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Functional and practical importance of AMF mixed inoculants for plant development

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Abstract

Until a recent past, arbuscular mycorrhizal fungi (AMF) were generally used as single species inoculants carefully selected to enhance plant growth. However, in ecological perspective, it is expected that a mixture of different AMF species would be more efficient to occupy the ecological niches of the symbiosis and would adapt better to environmental fluctuations. A limited number of studies have reported complementary effects of different AMF taxa on plant development. Experiments were performed in different conditions. In most cases, combinations of AMF showed better results than single species. Particularly clear results were obtained in ultramafic soils characterized by multiple stress factors: low level of main mineral elements, high concentrations of potentially toxic metals (Ni, Co, Cr, Mn), and critically low Ca/Mg ratio. These studies reported synergistic effects of mixed isolates belonging to different families. The most performant mix showed quantities of P and K absorbed per plant more than 10 times higher than non-inoculated plants and more than two times higher than the most performant AMF isolate. Inversely, the translocation factors for potentially toxic metals (particularly Ni and Co) were reduced three to ten times. Mathematical modelization of the effects of 12 AMF inoculants, including six mixes on plant growth, revealed that the predicted value of the performance based on the properties of the different AMF was very high ($R^2 = 0.90$; $p < 0.00001$), indicating that the improvement of plant biomass was nearly totally explained as a resultant of the complementary effects of the 5 isolates. Field experiments in natural conditions and commercial trials confirmed the greenhouse results obtained in sterilized soils and revealed large positive effects on plant development of selected AMF mixes. For such success, it is suggested that the isolates used for the mixed inoculants must belong to different families, and must be selected for their synergistic effects on plant development.

40

41 **KeyWords** Arbuscular mycorrhiza; AMF mixture; multispecies inoculum; functional
42 complementarity; commercial inoculant

43 **1 Introduction**

44 Arbuscular mycorrhizal fungi (AMF) are ubiquitous plant symbionts with great potential as
45 biofertilizers for biotechnological improvement of plant development in environmental and
46 agricultural sustainable practices. The mastery of AMF inoculants is an important goal for the coming
47 decades (Vosátka et al. 2012; Berruti et al. 2016). This mastery can help solve global problems such
48 as increasing soil degradation, concerns related to chemical fertilizers such as water pollution and
49 phosphorus depletion, and adaptation to climate change. However, due to the high complexity and
50 diversity of the soils, different difficulties are still limiting commercial use of AMF to optimize
51 agroecosystem services (Sportes et al. 2021).

52 The effects of AMF on plant development are highly variable in relation to plant taxa, fungal
53 isolates, and substrate composition (Wang and Qiu 2006; Hoeksema et al. 2010; Smith et al. 2010;
54 Faye et al. 2013; Koch et al. 2017). The efficiency of the isolates in plant growth promotion can vary
55 from negative to highly positive effect (Smith and Smith 2012). The number of studies concerning
56 the efficiency of AMF inoculants has increased in the last decade. A search on Google Scholar with
57 the keywords “arbuscular mycorrhizal inoculants” and “arbuscular mycorrhizal inoculum” from 2000
58 to 2010 gives a result of 402 papers; from 2011 to 2021 the search leads to 579 papers. The number
59 of companies producing AMF inoculants grew from 10 in 1990 to 75 in 2017 (Sportes et al. 2021).
60 Thus, from the 2000s onwards, more and more companies developed end-use formulations with
61 varied success (Duffy and Cassels 2000; Ijdo et al. 2011; Faye et al. 2013; Berruti et al. 2016; Hart et
62 al. 2017; Srivastava et al. 2021). Srivastava et al. (2021) reported an analysis of 696 patents published
63 in the two last decades showing that AMF have been used consistently as biofertilizers and
64 bioremediators. However, in a meta-analysis of 631 papers relating to mycorrhiza inoculation, from
65 1970 to 2014, Hart et al. (2017) showed that there is yet a lot to do before mastering correctly
66 commercial inoculants at large scale. The inefficiency of different commercial AMF inoculants is
67 sometimes simply due to the absence of viable propagules (Salomon et al. 2021) related to bad
68 technical practices. But there are also more complex difficulties that need new investigations (Berruti
69 et al. 2016; Hart et al. 2017).

