Life of Microbes Inside the Plant: Beneficial Fungal Endophytes and Mycorrhizal Fungi

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1 **Life of microbes inside the plant: beneficial fungal endophytes and mycorrhizal**

2 **fungi**

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Abstract

Fungi are notably diverse and proliferate in different environments (soil, air, water) but, remarkably, the highest numbers of fungi are found in the soil or associated with plants. Fungal endophytes, the ones living within plant tissues, are important members of the plant microbiota, but the nature of the relationship established with the host can be variable. The majority of endophytes do not affect the plant at all; some are pathogenic and some may even be beneficial, influencing plant growth and health by modulating different biological processes.

In this chapter we will consider fungal endophytes with a recognized beneficial impact on plants, and on a special group of endophytes, the mycorrhizal fungi. We will describe the interaction at the cellular level, highlight the functional significance of the associations and the impact of these fungal symbionts on the host plant, and illustrate potential applications.

The plant-endophyte alliance may increase plant phenotypic plasticity, which is perhaps an advantage in fluctuating environments such as those emerging from global climate change. Understanding the complex interactions between fungal endophytes and plants is therefore of relevance for the development of tailored microbial inocula to improve crop production and quality under varying environmental conditions. This appears to be a promising means of developing more environmentally friendly agricultural crop production systems and increasing global food security.

Introduction

The plant microbiota

Plants are not alone: in many ecosystems, plants are greatly outnumbered by microorganisms such as bacteria and fungi that may live in close proximity or even in strict association with plant hosts (Vandenkoornhuyse et al., 2015; Müller et al., 2016). Complex microbial communities thrive on the outer surfaces as well as in internal tissues of plants; this microbial component, referred as the plant microbiota (all microorganisms) or plant microbiome (all microbial genomes), may influence plant growth and health by modulating a wide range of biological processes (Trivedi et al., 2020). Pioneering reports on the plant microbiota focused on the characterization of bacterial assemblages in the model species *Arabidopsis thaliana* (Bulgarelli et al., 2012), but more recent papers also consider fungi so that the term plant *mycobiota* is also currently used (Bonfante et al., 2019). High through-put sequencing techniques applied to environmental samples have revealed that fungi proliferate in many different environments (soil, air, water) but, remarkably, the greatest numbers of fungi are found in the soil or associated with plants, at an estimated fungal / plant species ratio of 17 / 1 (Nilsson et al., 2019).

Living together, which has been described as a symbiosis (Martin and Schwab, 2012), implies different possible types of interactions ranging from parasitic or pathogenic, where one partner gains benefit at the expense of the other, to mutualistic, where reciprocal benefits occur. The knowledge of the nature of these interactions and on how these fungal symbionts can influence plant physiology and metabolism is of fundamental relevance because it is recognized nowadays that plants likely host a still overlooked reservoir of beneficial fungi that may contribute to support their survival and growth under biotic and abiotic stresses. Some of these plant-fungal associations may function as key elements in the development of sustainable programs of crop production and environmental protection which, in the context of global climate global change, represent emerging challenges to modern societies.

In this chapter we focus on fungal endophytes with a recognized beneficial impact on plants and on a special group of endophytes, the mycorrhizal fungi. Mycorrhizal fungi are root endophytes which form specialized plant-fungal interfaces for resource exchange. In addition, they are phylogenetically distinct from most groups of endophytes (Smith and Read 2008; Porras-Alfaro and Bayman, 2011).

92 We will describe here the interaction at the cellular level, emphasize the functional
93 significance of the associations and the impact of the fungal symbionts on the host plant,
94 and illustrate potential applications.
95

96 **Fungal endophytes of plants**
97 Notwithstanding the knowledge of plant-associated microbes has been significantly
98 advanced by molecular high-throughput sequencing techniques (Nilsson et al., 2019), the
99 study of plant microbiota has very ancient roots. Anton de Bary, in 1886, coined the term
100 *endophytes* to define microorganisms, normally bacteria and fungi, that live within plant
101 tissues (Wilson 1995; Martin and Schwab, 2012). The term endophyte therefore refers to
102 the location, *i.e.* inside plant tissues, irrespective of being inside the cells or among the
103 cells, and does not specify the nature of the relationship established with the host plant.
104 Indeed, the majority of endophytes do not affect the plant at all; some are pathogenic and
105 others may even be beneficial. The outcome of the association may depend on the
106 partners involved, and the functional roles can also vary during the life cycle of the
107 microbe or in response to plant or environmental factors (Porras-Alfaro & Bayman
108 2011).
109 It has been shown that each plant species has a highly variable and in some cases
110 impressively rich community of fungal endophytes - up to 90 species have been
111 described within a single leaf of a tropical tree (Bayman 2006). Plant organs of a single
112 plant also often differ greatly in the dominant taxa of endophytes present, but we are far
113 from having a global picture of their functional significance.
114 Considering this heterogeneity, we will here give examples of some of the most well
115 characterized fungal endophytes known to have a positive impact on the host plant. They
116 include the shoot grass endophytes of the genus *Epichloë spp.* (Ascomycota,
117 Sordariomycetes, Clavicipitaceae), the well known biocontrol agents belonging to
118 *Trichoderma* spp. (Ascomycota, Sordariomycetes, Hypocreaceae), the generalist root
119 endophyte *Serendipita indica* (Basidiomycota, Sebacinales) and the recently described
120 root/systemic endophyte *Colletotrichum tofieldiae* (Ascomycota, Sordariomycetes,
121 Glomerellaceae) identified in natural samples of the model species *Arabidopsis thaliana*.
122

123 *Epichloë* **spp.**
124 The genus *Epichloë,* belonging to the family Clavicipitaceae within Ascomycota, forms
125 symbiotic relationships with many grasses of the Pooideae subfamily (Schardl et al.,

126 2004). Phylogenetic analysis places *Epichloë* species within a group of endophytic and
127 plant pathogenic fungi, with a common ancestor probably derived from an animal
128 pathogen (Spatafora et al., 2007).

