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1 **Phosphorus is a critical factor of the *in vitro* monoxenic culture method for a**
2 **wide range of arbuscular mycorrhizal fungi culture collections**
3
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10
11 **Abstract**
12 Establishing an effective way to propagate a wide range of arbuscular mycorrhizal
13 (AM) fungi species is desirable for mycorrhizal research and agricultural
14 applications. Although the success of mycorrhizal formation is required for spore
15 production of AM fungi, the critical factors for its construction in the *in vitro*
16 monoxenic culture protocol remain to be identified. In this study, we evaluated the
17 growth of hairy roots from carrot, flax, and chicory, and investigated the effects of
18 the phosphorus (P) concentration in the mother plate, as well as the levels of P,
19 sucrose, and macronutrients in a cocultivation plate with a hairy root, amount of
20 medium of the cocultivation plate, and location of spore inoculation, by utilizing the
21 Bayesian information criterion model selection with greater than 800 units of data.
22 We found that the flax hairy root was suitable for *in vitro* monoxenic culture, and
23 that the concentration of P in the cocultivation plate was a critical factor for
24 mycorrhizal formation. We showed that an extremely low concentration of P (3 μM)
25 significantly improved mycorrhizal formation for AM fungi belonging to the
26 Glomerales order, while a high concentration of P (30 μM) was suitable for
27 Diversisporales fungi. Therefore, we anticipate that the refining the P concentration
28 will contribute to future culture collections of a wide range of AM fungi.
29

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30 **Introduction**

31 Arbuscular mycorrhizal (AM) fungi are obligate biotrophs that develop symbiotic
32 relationships with the vast majority of land plants, to create arguably the world's
33 most prevalent mutualism (Brundrett, 2009; Smith and Read, 2008). Through
34 symbiosis, AM fungi allow plants to obtain soil resources effectively, while receiving
35 carbon in the form of sugars and lipids from the host plants (Luginbuehl et al., 2017).
36 These fungi also confer tolerance against environmental stresses such as water,
37 salinity, and heavy metal levels to the hosts (Augé et al., 2014; Díaz et al., 1996;
38 Porcel et al., 2012), and enhance disease resistance and photosynthetic activity
39 (Boldt et al., 2011; Jung et al., 2012; Pozo & Azcón-Aguilar, 2007). Because of these
40 beneficial effects to plants, substantial efforts have been made to establish ways to
41 propagate AM fungi for agricultural applications (Berruti et al., 2016; Sawers et al.,
42 2008).

43 AM fungi belong to the subphylum Glomeromycotina and originated in the
44 Ordovician period, approximately 480 million years ago (Redecker et al., 2000;
45 Spatafora et al., 2016). Approximately 342 AM fungal species have been reported
46 and classified into four orders (Diversisporales, Glomerales, Archaeosporales, and
47 Paraglomerales), 11 families, and 43 genera (Redecker et al. 2013, http://www.amf-
48 phylogeny.com/amphylo_species.html). Since the majority are unculturable under
49 *in vitro* conditions, their symbiotic functions in host plants are largely unknown.
50 Since AM fungi and their host plants interact in complex underground networks
51 involving multiple partners, an *in vitro* pure culture method for an isolated AM
52 fungus is required to directly assess the symbiotic interaction between AM fungi and
53 their hosts.

54 One of the most successful methods of *in vitro* pure culture for AM fungi is
55 the use of Ri T-DNA transformed roots (hairy roots), known as *in vitro* monoxenic
56 culturing (Bécard & Fortin, 1988a; Chabot et al., 1992; Cranenbrouck et al., 2005;
57 Mugnier & Mosse, 1987). In the past three decades, *in vitro* monoxenic culturing
58 with hairy roots has become an important technique for investigating the physiology
59 of AM fungi and their associations with host plants (Fortin et al., 2002). Using this
60 method, spores of the AM fungus *Rhizophagus irregularis* can be propagated *in vitro*
61 for laboratory and greenhouse use and as a commercial inoculum for increasing crop
62 yields (Berruti et al., 2016), such as that of potato (Hijri, 2016), wheat (Al-Karaki et