70 Until a recent past, AMF were generally commercialized as single species inoculants carefully
71 selected to enhance plant growth and concerned only a few well-studied fungal species relatively easy
72 to produce, such as *Rhizophagus intraradices*, *R. irregularis*, *Funneliformis mosseae*, and
73 *Claroideoglossum etunicatum* (Ijdo et al. 2011; Vosátka et al. 2012; Berruti et al. 2017; Basiru et al.
74 2021). However, as stressed by Hart et al. (2017), there is no evidence that a given selected AMF
75 could be “superior” to the others in diverse soil and environmental conditions. This use of a single

76 “performant” isolate is now undergoing change because in ecological perspective, it is expected that
77 a mixture of different AMF species would be more efficient to occupy the ecological niches of the
78 symbiosis and adapt better to environmental fluctuations. This appeared clearer when it was
79 evidenced that each plant can host many AMF species (Jansa et al. 2003; Vandenkoornhuysen et al.
80 2007). From 2001 to 2015, about 21% of AMF inoculation experiments concerned multispecies
81 inoculants, and 18 % tested multispecies and monospecies inoculants (Berruti et al. 2016). However,
82 a limited number of studies reported complementary effects of different AMF taxa on plant
83 development. Kiers et al. (2011) suggested that plant species select AMF partners by transferring
84 more carbohydrates to the symbionts that are better for their development. Inversely, AMF reinforce
85 this selected relation by transferring more nutrients to plants that feed them better. These selective
86 interactions suggest how AMF diversity linked to one plant can reach a balanced state optimizing the
87 functional traits of the symbiosis. In practice, only a part of the comparative studies showed that AMF
88 mixed inoculants were more efficient than single inoculants (Jin et al. 2013; Chen et al. 2017; Yang
89 et al. 2017; Crossay et al. 2019; Fathalla 2020; Martignoni 2021). Different factors can explain the
90 variations in conclusions concerning the AMF-mixed inoculants in relation to soil or substrate
91 variability, specific conditions of the experiments, AMF origin, and selection criteria of the isolates.
92 It is then of major importance to synthesize studies that can allow to understand more clearly these
93 variations and their consequences on the efficiency of AMF inoculants.

94 This chapter aims to highlight the interest of AMF mixed inoculants and how they should be
95 selected. A particular focus is made on using mixed inoculants in extreme soils for plants facing
96 multi-stress environments. These environments are considered preferential targets for commercial
97 use of AMF inoculants because of their clear effects in these conditions (Vosátka et al. 2012; Hart et
98 al. 2017). This is particularly the case of ultramafic soils characterized by nutrient deficiencies and
99 high concentrations of potentially toxic metals (Brooks 1987; Jaffré and L’Huillier 2010).

100

101 **2 Interest of mixed inoculants**

102 ***2.1 Main problems posed by inoculation***

103 In natural conditions, AMF inoculant faces niche competition with local AMF communities for root
104 colonization (Jefwa et al. 2009; Faye et al. 2013) and can also be affected by other microbial taxa in
105 the rhizosphere, a very dense microenvironment with a high level of antagonism and stress (Hinsinger
106 et al. 2009; Miransari 2010; Velásquez and Cabello 2010). Thus, the success in the field of a selected
107 AMF inoculant is not guaranteed, particularly for mono-species ones and non-native isolates