129 *Epichloë* endophytes grow asymptomatically in the intercellular spaces of the aerial
130 tissues of the host plant. Notably, the expansion of hyphae occurs in synchrony with the
131 growth of the newly developing leaf, while the root is essentially uncolonized (Herd et al.,
132 1997; Tan et al., 2001). These endophytes, in most cases, are exclusively seed-
133 transmitted from previously infected hosts (Schardl et al., 2004), a mode of transmission
134 that represents another signature with ecological relevance of the intimate plant-fungus
135 association.

136 The nature of this symbiosis has been described as 'defensive mutualism' (Bastias et al.,
137 2017): *Epichloë* endophytes are indeed able to produce several alkaloids, bioactive
138 secondary metabolites that are detrimental for herbivores (Schardl et al., 2004; 2013).
139 Genomic studies and chemical analyses have been instrumental in deciphering the
140 alkaloid biosynthetic pathways (Schardl et al., 2013; Young et al., 2015); it is now well
141 established that alkaloid profiles depend not only on the specific fungal strain but also on
142 the host plant phenology and on environmental factors (Saikkonen *et al*., 2013; Ball *et al*.,
143 1995; Justus et al., 1997). Moreover, the quali- and quantitative alkaloid profile (i.e.
144 chemical structure and amount of specific compounds) is a key determinant in the
145 defense efficiency against a given attacker.

146 In the late 70s, *Epichloë* endophytes were considered undesirable, as they were found to
147 lead to serious health problems in livestock (Bacon *et al*., 1977; Fletcher and Harvey,
148 1981). Later, however, since the yield of endophyte-free grasses was lower compared to
149 endophyte-infected grasses, a major effort was undertaken to select livestock-friendly
150 endophytes that still produced alkaloids with insecticidal and nematocidal activities
151 (Cagnano et al., 2019) and artificially infected grass cultivars were developed for the
152 market (Johnson et al., 2013).

153 In order to gain a deeper understanding of the biological system, the *Epichloë festucae* -
154 ryegrass *(Lolium perenne)* model was characterized at the cellular and molecular levels.
155 The benefits provided by *E. festucae* were found to extend far beyond protection against
156 herbivores (the only issue that has been mechanistically explained). Improvement in
157 pathogen resistance (Tian et al., 2008), drought tolerance and enhanced growth (Hahn et
158 al., 2008), and better ability to compete with other plant species (Quigley 2000) have also
159 been reported.

5

160 To gain insight into the molecular changes induced by endophyte infection, a comparison
161 of the gene expression profiles of perennial ryegrass infected or not with *E. festucae* was
162 carried out (Dupont et al., 2015). Endophyte infection triggered a strong reprogramming
163 of host genes, in particular favouring secondary metabolism at a cost to primary
164 metabolism. This is similar to what found in plant pathogenic interactions but in stark
165 contrast to what has been observed in response to fungal colonization by mycorrhizal
166 fungi where only a modest host gene modulation occurs.

167 A transcriptomic analysis of the *E. festucae - L. perenne* interaction also was carried out
168 with a focus on the developmental stages of different host tissues (Schmid *et al*., 2017).
169 Notably, fungal genes annotated as encoding small secreted proteins (SSP), which can
170 function to control colonization of host tissue in pathogenic and mutualistic symbioses
171 (Toruño *et al*., 2016; Plett *et al*., 2011; Kloppholz *et al*., 2011), were highly expressed in
172 hyphae within emerging leaves. By contrast, genes involved in synthesizing anti-
173 herbivore compounds are highly expressed in the fungal mycelium of mature plant
174 tissues, in agreement with the alkaloid profiles. But *E. festucae*-infected plants can have a
175 second line of defence: endophyte colonization induces a systemic effect in the plant.
176 young as well as mature plant tissues display the activation of genes mainly involved in
177 hormone biosynthesis and signalling and in stress and pathogen resistance (Schmid *et al*.,
178 2017; Dupont *et al*., 2015). Considering, in particular, defence against chewing insects, it
179 has been proposed that *Epichloe* endophytes can use not only alkaloids but also can
180 activate a level of intrinsic immunity in the plant by promoting innate defense responses
181 (Bastias *et al*., 2017). These data suggest that *E. festucae* is able to trigger an activated
182 status (Martinez-Medina *et al*., 2016), in analogy to a mechanism that has been proposed
183 for mycorrhizal fungi (Pozo and Azcón-Aguilar, 2007). This activated status could be the
184 basis of the increased resilience of the host plants to a broad variety of stresses (Fig. 1).

185

186 **Trichoderma spp.**

187 Filamentous fungi belonging to the genus *Trichoderma* thrive in the rhizosphere (the soil
188 adjacent to and influenced by plant roots), and are able to colonize the root system from
189 early seedling developmental stages (Harman *et al*., 2004). As early as 90 years ago,
190 *Trichoderma* species were recognized as pathogens of other soil fungi (Weindling,
191 1932). Since then, the knowledge of *Trichoderma* biology has evolved continuously,
192 leading to the successful development and marketing of *Trichoderma*-based products to
193 be used in agriculture as biocontrol agents to control plant pathogenic fungi (Fraceto *et al*.

6

194 2018).