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63 al., 2004), and cassava (Ceballos et al., 2013). Although there have been intensive
64 efforts to improve spore production of AM fungi in the protocols for *in vitro*
65 monoxenic culture (Douds, 2002; Rosikiewicz et al., 2017), the success rate of
66 mycorrhizal formation, which is a prerequisite for spore production, remains to be
67 determined. In particular, there are no known reports assessing the critical factors
68 for mycorrhizal formation in the *in vitro* monoxenic culture protocol for several AM
69 fungal species.
70 In this study, we investigated six factors (the concentration of P in the
71 mother plate; the concentration of P, sucrose, and macronutrient medium in a
72 cocultivation plate with hairy root; the amount of medium in the cocultivation plate;
73 and the location of spore inoculation) modified by the standard method provided by
74 the Glomeromycota *in vitro* collection (GINCO, http://www.mycorrhiza.be/ginco-
75 bel/) to identify critical factor(s) for mycorrhizal formation in the *in vitro* monoxenic
76 culture. We found that the concentration of P in the cocultivation plate is a critical
77 factor for mycorrhizal formation by regression analysis and showed that extremely
78 low concentrations using flax hairy roots significantly improved the mycorrhizal
79 formation of *R. irregularis*. In addition, we demonstrated that the modification the
80 success rate of mycorrhizal formation for other AM fungi belonging to Glomerales
81 can be higher than that of the standard method. Thus, altering the P concentration
82 of the *in vitro* monoxenic culture method can contribute to a wide range of AM fungi
83 culture collections.
84
85 **Materials and methods**
86
87 **Biological materials**
88 *Agrobacterium rhizogenes*-transformed roots of carrot (*Daucus carota* strain carrot
89 DC2) flax (*Linum* sp. L. strain flax NM) and chicory (*Cichorium intybus* L. strain
90 ChicoryA4NH), known as hairy roots, were obtained from GINCO. All were cultured
91 on a modified Strullu–Romand (MSR) medium used as a cocultivation plate for AM
92 fungi (Cranenbrouck et al., 2005) in Petri dishes (Asnol sterilization Petri dish,
93 GD90-15, 90 mm diameter). The plates for culturing hairy roots were incubated in
94 an inverted position in the dark at 25 °C and maintained by sub-culturing every 4
95 weeks.

