, a
978 synthetic strigolactone analogue (courtesy of Dr. Mara Novero).

979 **Fig. 5.** A look at the wall of an AM fungus under transmission electron microscope. A detail of the
980 cell wall of a spore of *Glomus versiforme* reveals a 3D helicoidal architecture created by highly
981 cristalline nano-chitin fibrils (arrows), which are laid down in planes which progressively rotate of a
982 certain angle (a). The optical effects of this 3D architecture after sectioning are fibrillar arcs. This

983 architecture provides strength and resistance to the spores. Such organization is lost during the
984 germination. However, the nanocrystals of fibrillar chitin (arrow heads) are easily detected in the
985 wall of extraradical (b) but not of intracellular (c) hyphae, and of thin arbuscule branches (d). Here,
986 the cell wall structure is amorphous, but Wheat Germ Agglutinin coupled to gold granules still
987 reveals the presence of chitooligosaccharides. The molecular control of such hierarchical
988 organization is at the moment not known.

989 **Fig. 6.** Schematic view of the interface zone in an ectomycorrhizal (A) and AM (B) symbiosis. In C
990 several of the molecules so far determined through in situ labeling experiments are listed. Modified
991 from Bonfante 2001 and Balestrini and Bonfante 2014.

992 **Fig. 7.** Electron microscopy observations in the seventies revealed the cellular re-organization of
993 plant cells following the fungal colonization. On the right: a *Lotus japonicus* cortical cell is
994 colonized by *Gigaspora margarita*. The cortical cell is dominated by an enlarged nucleus with a
995 prominent nucleolus and a loose chromatin. The fungal branches of different size (from the trunk to
996 the collapsed terminal branches) fill up the plant cell lumen. At the left, an epidermal Calluna cell is
997 colonized by *Pezizella ericae*, which forms a coil. Also in this case the coil is surrounded by the
998 invaginated host membrane and the plant nucleus occupies a central position. These observations
999 reveal a similar cell-reorganization, irrespectively of the different fungal physiology.