195 Studies focused mainly on *Trichoderma harzianum* have demonstrated that parasitism by

196 fungi, even beneficial ones, relies on the production of many hydrolytic enzymes, which

197 act in synergy with fungitoxic metabolites (Lorito *et al.*, 2010). However, these may not

198 be the primary mechanisms of biocontrol, since root colonization also stimulates plant

199 defense mechanisms initiated by extensive physiological and metabolic changes

200 mediated mainly by jasmonic acid and another plant hormone, ethylene (Contreras-

201 Cornejo *et al.*, 2015; Martínez-Medina *et al.*, 2017). A strain of *T. harzianum* also has

202 been found to produce a nitrogen heterocyclic compound called harzianic acid, which has

203 various biological properties including plant growth promotion, antimicrobial activity

204 and the ability to chelate soil iron, facilitating its solubilisation and, as a consequence, its

205 availability to plants (Vinale *et al.*, 2013). Thus, the multiple benefits provided to the

206 host plant encompass not only protection from pathogens, but also improvement of plant

207 growth and vigor and enhanced defense responses against various stresses (Fig. 1).

208 Collectively, these reasons explain why the new generations of *T. harzianum*

209 formulations are therefore described as plant protectants and biostimulants of crop yield

210 and quality.

211

212 ***Serendipita indica***

213 Another plant endophyte that has been well characterized in recent decades is *Serendipita*

214 *indica* (syn. *Piriformospora indica;* Varma *et al.*, 1999), a filamentous fungus belonging

215 to Sebacinales (Basidiomycota). It is worth noting that the order Sebacinales include

216 endophytes that associate with roots (*i.e. S. indica, S. vermifera* and *S. herbamans*),

217 mycorrhizal fungi forming symbioses with orchids, as well as saprotrophs, but not

218 pathogenic fungi. The root associated Sebacinales likely evolved from saprotrophic

219 ancestors (Garnica *et al.*, 2016) and are described as ubiquitously distributed generalists.

220 In particular, *S. indica* colonizes the intercellular spaces of plant roots and the

221 intracellular regions of epidermal and cortical cells, but never the central cylinder of the

222 root. In early colonization stages the fungus is surrounded by a plant-derived plasma

223 membrane, indicating that the colonized cells are viable. Later, it switches to a host cell

224 death-associated phase, where thin fungal hyphae are found in dead or dying cells,

225 although massive necrotic areas are never observed (Deshmukh *et al.*, 2006; Fesel and

226 Zuccaro, 2016).

227 Benefits for the host plant include increased biomass, possibly as a consequence of
228 enhanced assimilation of mineral nutrients such as phosphate (Yadav *et al*., 2010), and a
229 higher tolerance against biotic and abiotic stresses (Sherameti *et al*., 2008; Fig. 1). The *S.*
230 *indica-Arabidopsis thaliana* interaction has emerged as a model to study the systemic
231 effect leading to an increase tolerance to leaf pathogens. In that specific case, the
232 protection against the powdery mildew *Golovinomyces orontii* was demonstrated to be
233 mediated by the jasmonate hormonal signaling pathways (Stein *et al*., 2008).
234
235 ***Colletotrichum tofieldiae***
236 Another intriguing endophyte, the recently characterized *Colletotrichum tofieldiae*, was
237 originally isolated from natural populations of asymptomatic *A. thaliana* plants in Spain
238 (García *et al*., 2013). It was also detected in trees and orchids across the Eurasian
239 continent (Damm *et al*., 2009; Tao *et al*., 2013) suggesting a very broad host range. The
240 fungus colonizes the root and occasionally spreads into the aerial parts of the plant
241 through the central cylinder, reminiscent of a pathogenic association; indeed *C. tofieldiae*
242 is closely related to pathogenic *Colletotrichum* species. *C. tofieldiae* was shown to
243 promote plant growth as a consequence of improved mineral nutrition, thanks to
244 endophyte-mediated transfer of phosphorus. Remarkably, phosphate transfer only occurs
245 under low phosphorus conditions (Hiruma *et al*., 2016), which is similar to what is
246 observed in the arbuscular mycorrhizal symbiosis (Fig. 1). This result has opened a
247 window on how non-mycorrhizal plants cope with low nutrient availability, since *A.*
248 *thaliana*, like most Brassicaceae, has lost the ability to form mycorrhizal symbioses
249 during evolution.
250 The comparison between the genomes of *C. tofieldiae* and its pathogenic relative, *C.*
251 *incanum*, and the between the transcriptomes of both fungi and the host plant
252 *Arabidopsis,* was instrumental in highlighting the genomic traits related to the transition
253 from pathogenicity to mutualism (Hacquard *et al*., 2016). The *C. tofieldiae* genome has a
254 narrowed repertoire of genes encoding secreted proteins, chitin-binding and secondary
255 metabolism-related proteins compared to that of *C. incanum*. Moreover, *C. tofieldiae*-
256 colonized plants show limited activation of pathogenicity-related genes compared to *C.*
257 *incanum*-infected plants. It is worth noting that *A. thaliana* roots induced defence genes
258 when colonized by *C. tofieldiae* under conditions of high phosphorus concentrations
259 (Hacquard et al., 2016). This finding pointed for the first time to an interdependency
260 between plant immunity and plant nutrition. A similar activation of plant defence-related