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96 Sterile spore suspensions of the AM fungus *Rhizophagus irregularis*
97 DAOM197198 and the inoculum of National Agriculture and Food Research
98 Organization (NARO) AM fungi *Claroideoglomus etunicatum* MAFF520053
99 *Scutellospora cerradensis* MAFF520056, *Ambispora callosa* MAFF520057,
100 *Acaulospora longula* MAFF520060, *Gigaspora rosea* MAFF520062, *Acaulospora*
101 *morrowiae* MAFF520081, *Rhizophagus clarus* MAFF520086, *Paraglomus occultum*
102 MAFF520091, and *Claroideoglomus claroideum* MAFF520092 were obtained from
103 Premier Tech (Quebec, Canada), and NARO Genebank
104 (https://www.gene.affrc.go.jp/index_j.php), respectively. The strains of NARO AM
105 fungi were propagated with Welsh onion (*Allium fistulosum* L. 'Motokura'), sorghum
106 (*Sorghum bicolor* (L.) Moench. 'Ryokuhiyou sorugo'), and white clover (*Trifolium*
107 *incarnatum* L. 'Dixie') grown in pots with quartz sand (Mikawakeisa Nomal No.5,
108 Mikawakeisa Corp., Aichi, Japan). Centrifuge tubes (50 mL, Labcon North America,
109 Petaluma, CA, USA) with an 8-mm-diameter hole with cotton (Φ8×25 mm, Safe
110 Basic cotton roll, A.R. Medicom Inc. (Asia) Ltd., Hyogo, Japan) at the bottom were
111 used as pots. Each spore was isolated from each inoculum of AM fungi by wet sieving
112 (500, 100, and 45 μm meshes) and decanting (Gerdemann, 1955). Twenty spores of
113 each AM fungus were placed between 30 g and 35 g of quartz sand in the pot, and 3–
114 4 seeds of each host were sown on the upper layer of the sand. Each pot was irrigated
115 with 10 mL of reverse osmosis water, covered with plastic wrap until seeds were
116 germinated and thinned to three plants per pot. Every other day for 12 weeks after
117 sowing, each pot was irrigated with 10 mL of half-strength Hoagland's solution
118 containing 50 μM phosphorus (P). At 12 weeks after sowing, irrigation was stopped
119 for the host plants to senesce. Air-dried soil containing each spore of AM fungi was
120 stored at 4 °C before use.
121 The half-strength Hoagland's solution contained 126.4 mg KNO3; 295.2 mg
122 $Ca(NO_3)_2 \cdot 4H_2O$; 123.3 mg $MgSO_4 \cdot 7H_2O$; 34 mg KH_2PO_4 (corresponding to 50 μM
123 P); 196 mg K_2SO_4; 8.2 mg Fe (III)-EDTA; 0.72 mg H_3BO_3; 0.45 mg MnSO4・$4H_2O$; 0.06
124 mg $ZnSO_4 \cdot 7H_2O$; 0.02 mg $CuSO_4 \cdot 5H_2O$; and 0.0063 mg $Na_2MoO_4 \cdot 2H_2O$ in 1 L
125 distilled water.
126 The MSR medium contained 739 mg $MgSO_4 \cdot 7H_2O$; 76 mg KNO_3; 65 mg
127 KCl; 4.1 mg KH_2PO_4 (corresponding to 30 μM P); 359 mg $Ca(NO_3)_2 \cdot 4H_2O$; 0.9 mg

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128 calcium panthotenate; 1 × 10^{-3} mg biotine; 1 mg nicotinic acid; 0.9 mg pyridoxine; 1
129 mg thiamine; 0.4 mg cyanocobalamine; 1.6 mg Na Fe (III)-EDTA; 2.45 mg $MnSO_4 \cdot$
130 $4H_2O$; 0.29 mg $ZnSO_4 \cdot 7H_2O$; 1.86 mg H_3BO_3; 0.24 mg $CuSO_4 \cdot 5H_2O$; 0.0024 mg
131 $Na_2MoO_4 \cdot 2H_2O$; 0.035 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$; and 10,000 mg sucrose, solidified
132 with 4,000 mg Phytagel in 1 L distilled water.
133 All chemicals were provided by Wako Pure Chemical Corp (Osaka, Japan),
134 except for FE(III)-EDTA, Na Fe (III)-EDTA, and Phytagel, which were received from
135 Sigma-Aldrich (USA, MA, Burlington).
136
137 **Experiment 1—the evaluation of hairy root growth**
138 Experiment 1 was designed to compare the growth of hairy roots of carrot, flax, and
139 chicory. Root fragments of 1 cm length were cut from the tips of hairy roots after 3
140 weeks of subculture, transferred onto the MSR medium, and incubated in the dark
141 at 25 °C for 4 weeks. The flesh weights of the hairy roots were then measured (n =
142 12).
143
144 **Experiment 2—the evaluation of the success rate of AM fungal colonization in**
145 **different culture conditions.**
146 Experiment 2 was designed to evaluate different culture conditions and their
147 success rate for AM fungal colonization using flax hairy roots and *R. irregularis*
148 DAOM197198. A list of all culture conditions evaluated in Experiment 2 is presented
149 in Table 1. Using the same conditions as Experiment 1 for a control, 1 cm-long flax
150 hairy root fragments were transplanted onto the MSR medium and inoculated with
151 approximately 100 spores of *R. irregularis* DAOM197198 at a distance of 2.5 cm from
152 the root fragment, and incubated in the dark at 25 °C. The standard culture
153 conditions were modified in the following order: mother plate P concentration (3
154 and 30 μM), macronutrients in the MSR medium (the original and half of the
155 concentration of N, P, K, Mg, Ca, and S in the MSR medium), P concentration (0.3, 3,
156 15, and 30 μM) in the MSR medium, sucrose concentration (10 and 20 g/L) in the
157 MSR medium, amount of MSR medium (10, 20, and 40 mL/plate), and location of the
158 spore inoculation (distances of 1, 1.5, 2, 2.5, and 3 cm away from the root tip). Five
159 plates were prepared as replicates, and 2–15 replicates for each condition were
160 tested (Table 1). The success rate of AM fungal colonization was determined by the