108 generally less adapted to local conditions (Henkel e al. 1989; Faye et al. 2013; Pellegrino and Bedini
109 2014). To reduce this difficulty, AMF inoculation is generally performed with a sterile or poorly
110 colonized substrate in a nursery (Velàszquez and Cabello, 2010; Amir et al. 2019) before transferring
111 the plants to the field. Even in these conditions, AMF inoculant can decline in roots of inoculated
112 plants, few months to few years after transfer (Pellegrino et al. 2012; Amir et al. 2019). A progressive
113 decline of the AMF inoculated population was also reported in greenhouse conditions in the presence
114 of a pre-established arbuscular mycorrhizal fungal community (Janoušková et al. 2013). According
115 to this latter study, the decline varied with AMF species and was observed 12 to 24 weeks after
116 inoculation. A combination of different AMF taxa can allow a better adaptation and then better
117 stability in the ecosystem with the occupation of more ecological niches (Maherali and Klironomos
118 2007; Yang et al. 2017; Hart et al. 2017; Crossay et al. 2019). Indeed, as suggested by Kiers et al.
119 (2011), the interactions between AMF communities and plant host result in a selection of a number
120 of AMF taxa that can optimize the symbiosis. Furthermore, Bennett and Bever (2009) found that
121 AMF isolates that have a high competitive ability can be bad mutualists, and good mutualists can
122 have low competitive ability. A trade-off between these two abilities may then occur between AMF
123 communities during the competition for root colonization. These selective interactions vary
124 depending on soil, plant, and environment (Bever 2002; Hart et al. 2017). For all these reasons, it is
125 important to test the performance of AMF inoculants, not only in simplified conditions but also in
126 varied natural conditions before their use on a large scale, as also suggested by other authors (Faye et
127 al. 2013; Hart et al. 2017).

128 Several types of difficulties can explain the variations in conclusions of experiments that compare
129 the efficiency of AMF inoculants. Differences in symbiosis performance can be due to variations in
130 selection criteria used to perform the AMF mixtures. Thus, Maherali and Klironomos (2007) found
131 that the more the phylogenetic distance between AMF of the inoculant consortium was, the more the
132 efficiency of the symbiosis was, due to functional complementarity and reduced competition between
133 the isolates. Chen et al. (2017) and Crossay et al. (2019) also confirmed that AMF consortium
134 consisting of phylogenetically distant AMF species have a better effect than a single isolate or closely
135 related ones. Fathallah (2020) found that an inoculant with two AMF isolates belonging to different
136 families showed better root colonization and was more efficient in increasing wheat grain production
137 than single species or close species. In a meta-analysis, Yang et al. (2017) reported that the
138 performance of the symbiosis was positively correlated to AMF family richness and not to their
139 species richness. According to Gosling et al. (2016), the failure of mixed inoculants compared with
140 single ones is generally linked to functional redundancy of phylogenetically close species.
141 Conversely, Martignoni et al. (2021) studied the effects on plant productivity of two AMF isolates

142 differing for their carbon demand (carbon sink strength) and showed their functional complementarity
143 when mixed. The isolate with high carbon sink strength was able to establish a fungal community
144 more quickly and improved the nutrition and the growth of the plant, whereas the isolate with low
145 carbon sink strength inflicted lower carbon costs to the host plant and supported maximal plant
146 productivity once the plant biomass was large.

147 However, the only consideration of taxonomic and functional resolution may not be sufficient to
148 explain the relative success or failure of a mixed inoculant. For example, Malicka et al. (2021)
149 compared the effects of three native AMF isolates belonging to three different families and their mix
150 on the growth of *Lolium perenne* in pots with a mixture of sand and bentonite contaminated with
151 phenol and polynuclear aromatic hydrocarbons. They showed that mixed inoculant was less
152 performant than the single ones. Here, we can suspect that using a simplified substrate instead of the
153 soil from where AMF were isolated could be a cause of these results. Indeed, the abiotic and biotic
154 conditions, and consequently the substrate's ecological niches, are very different from the original
155 soil. Thus, differences in experimental conditions, particularly AMF origin and soils or substrates
156 used to test them, can influence the relative performance of the inoculants. The relevance of
157 comparisons on a unique soil of AMF isolates of different origins is also questionable for the same
158 reasons (Faye et al. 2013). Gosling et al. (2016) suggested that soils characterized by multiple stress
159 factors would reveal the benefits of AMF diversity more clearly. All these reasons can explain why
160 the effectiveness of commercial inoculants is often not apparent and vary depending on conditions
161 (Faye et al. 2013; Hart et al. 2017).