261 genes mirrored by alterations in the root-inhabiting fungal community was observed in
262 maize plants grown under high phosphorus concentrations (Yu *et al*., 2018). Lowering
263 plant defences under low phosphorus availability therefore appears to be a strategy to
264 favor the recruitment of beneficial soil microbes that can help to overcome the nutritional
265 stress.
266
267 Fungal endophytes are thus ubiquitous and taxonomically very diverse organisms.
268 Phylogenetic analyses and data from whole genome sequencing, although still limited to
269 only a few taxa, have given important insights into the evolutionary origin of
270 endophytism. As mentioned before, some of endophytes, such as *Epichloë* and *C.*
271 *tofieldiae,* are clearly derived from fungi with a pathogenic life style, whereas the root-
272 associated Sebacinales (i.e. *S. indica*) are most probably derived from a saprotrophic
273 ancestor (Lahrmann *et al*., 2015; Fesel and Zuccaro, 2016). The genomic signature of
274 this origin is a reduced repertoire in *S. indica* of genes involved in secondary metabolite
275 production compared to that found in the genomes of *Epichloë spp.* and *C. tofieldiae.*
276 Despite these differences, colonization of the roots of *A. thaliana* by *S. indica* or *C.*
277 *tofieldiae* shares common features: both fungi initially penetrate the epidermis by
278 undifferentiated hyphae and spread in the cortical tissue intercellularly and
279 intracellularly; at later stages of both interactions cell death, maily limited to the
280 epidermal layer, is observed. Moreover, in both cases, to restrict fungal growth inside the
281 root and keep a well-balanced endophytic interaction, an intact plant immune system is
282 required (Fesel and Zuccaro, 2016). Further comparative analysis of additional
283 interactions will help to define commonalities and unicities.
284
285 A further level of complexity in the plant-fungal endophyte interaction is the presence
286 within the microbial symbiont of endobacteria (Bonfante et al., 2019) and viruses (Bao
287 and Roossinck, 2013). Notably, these bacteria can influence several aspects of the fungal
288 biology including the outcome of plant-fungal symbioses. One example from the
289 prokaryotic world is *Rhizobium radiobacter* strain F4, which was detected inside *S.*
290 *indica*; the endobacterium improves the fitness of *P. indica* and contributes to the
291 beneficial effects exerted by the fungal endophyte on the host plant (Guo *et al*., 2017).
292 Fungal viruses, also called mycoviruses, also are emerging as an abundant component of
293 the microbiota of fungal endophytes (Bao and Roossinck, 2013) that may have an impact
294 on fungal fitness and, as a consequence, on the plant host, in a tripartite interaction. A

295 historic case is the virus that makes the fungal pathogen *Cryphonectria parasitica* less
296 virulent for the host plant, which led to the identification of a powerful biocontrol agent
297 (Choi and Nuss, 1992). Another example refers to thermotolerance: a mycovirus, living
298 inside the fungal endophyte *Curvularia protuberate* was shown to confer heat tolerance
299 to the fungus and to the host plant *Dichanthelium lanuginosum,* allowing their growth in
300 the geothermal soils of Yellowstone National Park (Márquez et al., 2007). The precise
301 molecular mechanisms at the basis of the phenomenon are not known but osmolytes and
302 reactive oxygen species were differentially accumulated in plants colonized with the
303 virus-infected endophyte.
304 From these few examples it is clear that plants can host a taxonomically broad spectrum
305 of fungal endophytes; this heterogeneity inevitably mirrors functional diversity
306 potentially leading to a range of significance for the host plant growth and health.
307 Digging into this world is indispensable to expanding our knowledge on endophytism
308 and its many beneficial applications to agriculture.
309
310 **Mycorrhizal fungi: ancient allies of plants**
311 Under the word mycorrhizas - literally fungus-root interaction - we include several types
312 of interactions which have originated over 400 million years of co-evolution between
313 plants and fungi (Genre *et al*., 2020). About 85% of vascular and non-vascular plants
314 develop mycorrhizas (Brundrett and Tedersoo, 2018). This number and their scattered
315 geographical distribution are clear indication of their evolutionary and ecological success
316 (Davison *et al*., 2015). Four main mycorrhizal types are recognized - ectomycorrhiza,
317 arbuscular, orchid and ericoid mycorrhizas - that feature specific morphological traits and
318 different combinations of partners (Fig. 2).
319 Ectomycorrhizas (ECM) are established between soil-borne fungi (predominantly
320 belonging to Basidiomycota and Ascomycota) and shrubs and forest trees including
321 woody species with commercial value, such as poplar, birch, oak, pine and spruce
322 (Wiensczyk *et al*., 2002). In ECM the young root tips are enwrapped by fungal hyphae
323 that generate a pseudoparenchymatous tissue known as the 'sheathing mantle'. The
324 mantle's inner hyphae further develop between epidermal and cortical root cells, leading
325 to the so-called 'Hartig net', but intraradical hyphae never penetrate the cell lumen (Fig.
326 2). Together with the extraradical mycelium, these fungal structures are directly involved
327 in the mobilization, absorption, and translocation of mineral nutrients and water from soil
328 to roots. ECM has a massive potential in forest management, including reforestation,