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161 presence or absence of elongation of the extraradical hyphae (EH) and spore
162 formation (SF) at 30 and 60 days after inoculation. In addition, the number of spores
163 in 3 and 30 μM P in the MSR medium and in the 20 and 40 mL samples of the MSR
164 medium were counted at 11 months after inoculation.
165

166 **Experiment 3—the evaluation of the success rate of colonization for different**
167 **species of AM fungi in the optimized culture condition.**

168 Experiment 3 was designed to evaluate whether the concentration of P in the MSR
169 medium is a critical factor based on Experiment 2 using *R. irregularis*, for the success
170 rate of colonization of different AM fungal species. Spores of the NARO AM fungal
171 species were collected from the propagated inoculum by wet sieving and the
172 decanting technique described above. The collected spores were surface-sterilized
173 using a modified method described by Bécard and Fortin (1988). Briefly, spores
174 were sonicated with sterile 0.05% Tween 20 solution three times, soaked in 2%
175 (w/v) chloramine T solution for 10 min, and rinsed three times with sterile 0.05%
176 (w/v) Tween 20 solution. The surface-sterilized spores were stored in a sterile
177 solution containing 0.02% (w/v) streptomycin and 0.01% (w/v) gentamycin at 4 °C
178 until use. Each spore was inoculated onto the MSR medium with 1-cm-long flax hairy
179 root fragments, inoculated with 5 spores at a distance of 2.5 cm from the root
180 fragment, and incubated in the dark at 25 °C. The germinated spores were
181 transferred from MSR medium with a scalpel and tweezers to 3 or 30 μM P MSR
182 medium. As previously mentioned, the success rates of AM fungal colonization and
183 the presence or absence of elongation of EH and SF for different AM fungal species
184 were determined at 30, 60, and 90 days after inoculation.
185

186 **Statistical analysis**

187 All statistical analyses were performed using R version 3.4.0. The data on the success
188 rate of AM fungal colonization for all treatments were pooled and used for logistic
189 regression analysis with model selection using the 'bestglm' utility of the R package
190 (McLeod et al., 2020) to identify the main factors that contribute to the success rate
191 of AM fungal colonization, and the mathematical functions that describe the
192 methods these factors use to explain the success rate. By default, 'bestglm' uses the
193 Bayesian information criterion (BIC) for model selection. Since BIC is appropriate

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194 for controlled experiments with a limited number of important explanatory
195 variables (Aho et al., 2014), it was deemed acceptable for identifying relevant
196 common factors among the different criteria for the success of AM fungal
197 colonization. To examine the differences among experimental groups, data were
198 analyzed with Welch's *t*-test, Tukey's honest significant difference test, and the
199 Games-Howell test ($P < 0.05$).
200

201 **Results**

202 **Flax hairy root is suitable for *in vitro* monoxenic culture of AM fungi**

203 Carrot hairy roots have been widely used for *in vitro* monoxenic cultures of AM fungi
204 (Cranenbrouck et al., 2005; Fortin et al., 2002; Kokkoris & Hart, 2019a). Recently,
205 GINCO has provided flax and chicory hairy roots in addition to carrot hairy roots,
206 and we assessed the flesh weight for the three varieties. The results showed that the
207 flax hairy roots had significantly higher growth rates than chicory and carrot hairy
208 roots during the four weeks of incubation (9.44- and 1.88-times, respectively) (Fig
209 1A). In addition, the flax hairy root revealed the lowest coefficient of variation in dry
210 weight (Fig 1B). This result indicates that flax hairy roots are suitable for *in vitro*
211 monoxenic cultures of AM fungi because of their vigorous growth and robustness
212 against experimental conditions.
213