162

163 **2.2 Case studies on ultramafic soils**

164 The use of AMF inoculants in stressful environments, particularly for restoring degraded ecosystems
165 and soil remediation, is considered as important (Valliere et al. 2020) and commercially less difficult
166 (Vosátka et al. 2012; Hart et al. 2017). It could have different advantages, not only for plant growth
167 promotion and adaptation but also to reduce soil erosion, due to soil binding capacity of extraradical
168 mycelium (Mozafar et al. 2002; Vosátka et al. 2012) and glomalin production (Rilling et al. 2004;
169 Vodnik et al. 2008). As stressed by Gosling et al. (2016), the benefits of AMF mixed inoculants,
170 compared with single species ones, could become clearly apparent when the plant host faces multiple
171 stress factors. Ultramafic soils can then be considered as good models to highlight the interest of
172 mixed inoculants, with at least three types of abiotic stresses (Brooks 1987; Proctor 2003; Jaffré and
173 L'Huillier 2010): i) main element deficiencies (particularly P and K), ii) potentially toxic metals (Ni,
174 Co, Cr, Mn), and iii) low Ca/Mg value (< 1) which is a limiting factor because Ca absorption is

175 restricted by the competition with Mg cations. In these ecosystems, AMF symbiosis has been shown
176 to be abundant and essential (Perrier et al. 2006; Doubkova et al. 2012, 2013; Amir et al. 2013; Amir
177 and Ducouso 2010; Lagrange et al. 2013).

178 Five new species of AMF from New Caledonian ultramafic soils, recently
179 described, *Rhizophagus neocaledonicus*, *Acaulospora saccata*, *A. fragilissima*, *Scutelospora*
180 *ovalis*, and *Pervetustus simplex* (Blaszkowski et al. 2017; Crossay et al. 2018), and a native isolate
181 of *Claroideoglomus etunicatum*, have been tested on host plants (Crossay et al. 2019, 2020). Crossay
182 et al. (2019) have compared the effects of these 6 isolates inoculated separately, or in combinations,
183 on the development of *Metrosideros laurifolia* (an endemic shrub), in greenhouse conditions. The
184 tested isolates differed for their performance features: root colonization, sporulation, effects on N, P,
185 K, and Ca plant uptake, influence on Ca/Mg value of plant organs, and effect on potentially toxic
186 metal translocation. Two AMF mixes showed synergistic effects on the growth and adaptation of the
187 plant (Fig. 1a). The most performant treatment (mix 5) was a consortium of 5 isolates belonging to 5
188 different families (Glomeraceae, Claroideoglomeraceae, Acaulosporaceae, Gigasporaceae and
189 Pervetustaceae), confirming that combinations with phylogenetically distant isolates are more
190 efficient, as suggested by other authors (Chen et al. 2017; Maherali and Kliromonos, 2007; Yang et
191 al. 2017). Mix 5 was more efficient than the other mixes for most of the tested properties (Fig. 1b,
192 1c). For this mixed inoculum quantities of P and K absorbed per plant were more than 10 times higher
193 than non-inoculated plants and more than two times higher than the most performant AMF isolate.
194 Ca/Mg values of plant organs were also significantly higher than plants inoculated with a single AMF.
195 The translocation factors for potentially toxic metals (particularly Ni and Co) were reduced three to
196 ten times. Moreover, Crossay et al. (2020) showed that this last effect was clearer for sorghum (a low
197 metal-tolerant plant), than for *M. laurifolia*. This result reinforces the conclusion that the symbiosis
198 involving AMF efficient consortium can optimize plant adaptation to the soil in which it develops.
199 Crossay et al. (2019) also reported a mathematical model demonstrating the synergistic effects of the
200 native AMF isolates (Fig. 1 d). The correlation between plant biomass (variable to be explained) and
201 the predicted value of the performance based on the properties of the different AMF was very high
202 ($R^2 = 0.90$; $p < 0.00001$), indicating that the improvement of plant biomass was nearly totally
203 explained as a resultant of the complementary effects of the 5 isolates.