329   phytostabilization and arboriculture activities. The promotion of abiotic stress tolerance
330   provided by ECM to the host plants may also have a positive impact on the likelihood of
331   plant adaptation to climate changes (Bennet and Classen, 2020).
332   Furthermore, a growing interest in ECM fungi lies in the production of worldwide-
333   appreciated fruitbodies such as boletes, chanterelles and truffles (Danell *et al.*, 1997;
334   Mello *et al.*, 2012; Murat *et al.*, 2015).
335   The other mycorrhizal types are all endomycorrhizas, since they are characterized by the
336   occurrence of fungal intracellular structures (Fig. 2). In the ericoid mycorrhizas (ERM),
337   some fungi belonging to Leotiomycetes (Ascomycota) or some Basidiomycota penetrate
338   the epidermal cell walls of Ericaceae (heather) roots and form intracellular hyphal coils.
339   The ERM fungi help the host species to colonize low mineral and acid soil (i.e.
340   heathland), since they play a key role in releasing soil nutrients through the efficient
341   decomposition of a wide variety of complex and recalcitrant organic substrates (Smith
342   and Reads, 2008; Perotto *et al.*, 2018).
343   In orchid mycorrhizas (ORM), plants belonging to the Orchidaceae family establish with
344   fungal taxa from several similar groups of Basidiomycota in a peculiar symbiotic
345   interaction (Dearnaley *et al.*, 2012). In the early stages of their development, orchids
346   show a mycoheterotrophic phase, since instead of fixing carbon themselves via
347   photosynthesis, they rely on their mycorrhizal partners to obtain carbohydrates, likely
348   extracted by the fungus from soil organic matter (Merckx 2012). ORM fungi colonize the
349   functional cells of the host by forming dense hyphal coils called pelotons (Peterson *et al.*,
350   2004; Fig. 2). In the adult phase several orchids switch exclusively to photosynthesis;
351   however, at maturity some of them continue to obtain carbon from mycorrhizal fungi or
352   utilize both strategies (Dearnaley *et al.*, 2016; Merckx 2012). Beside ensuring the orchids'
353   livelihood, ORM fungi also play a pivotal role in orchid propagation and conservation
354   (Batty et al., 2002).
355   The arbuscular mycorrhizal (AM) symbiosis is established between a small group of soil
356   fungi that belong to the Glomeromycotina subphylum (Spatafora *et al.*, 2016) and an
357   extremely wide range of land plants, including the majority of staple crops. Among
358   mycorrhizal types, the AM symbiosis is therefore the dominant association (72%),
359   followed by ORM (10%), ECM (3%) and ERM (1%) (Brundrett and Tedersoo, 2018).
360   AM fungi are characterized by peculiar features such as multinucleate hyphae and spores
361   and the apparent absence of a sexual cycle (Kamel *et al.*, 2016; Lanfranco *et al.*, 2016;
362   Corradi and Brachmann, 2017). They are also obligate biotrophs, that is, uncultivable in

363 the absence of host plants, with very large genomes extremely rich in transposable
364 genetic elements (Chen *et al*., 2018; Kobayashi *et al*., 2018; Morin *et al*., 2019; Sun *et al*.,
365 2019; Venice *et al*., 2019). A further level of complexity is given by their microbiota,
366 which include endobacteria (Bonfante and Desirò, 2017) and viruses (Ikeda *et al*., 2012;
367 Turina *et al*., 2018).
368
369 Although mycorrhizal fungi are highly diverse in terms of their evolutionary history, the
370 genome sequencing data (see the database MycoCosm) has highlighted some symbiotic
371 signatures (Genre *et al*., 2020). A common trait found in the genome of all mycorrhizal
372 fungi is the presence of several genes encoding small secreted proteins (SSP) that may
373 serve to manipulate host plant metabolism and enhance colonization. A few such proteins
374 have been characterized and indeed shown to interfere with plant defence responses
375 (Plett *et al*., 2011; Kloppholz *et al*., 2011). A limited repertoire of genes encoding plant
376 cell wall-degrading carbohydrate-active enzymes (CAZymes) characterizes the genomes
377 of AM and ECM fungi (Kohler *et al*., 2015; Martin *et al*., 2016), which are evolutionarily
378 oldest. The low ability to attack plant cell wall polysaccharidic components has been
379 interpreted as a strategy to limit the production of degradation products that are well
380 known activate plant defence responses. These common features may have originated by
381 processes of convergent evolution, possibly related to adaptation to a lifestyle dependent
382 upon living host tissues as food sources (Genre *et al*., 2020).
383 The current knowledge on mycorrhizal symbioses, especially with regard to cellular and
384 molecular aspects, mainly refers to the AM symbiosis because it has been investigated
385 more due to its wide host range and geographical distribution. For this reason, in the
386 following paragraphs we will more deeply consider some aspects of this mycorrhizal
387 association.
388
389 **The AM symbiosis: from the origin to the present**
390 The origin and the evolution of mycorrhizal symbioses have been deciphered in the light
391 of palentological and, more recently, phylogenomic data (Strullu-Derrien *et al.,* 2018;
392 Genre *et al*., 2020). These data identify the AM symbiosis as the most ancient
393 mycorrhizal type. The co-evolution of land plants with AM fungi is witnessed by fossil
394 records of early land plants from the Rhynie chert dated 407-million-year-old, where
395 arbuscule-like structures were identified in the rhizomes of extinct plants such as
396 *Aglaophyton majus* (Remy *et al*., 1994; Strullu-Derrien et al., 2018). To support the

397 hypothesis that the evolutionary history of land plants and AM fungi are inextricably
398 linked, phylogenetic analyses indicate that plant genes required for AM formation are
399 present in the genomes of the closest algal relatives to land plants (Delaux *et al.*, 2015).
400 The occurrence of AM symbiosis in liverworts, that are the earliest diverging group
401 among living plants, clearly shows that this mutualistic association predates the
402 development of true roots (Brundrett, 2002; Field *et al.*, 2012) and suggests that AM
403 fungi assisted the early land plants to cope with nutrient acquisition from a new
404 environment, the harsh and primeval soil (Pirozynski and Malloch, 1975).