214 **Success rate of AM fungal colonization in different culture conditions**

215 To identify the critical factor(s) for mycorrhizal formation in the *in vitro* monoxenic
216 culture using flax hairy roots, we investigated the effects of different culture
217 conditions (the concentration of P in the mother plate, the concentration of P,
218 sucrose, and macronutrients in the cocultivation MSR medium with hairy root, the
219 amount of cocultivation MSR medium, and the location of spore inoculation) on the
220 success rate of AM fungal colonization, through the presence or absence of
221 elongation of extraradical hyphae (EH), and the formation of spores (SF). We then
222 compared the results to the standard method provided by GINCO (Fig. 2), and
223 significant differences (Welch's *t*-test for two parameters; Games-Howell test for
224 more than three parameters, $P < 0.05$) were detected for all parameters except for
225 sucrose concentration at 30 and 60 DAI and location of spore inoculation at 60 DAI
226 (Fig. 2). To estimate the significant parameters for predicting the success rate of AM

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227 fungal colonization, we performed a regression analysis using all the data collected
228 in this study. For each of the binary variables based on the four success criteria as
229 response variables, we identified a combination of explanatory parameters that
230 minimized the BIC and their relative contributions (Table 2). The only factor
231 included in all models was the concentration of P, and it had the largest contribution
232 in all cases. In addition, the best model was obtained when the concentration of P
233 was squared after non-linear logarithmic transformation in all cases (Table 3). Given
234 that the coefficient of $\log(x)^2$ (x represents the concentration of P) was negative for
235 all models, the colonization success rate peaked at a low P concentration (Fig. 3).
236 The modification with 3 μM P in the MSR medium significantly improved the success
237 rate of AM fungal colonization by 2.05–3.25 times for EH and 1.50–8.00 times for SF
238 compared with the standard method, suggesting that 3 μM P in the MSR medium
239 was an optimal concentration for the success rate of *R. irregularis* colonization (Fig.
240 2).

241 To validate the improvement of AM fungal colonization prior to
242 sporulation, we observed that the conditions of 3 μM P in the MSR medium produced
243 2.3 times more spores than that of the control (Welch's *t*-test, $P < 0.001$, Fig. 4).
244 However, the increased amount of the MSR medium (40 mL), which presented the
245 second greatest contribution in the model selection (Table 2), produced fewer
246 spores than that of the control (20 mL, Fig. 4), which might be because the increased
247 growth of hairy roots provided more opportunities for AM fungal colonization, but
248 the medium did not contain sufficient factors for sporulation.
249

250 **Success rate of colonization for different species of AM fungi in the optimized**
251 **culture condition**

252 To test whether the modification of P in the MSR medium improved the success rate
253 of AM fungal colonization by other strains, *C. etunicatum* MAFF520053, *S.*
254 *cerradensis* MAFF520056, *Am. callosa* MAFF520057, *Ac. longula* MAFF520060, *G.*
255 *rosea* MAFF520062, *Ac. morrowiae* MAFF520081, *R. clarus* MAFF520086, *P.*
256 *occultum* MAFF520091, *and C. claroideum* MAFF520092 were cultured under the
257 modified conditions (3 μM P) and the standard method (30 μM P). The low
258 concentration of P significantly improved the success rate of AM fungal colonization
259 for *C. etunicatum* MAFF520053 (from 0% to 20% in EH at 60 DAI; Table 4). The same

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260 tendency was observed for *Am. callosa* MAFF520057 (from 0% to 12% in EH at 30
261 DAI; Table 4) and *R. clarus* MAFF520086 (from 0% to 28% in EH and from 0% to
262 16% in SF at 30 DAI; from 20% to 44% in EH and from 20% to 40% in SF at 60 DAI;
263 and from 24% to 44% in EH and from 24% to 40% in SF at 90 DAI; Table 4). In the
264 case of *G. rosea*, we observed the opposite trend (from 100% to 60% in EH at 30 and
265 60 DAI; Table 4).