204 As noted before, the inoculated AMF are generally progressively replaced by native taxa in the
205 field. However, contrary to the assertion of Hart et al. (2017), this could not be a problem. Indeed,
206 the inoculant primary effect can continue indirectly to stimulate plant development because the better
207 health of the inoculated plants allows a higher metabolism and photosynthesis and then a better ability
208 to feed AMF communities (Amir et al. 2019). These latter authors performed a field experiment to

209 restore an ultramafic area. They used degraded topsoil containing a low initial level of AMF viable
210 spores. *Metrosideros laurifolia* plants, inoculated with a mix of three selected AMF isolates showed
211 a clearly higher level of mycorrhizal colonization than non-inoculated ones after 528 days of field
212 plantation, while other taxa have mainly replaced AMF inoculant. Indeed, after only 335 days, the
213 inoculated taxa represented not more than 9% of AMF communities inside plant roots. However,
214 AMF diversity was significantly higher in the roots of inoculated plants, which weighed four times
215 more than in control plots.

216 The precedent studies allowed the selection of the better AMF consortium improving plant
217 adaptation and growth on ultramafic soils. After this work, a company developed a dixenic production
218 protocol for the selected AMF species to market a suitable product (Fig. 2). Today, different
219 companies use the AMF commercial inoculant for the restoration of mine-degraded areas.

220 These case studies clearly show the interest of AMF native mixed inoculants with isolates
221 phylogenetically distant. The main practical conclusion is that the selection of AMF isolates aiming
222 to perform as an efficient mixed inoculant must be based on their complementary features and effects
223 on different aspects of plant development. However, more field trials must be assessed to evaluate
224 the effect of AMF selected consortia in diverse situations. More generally, rigorous field experiments
225 are still needed to demonstrate the benefit of AMF inoculation, at a large-scale, for plantations and
226 ecosystems (Vosátka et al. 2012; Berruti et al. 2016, Hart et al. 2017), and check the absence of
227 ecosystemic perturbations linked to interactions between introduced and native AMF over several
228 years (Antunes and Koyama 2017, Hart et al. 2017).

229

230 **3 Towards more complexity in practices**

231 As just illustrated, there are different advantages of using multispecies inoculants; however, there are
232 also some difficulties. The most important is that only a reduced number of AMF species can be
233 produced aseptically (Vosátka et al. 2012; Berruti et al. 2016), particularly with root-organ
234 monoxenic culture. Therefore, it could be challenging to guarantee the total safety of the inoculants
235 in terms of deleterious microorganisms (Ijdo et al. 2011; Vosátka et al. 2012; Berruti et al. 2016)
236 when at least a part of AMF consortium was not produced in that safety conditions. However, this
237 problem will likely be solved with the improvement of AMF culture technologies. The other difficulty
238 is the more complex and longer selection strategy of complementary AMF isolates compared to single
239 inoculants. Nevertheless, this constraint could be easily accepted if the superiority of the mixed
240 inoculants, when correctly selected, is demonstrated in diverse conditions.

241 If the ecological point of view supports the use of inoculants containing functionally and
242 phylogenetically distant mixed AMF, it would also suggest, for the future, the use of more complex
243 inoculants affecting more ecological niches. Indeed, different studies (Vasquez et al. 2000; Miransari
244 2010; Vosátka et al. 2012; Bizoš et al. 2020; Bourles et al. 2020) suggest the use of AMF isolates
245 mixed with other microorganisms, such as mycorrhiza helper bacteria (MHB), plant growth
246 promoting rhizobacteria (PGPR), and/or saprophytic or endophytic performant fungi such
247 as *Trichoderma* and *Fusarium*.