405 Nutrient transfer appears to be a dominant function, as AM fungi provide access to
406 phosphorus, which is poorly mobile in soil, and also, to a lesser extent, nitrogen, sulfur
407 and other essential nutrients (Wang *et al.*, 2017). As a consequence, under limiting
408 nutrient availability, mycorrhizal plants often display a growth effect compared to non-
409 mycorrhizal plants. But the AM symbiosis is multifunctional, as the benefits at at the
410 level of the entire plant extend beyond improved mineral nutrition. AM fungi offer plant
411 protection against pathogens (Pozo and Azcon Aguilar, 2007; Cameron *et al.*; Fiorilli *et
412 al.*, 2018; Miozzi *et al.*, 2020), positively influence plant water potential (Porcel & Ruiz-
413 Lozano, 2004) and can enhance drought tolerance (Ruiz-Lozano *et al.*, 2015; Chitarra *et
414 al.*, 2016; Bahadur, *et al.*, 2019; Fracasso *et al.*, 2020; Balestrini *et al.*, 2020). These
415 benefits are not only a mere consequence of a better nutritional status, but involve several
416 metabolic and physiological changes at local and systemic levels (Fiorilli *et al.*, 2018;
417 Miozzi *et al.*, 2020; Fracasso *et al.*, 2020). The AM symbiosis acts therefore as a natural
418 mechanism against biotic and abiotic stresses.

419 In addition, AM fungi confer several ecosystem benefits in natural and agricultural
420 environments (van der Heijden *et al.*, 2015). They are the main drivers of carbon
421 sequestration in rooting zone (Johnson *et al.*, 2016), and, thanks to their hyphal network,
422 they improves soil quality by promoting particle aggregation, thereby limiting erosion by
423 wind and water, and they decrease the risks of ground water contamination (Chen *et al.*,
424 2018).

425 AM fungi have a major role in the composition of plant communities (van der Heijden *et
426 al.*, 2015). Moreover, each fungal mycelium can colonize several host plants of the same
427 or different species, leading to the formation of the so-called common mycorrhizal
428 networks (CMNs). A strongly interconnected plant community can potentially gain
429 stability, because weaker individuals might profit from the mineral nutrient supply of the
430 CMN at the expense of older plants, a phenomenon known as facilitation. These multiple

431   benefits of AM fungi translate into significant ecological services not only in natural

432   contexts, but also in agricultural sites. In the current context of growing concerns about

433   environmental issues, AM fungi are proposed as crucial actors of ecosystem functioning

434   and in the so-called Microbial Revolution, *i.e.* the route to increase, through the

435   exploitation of the plant microbiota, of sustainable crop productivity (Baez-Rogelio *et*

436   *al.*, 2017).

437

438   **How to establish a mutualistic AM relationship**

439   The establishment of a functional AM requires a fine coordination at the cellular and

440   metabolic levels of both partners; a number of signaling molecules and genes play a role

441   in this inter-kingdom communication (MacLean *et al.*, 2017; Lanfranco *et al.*, 2018;

442   Pimprikar and Gutjahr, 2018). Under conditions of limiting inorganic phosphorus, host

443   plant roots actively exude strigolactones, a class of compounds (Waters *et al.*, 2017) that

444   are perceived by AM fungi and promote spore germination, hyphal branching, nuclear

445   divisions and respiratory metabolism (Besserer *et al.*, 2008; Salvioli *et al.*, 2016). In turn,

446   the fungal partners release structural carbohydrate-derived molecules, including lipo-

447   chito-oligosaccharides (Maillet *et al.*, 2011) and short chitin tetra- and pentamers (Genre

448   *et al.*, 2013), which are perceived by the root surface epidermal cells, where they trigger

449   the activation of the so-called common symbiosis signaling pathway - the same pathway

450   involved in the nitrogen fixing legume symbiosis (Oldroyd, 2013; MacLean *et al.*, 2017),

451   which includes several evolutionarily conserved genes needed for fungal accommodation.

452   The CSSP induces the expression of a range of symbiosis-supporting genes (Schmitz and

453   Harrison, 2014; Hohnjec *et al.*, 2015) among which are factors that coordinate the

454   expression of a set of downstream genes involved in specific plant symbiotic functions

455   such as phosphate uptake and lipid synthesis and transport (Pimprikar and Gutjahr, 2018;

456   Hartmann *et al.*, 2019). Notably, a recent comparative survey of hundreds of plant

457   genomes and transcriptomes showed that a core set of CSSP members and downstream

458   genes are conserved in all plant lineages hosting intracellular symbionts including AM,

459   ORM and ERM, as well as nitrogen-fixing symbioses, but these genes have been lost in

460   plant lineages hosting extracellular symbionts (including ECM) or those that are not

461   symbiotic (Radhakrishnan *et al.*, 2020).

462   At a cellular level, fungal hyphae contact the surface cells of host roots through swollen

463   adhesion structures called hyphopodia. Penetrating hyphae develop inside the root cortex,

464   taking an intercellular and/or intracellular route. Once the inner root cortex is reached,