266 To validate the improvement of AM fungal colonization leading to
267 sporulation in *R. clarus*, the number of spores of *R. clarus* MAFF520086 was
268 measured only on the spore-forming plates (the number of spore-forming plates in
269 P30 and P3 were 6 and 10, respectively) and found that 3 μM P revealed 3.44 times
270 more spore formation compared to that of 30 μM P (Welch's *t*-test, $P < 0.001$, Fig. 5).
271

272 **Discussion**

273 Over the past three decades, there have been extensive efforts to improve *in vitro*
274 monoxenic culture using carrot hairy roots for AM fungi , with only P-free or 30 μM
275 P as the low-P treatment (Bécard & Fortin, 1988b; Declerck et al., 1998; Douds, 2002;
276 Karandashov et al., 2000; Olsson et al., 2002; Rosikiewicz et al., 2017). Our previous
277 study showed that an extremely low concentration of P (3 μM) in the cocultivation
278 plate using flax hairy roots increased AM fungal colonization (Sato et al., 2019), but
279 the success rate of mycorrhizal formation, which is a prerequisite for spore
280 production, remained to be assessed. This study screened critical factors for the
281 success rate of mycorrhizal formation and found that the P concentration in the
282 cocultivation plate is a critical factor for the mycorrhizal formation of several AM
283 fungi.

284 Glomerales (i.e., *Rhizophagus irregularis* DAOM197198, *Claroideoglomus*
285 *etunicatum* MAFF520053, and *Rhizophagus clarus* MAFF520086) showed higher
286 success rates of AM fungal colonization at lower concentrations of P (Table 4). In
287 addition, Archaeosporales (i.e., *Am. callosa* MAFF520057) developed extraradical
288 hyphae only at lower concentrations of P (Table 4). In contrast, Diversisporales (i.e.,
289 *Scutellospora cerradensis* MAFF520056 and *Gigaspora rosea* MAFF520062) may
290 prefer higher P concentrations in the MSR medium for the success rate of AM fungal
291 colonization. Although colonization of *Gigaspora margarita*, Diversisporales is
292 enhanced under low phosphorus conditions (Gutjahr et al., 2009; Navazio et al.,

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293 2007; Tawaraya et al., 1998) similar to Glomerales, further studies are required. The
294 optimal concentration of P may vary depending on the phylogenetic lineage, since P
295 concentration affects AM fungal colonization and sporulation in a species-specific
296 manner in pot cultures (Silva et al., 2005; Sylvia & Schenck, 1983).

297 Replacement of the cultivation medium and re-supply of glucose increased
298 the spore production of *Rhizophagus irregularis* (Douds, 2002). Given that the
299 increased sucrose concentration in the cocultivation medium at the beginning of
300 culture did not affect the success rate of mycorrhizal formation in this study (Fig. 2
301 and Table 2), replenishment of the carbon source after establishment of the plant-
302 AM fungi symbiotic connection would be critical for mycorrhizal formation.
303 Although the transition from the elongation of EH to SF is a gradual continuous
304 change in *R. irregularis*, we observed that the P concentration in the mother plate
305 affected the success rate of EH, but not that of SF, while P and sucrose in the
306 cocultivation medium, which were transferred from the mother plate, are important
307 to the success rate of SF (Table 2). These data suggest that AM fungi should have a
308 mechanism to sense external nutritional conditions, including host status, to
309 regulate their stage transition after colonization.