248 Vosátka et al. (2012) conclude their synthesis on mycorrhizal biotechnology and industry by
249 indicating that mycorrhizal inoculation is not a panacea and that “protection and proper management
250 of native AMF populations in soils is a primary tool to exploit positive effects of mycorrhizal
251 symbiosis phenomena.” It is indeed possible to optimize mycorrhizal symbiosis without inoculation.
252 For example, Brito et al. (2021) developed an agricultural strategy that stimulates AMF extension as
253 a soil health condition (Abbott and Lumley 2015) for a sustainable development perspective. The
254 authors (Brito et al. 2021) used plants that favor AMF development with non-tilled soil or appropriate
255 tillage methods that avoid perturbation of extra-radical mycelium. They showed that these conditions
256 induce a higher AMF diversity, with better plant growth and higher tolerance of plants to abiotic and
257 biotic stresses, including tolerance to manganese toxicity and fungal diseases. This is an excellent
258 example of integrated use of natural AMF diversity to improve plant production.

259

260 **4 Conclusion and perspectives**

261 Biotechnologies for commercial production of AMF inoculants are undergoing processes of
262 improvement. In the context of a fast-changing world in reaction to the global ecological crisis, their
263 use for ecosystem restoration, soil remediation, and sustainable agriculture may be of high interest.
264 However, different challenges are still running before large-scale use of these new practices. The
265 success of AMF inoculants as biofertilizers will thus depend on a few points that need more focalized
266 research. These points are mostly listed in different reviews (Idjo et al. 2011; Vosátka et al. 2012;
267 Berruti et al. 2016; Hart et al., 2017), and it is not necessary to report them here. Overall, the two
268 major difficulties of any microbial inoculation aiming plant development consist of i) an inoculant
269 adequation with functional and ecological niches that can receive the entering microorganisms; ii) a
270 rigorous selection of the isolates to stick with the set ends. These are highly challenging goals that
271 need quite complex studies for each inoculant before reaching the commercial stage. Here we want
272 to stress the aspects mainly discussed in this text. Technologies generally use very tight approaches,

273 but, in this case, it is necessary to follow a systemic approach to answer correctly the difficulties we
274 just outlined. In this perspective, the use of AMF consortia correctly selected for their synergistic
275 effects may significantly improve the inoculant efficiency. More molecular research is also needed
276 to track correctly each isolate of the consortium among soil AMF communities and monitor their
277 persistence over several years (Thonar et al. 2012; Berruti et al. 2013; Hart et al. 2017).

278 As suggested by some reviews (Vosátka et al. 2012; Berruti et al. 2016), commercial inoculants'
279 production probably needs to be more localized, with selection of AMF isolates for a type of soil or
280 ecosystem in a given region. This will allow a better adaptation of the inoculant and prevent a possible
281 ecosystem transformation in relation to changes in mycorrhizal communities (Antunes and Koyama
282 2017; Islam et al. 2021). At least in some cases, producers may adapt their inoculants so that the
283 inoculant efficiency could be totally mastered and ecologically indisputable. In all cases, this will
284 need more research to develop practical but complex methods to perform such selection, particularly
285 for mixed inoculants. The AMF features could then be determined in relation to the type of expected
286 conditions and effects: type of environment and soil (mineral deficiencies, toxicity of particular
287 elements, drought limits, salinity, etc.), agricultural production to be optimized (whole plant, grains,
288 fruits or other organs), etc. Therefore, AMF isolates to be included in the mixed inoculant must be
289 selected for these features, and must present complementary performances so that their resultant
290 effect on plant health and production could be optimized. A mathematical modelization of the effects
291 of the different variables influencing the symbiosis efficiency could be produced as approached by
292 Crossay et al. (2019). The selection tests could then be standardized, and a software application able
293 to give a performance value for each AMF consortium could be created. This will allow adapting
294 more easily the inoculant production to the different conditions as noted above while keeping the
295 commercial inoculants in an acceptable bracket of prices.

296

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