465 hyphae penetrate single cells, where they give rise to highly branched structures called
466 arbuscules (Gutjahr & Parniske, 2013; Lanfranco *et al*., 2018; Fig. 2). Intracellular
467 hyphae, including large and fine branches of arbuscules, are surrounded by the perifungal
468 membrane, a plant derived specialized membrane, which functions to host the fungal
469 structures and separate them from the plant cell contents (Bonfante, 2018). Arbuscules
470 are believed to be the main sites for nutrient transfer between partners. Different studies
471 based on functional genomics analyses have provided evidence of these nutritional
472 exchanges between partners (Javot *et al*., 2007; Willmann *et al*., 2013, Rich *et al*., 2017;
473 Roth and Paszkowski, 2017; Wang *et al*., 2017; Keymer and Gutjahr, 2018). Mycorrhiza-
474 specific phosphate transporters, located at the membrane surrounding arbuscules and
475 responsible of the uptake of inorganic phosphorus ions released by the fungus, have been
476 pinpointed in different host species (Harrison *et al*., 2002; Paszkowski *et al*., 2002),
477 providing a mechanicistic basis for the well known benefit of phosphorus tranfer. Only
478 recently, a nitrate transporter from rice (OsNPF4.5), exclusively expressed in the cells
479 containing arbuscules, was shown to play a key role in mycorrhizal nitrate acquisition;
480 the up-regulation of related genes in maize and sorghum suggests that this symbiotic
481 route of nitrogen uptake might be conserved in cereal species (Wang *et al*., 2020).
482 On the fungal side, a six-carbon sugar transporter possibly involved in the uptake of
483 compounds from the host has been characterized (Helber *et al*., 2011). However, one of
484 the most important discoveries in the biology of AM fungi was obtained from genomic
485 analyses that showed that they lack genes encoding for the synthesis of fats (Wewer *et*
486 *al*., 2014). Their dependence on a living host for these molecules was then supported by
487 several other studies showing that AM fungi rely on lipids received from the host plant
488 (Jang *et al*., 2017; Luginbuehl, *et al*., 2017; Keymer *et al*., 2017). This finding has shed
489 light on the obligate nutritional nature of AM fungi and has suggested new strategies for
490 large scale production of AM inocula (Kameoka *et al*., 2019).
491 There is now great interest to decipher whether AM fungi prefer carbohydrates or fats
492 (Rich *et al*., 2017) derived from the host and to clarify the forces controlling the
493 exchange of nutrients in this mutualistic association. Toby Kiers has proposed the
494 "biological market" theory in which one partner preferentially allocates resources in
495 direct proportion to the relative amount of benefits it receives from the other partner
496 (Kiers *et al*., 2011; Werner *et al*., 2015). However, this simple mechanism does not
497 always apply and the issue deserves further investigations (Walder and van der Heijden,
498 2015; Kiers *et al*., 2016).

499

Genetic variation among the partners affects the outcome of the symbiosis

500 With the increasing availability of genomic resources, natural or induced (*via*
501 domestication) genetic variation has become a promising field of investigation that will
502 be instrumental to further explore the genetic determinants controlling the establishment
503 and functioning of mycorrhizal symbioses. It is well known that the outcome of the AM
504 symbiosis can be strongly influenced by the specific genetic makeup of the plant and the
505 fungal partners (Lanfranco *et al*., 2018). Despite the low morphological variation, AM
506 fungi show a high level of genetic variability and high functional diversity, even
507 considering isolates belonging to the same species (Koch *et al*., 2017). On the other hand,
508 responsiveness to the AM symbiosis can differ among plant cultivars of the same species
509 and, in addition, it is affected by soil nutritional status (Sawers *et al*., 2010; Chu *et al*.,
510 2013), pointing out a complex genotype-environment interaction. Sawers and colleagues
511 (2017) investigated AM-responsiveness in several American maize lines and identified a
512 correlation bewteen the performance of the symbiosis (in terms of shoot phosphorus
513 content and dry weight) with the amount of associated hyphae outside the root. The plant
514 molecular determinants at the basis of the fungal performance are still unknown; it has
515 been hypothesized that it could be related to the amount of carbohydrates and lipids
516 released by the plant.

517 Plant genetic variation also determines the root colonization level. In this context,
518 Lehnert *et al*. (2017) monitored root colonization on 94 bread wheat genotypes colonized
519 by a mixed inoculum of three AM fungal species. Genomic regions containing genes
520 related to defence and cell wall metabolism have been reported to be associated with the
521 low colonization level detected in some genotypes (Lehnert *et al*., 2017).

522 It has also been suggested that domestication, which was likely carried out under highly
523 fertilized conditions, may have decreased the ability of plants to be colonized by and to
524 respond positively to AM fungi (Lehmann *et al*., 2012). To address this issue, Stefani and
525 colleagues (2020) recently surveyed the AM fungal communities associated to different
526 durum wheat varieties released at different times in the history of durum wheat breeding.
527 Using deep 18S rDNA sequencing under field conditions, low variation in the AM fungal
528 diversity associated with the different cultivars was found. It has been hypothesized that
529 the genetic variation among plant genotypes seems to be too narrow to select for specific
530 plant-AM fungal associations; thus, in this case breeding has only marginally altered the

532   symbiotic signalling systems and the core of genes involved in mycorrhization (Stefani *et*
533   *al*., 2020).

534   In another interesting work, Martın-Robles *et al*. (2018) compared the AM-
535   responsiveness in different crops and in their wild progenitors. They showed that growth
536   benefits exerted by the AM symbiosis were dependent on inorganic phosphorus
537   availability; while wild progenitors positively responded to the AM symbiosis
538   irrespective of availability, in domesticated plants the growth effect became negligible
539   when phosphorus availability increased. These data suggest that domestication selected
540   for AM independence at high phosphorus concentrations, which might support the
541   possibility to increase yield in the absence of the fungus-associated carbon drain.

542   We envisage that further investigations on natural variations on both the fungus and the
543   plant side will have an impact on fundamental as well as on applied research.