310 AM fungi also control the transfer of nutrients depending on plant carbon
311 availability (Hammer et al., 2011), and host plants can distinguish the AM fungi that
312 provide greater P to the host and present them with more carbon sources (Kiers et
313 al., 2011). This bidirectional control for nutrient transfer in plant-AM fungi is
314 described by a theoretical framework reflecting experimental data (Jolicoeur et al.,
315 2002). The development of an effective way to propagate AM fungi must maintain
316 the optimal balance in material transfer to direct the spore production.

317 The 'minimal (M)' medium was developed and subsequently modified for
318 *in vitro* monoxenic culture for AM fungi (Bécard & Fortin, 1988; Pawlowska et al.,
319 1999), but the assessment of different concentrations of P in the medium have been
320 limited. This study showed that a simple modification in the concentration of P in
321 the cocultivation plate greatly improved the success rate of mycorrhizal formation
322 of AM fungi. This modification can be utilized in different types of culture systems
323 (Rosikiewicz et al., 2017), and can enhance AM fungi propagation. Given that long-
324 term *in vitro* propagation has the potential to promote the artificial evolution of AM
325 fungi (Kokkoris & Hart, 2019a, 2019b), the concentration of P would be one of the

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326 most important for selective pressure targets for evolution, and an optimal
327 concentration of P similar to the natural habitat would be required for maintaining
328 both the function and productivity of AM fungi collections.
329

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337

338 **Conflict of interest**

339 The authors declare that they have no competing interests.
340

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494 **Table 1** | List of all culture conditions evaluated in this study. Bold indicates culture
495 conditions of GINCOs standard protocol.

Experimenter	Mother P	Essential elements	P concentration	Sucrose concentration	Amount of MSR medium	Location of spore inoculation	Reprication
	μM	fold	μM	g L^{-1}	mL $plate^{-1}$	cm	
A	**30**	**1**	**30**	**10**	**20**	**1**	**10**
A	30	1	30	10	20	1.5	10
A	30	1	30	10	20	2	10
A	30	1	30	10	20	2.5	10
A	30	1	30	10	20	3	10
A	30	1	30	10	10	2.5	5
A	30	1	30	10	40	2.5	5
A	30	1	30	10	20	2.5	5
A	30	0.5	15	10	20	2.5	5
A	30	0.5	3	10	20	2.5	5
A	3	1	30	10	20	2.5	5
A	3	0.5	15	10	20	2.5	5
A	3	0.5	3	10	20	2.5	5
A	3	1	30	10	40	2.5	2
A	3	0.5	15	10	40	2.5	2
A	3	0.5	3	10	40	2.5	2
B	**30**	**1**	**30**	**10**	**20**	**1**	**5**
B	30	1	30	10	20	2.5	15
B	3	1	30	10	20	2.5	5
B	30	1	3	10	20	2.5	5
B	3	1	3	10	20	2.5	5
B	30	1	0.3	10	20	2.5	5
B	3	0.5	3	10	20	2.5	5
B	30	0.5	3	10	20	2.5	5
B	30	1	30	20	20	2.5	5
B	30	0.5	3	20	20	2.5	5

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Table 2 | BIC of the best model and the contribution of selected factors. The contribution of an explanatory variable is shown as the amount of decrease in BIC when that variable is excluded. A blank space means that the factor was not selected as the explanatory variable.

		BIC	△BIC					
Days after inoculation			Mother P	Macronutrient medium	Medium P	Medium C	Amount of medium	Inoculated position
30	EH	819.9	10.7		60.5	10		
	SF	538.6			121.9	8.9		
60	EH	995.8			41.7		21.6	6.6
	SF	824.2			56.4		24.7	7.8

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503 **Table 3** | Relationship between mathematical transformation of P concentration and
504 BIC, calculated by elongation of extraradical hyphae (EH) and spore formation (SF)
505 of each plate, for 0.3, 3, 15, and 30 μM P treatments at 30 and 60 days after
506 inoculation (DAI). The numbers represent BIC. In the first column, x represents no
507 processing, x^2 represents squaring, log(x) represents logarithmic transformation,
508 and $\log(x)^2$ represents squaring after logarithmic transformation.
509