544

545   **AM translational research: pitfalls and successes**
546   Due to the several benefits that mycorrhizal fungi can provide, ranging from the level of
547   the individual host plant to the entire ecosystem, these fungi are of high relevance for
548   applied purposes. The impressively wide host range, which includes major crops, implies
549   a large potential for AM fungi, especially in agroecosystems (Berruti *et al*., 2016; Chen
550   *et al*., 2018). However, results of the benefits provided by AMF to host plants obtained in
551   glasshouses may not directly be relevant in the field since, under these conditions,
552   several complex variables contribute to shape the outcome of the symbiosis. A very
553   important issue to consider is how benefit is defined; one parameter, often used under
554   controlled conditions, is the mycorrhizal growth response, MGR, the difference in
555   growth between colonized and non colonized plants at a defined level of resource supply
556   (Janos 2007; Johnson *et al*., 2015). However, under field conditions, crop yield may
557   represent a more interesting parameter. As commented by Ryan & Graham (2018) in a
558   field meta analysis study, a benefit in terms of increased yield is not often evident,
559   especially when cereals are considered. But to measure the activity of AM on crop
560   productivity in field conditions is not easy, mainly due to the need to have non
561   mycorrhizal (control) plants for comparison. Despite some inconsistencies, there are
562   many crops for which AM fungi have been clearly shown to increase yield, including
563   cassava (Ceballos *et al*., 2013; Rodriguez and Sanders, 2015), potato (Hijri, 2016) and
564   chickpea (Rocha *et al*., 2019). Moreover, increased temporal productivity stability (Yang
565   *et al*., 2014), enhanced yield quality (Lehmann *et al*., 2014; Bona *et al*., 2017; Torres *et*

566   *al*., 2018) and higher tolerance to biotic stresses (Fiorilli *et al*., 2018) have also been
567   observed (Fig. 1). Remarkably, even in the absence of a yield increase, the reduction in
568   the amount of water and fertilizer required to achieve the same yield, will influence
569   profitability and reduce the environmental impact. As advocated by Rillig *et al*. (2019)
570   all these aspects have to be considered: following the concept of agroecology, based on
571   the enhancement of ecosystem services to improve sustainable agricultural production
572   (Bender *et al*., 2016; DeClerck *et al*., 2016), the development of production systems,
573   where all the potential benefits provided by mycorrhizal symbionts as well as by other
574   beneficial soil microbes is maximized, needs to be pursued in the future.
575

Conclusions and perspectives

577   Plants represent a preferential ecological niche for many fungi which may provide
578   several benefits to the host (Fig. 1). If mycorrhizal symbiosis -at least the AM- has
579   received great attention, the world of plant endophytes is still overlooked. Based on the
580   current data on the description of novel fungi and the discovery of new metabolites,
581   endophytes seem to be an extremely valuable resource for bioprospecting.
582   There is also a clear need to integrate endophytic and mycorrhizal fungal research, as
583   important insights on how the host plant can recognize the symbiont, manage its
584   accommodation and the establishment of a beneficial interaction can be identified
585   (Hilbert *et al*., 2020). In many mycorrhizal studies, endophytes are ignored or reported as
586   contaminants, but we can envisage complex interactions with functional significance
587   (Lace *et a*l., 2015). Studying the plant microbiota as a whole system will be the next
588   challenge to gain a more comprehensive knowledge of the functions of the fungal
589   symbionts living inside a plant (van der Heijden and Hartmann 2016; Thomashow et al.,
590   2019).
591   Mycorrhizal fungi and endophytes enable plant hosts to survive in hostile environments
592   and can play a key role in improving plant responses to climate change. The development
593   of tailored microbial inocula for specific crops and environmental conditions appears to
594   be a promising contribution to solve urgent societal needs, an improvement of
595   environment-friendly agricultural crop production systems, and an increase of global
596   food security.
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Figure legends

Figure 1. Beneficial effects of plant fungal endophytes and mycorrhizal fungi. The plant-associated endophytes *Epichloë* (light pink), *Trichoderma* (yellow), *Serendipita indica* (turquoise), *Colletotrichum tofieldiae* (blue) and the AM fungi (purple) colonize different plant organs (dashed lines). They can provide several benefits to the host plant directly, via nutrient acquisition, or indirectly, through the modulation of plant hormones and the induction of plant defences to respond to pathogen attack. Each microorganism is represented by dot with corresponding color code.

Figure 2. Scheme of the main cellular features of ectomycorrhizas, arbuscular, orchid and ericoid mycorrhizas (from Genre *et al*., 2020). In arbuscular mycorrhizas, fungal hyphae contact epidermal cells through a flattened structure, the hyphopodium. Penetrating hyphae then develop *via* inter- and intracellular structures to reach the inner cortical cells, where they form highly branched tree-like structures called arbuscules. Fungal hyphae are always separated from the plant cell cytoplasm by an extracellular interface (green) surrounded by a membrane that develops from an extension of the host plasma membrane (orange). This interface is thought to be the main site of nutrient exchange. In ericoid mycorrhizas, hyphae penetrate the thick surface cell walls of ericaceae roots and produce dense hyphal coils inside each epidermal cell that also are surrounded by an interface compartment (green) and an extension of the plant membrane. In orchid mycorrhizas, the best described colonization process is in protocorms, the post-embryonic developmental phase after seed germination. Hyphae penetrate epidermal hair cells and reach the cortical tissue, where they form large hyphal coils, called 'pelotons', within a membrane-delimited symbiotic interface. In ectomycorrhizas, fungal hyphae form a tissue known as the 'sheathing mantle', which engulfs the whole root tip. Inner hyphae further develop between epidermal cells, reaching different depths into the cortical tissue depending on the host plant. These intraradical hyphae, called the Hartig net, never penetrate the cell lumen, and form an intercellular interface (green).