510

	30 DAI		60 DAI	
	EH	SF	EH	SF
x	836.4	547.5	1021.5	853.2
x^2	837.0	553.9	1023.3	859.5
lox(x)	856.1	576.7	1029.0	860.5
lox(x)^2	835.8	541.7	1019.4	847.1

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511 **Table 4** Success rate of arbuscular mycorrhizal (AM) fungal colonization of 9 AMF,
512 calculated by elongation of extraradical hyphae (EH) and spore formation (SF) of
513 each plate, for 30 and 3 μM P treatments at 30, 60, and 90 days after inoculation
514 (DAI).
515

				30 DAI		60 DAI		90 DAI	
AMF	Strain name	MAFF No.	P level	EH (%)	SF (%)	EH (%)	SF (%)	EH (%)	SF (%)
Claroideoglomus etunicatum	H1-1	520053	30	0	0	0	0	0	0
			3	0	0	20	0	24	0
Scutellospora cerradensis	TK-1	520056	30	40	0	40	0	40	0
			3	40	0	40	0	40	0
Ambispora callosa	OK-1	520057	30	0	0	0	0	0	0
			3	0	0	0	0	12	0
Acaulospora longula	F-1	520060	30	0	0	0	0	0	0
			3	0	0	0	0	0	0
Gigaspora rosea	C1	520062	30	100	0	100	0	100	0
			3	60	0	60	0	60	0
Acaulospora morrowiae	AP-5	520081	30	0	0	0	0	0	0
			3	0	0	0	0	0	0
Rhizophagus clarus	RF1	520086	30	0	0	8	4	24	24
			3	28	16	40	28	44	40
Paraglomus occultum	YC-1	520091	30	0	0	4	4	4	4
			3	0	0	4	0	4	4
Claroideoglomus claroideum	MI-1	520092	30	0	0	0	0	0	0
			3	0	0	0	0	0	0

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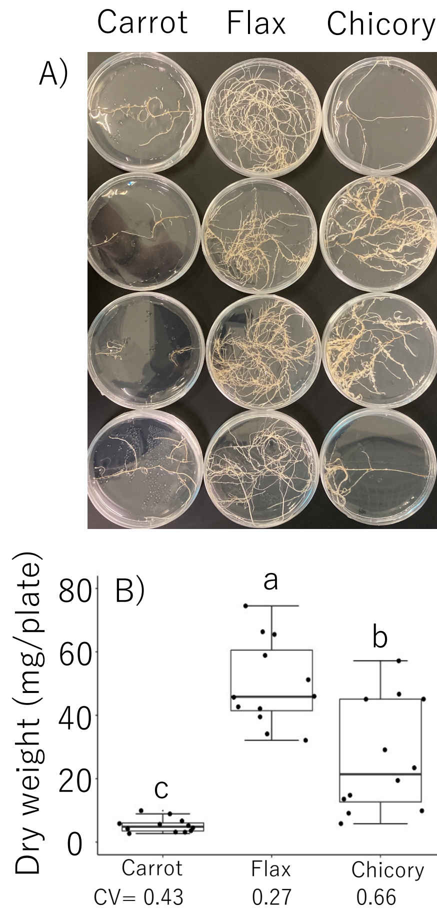
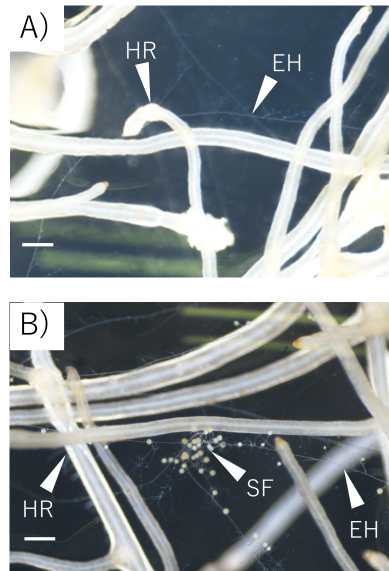
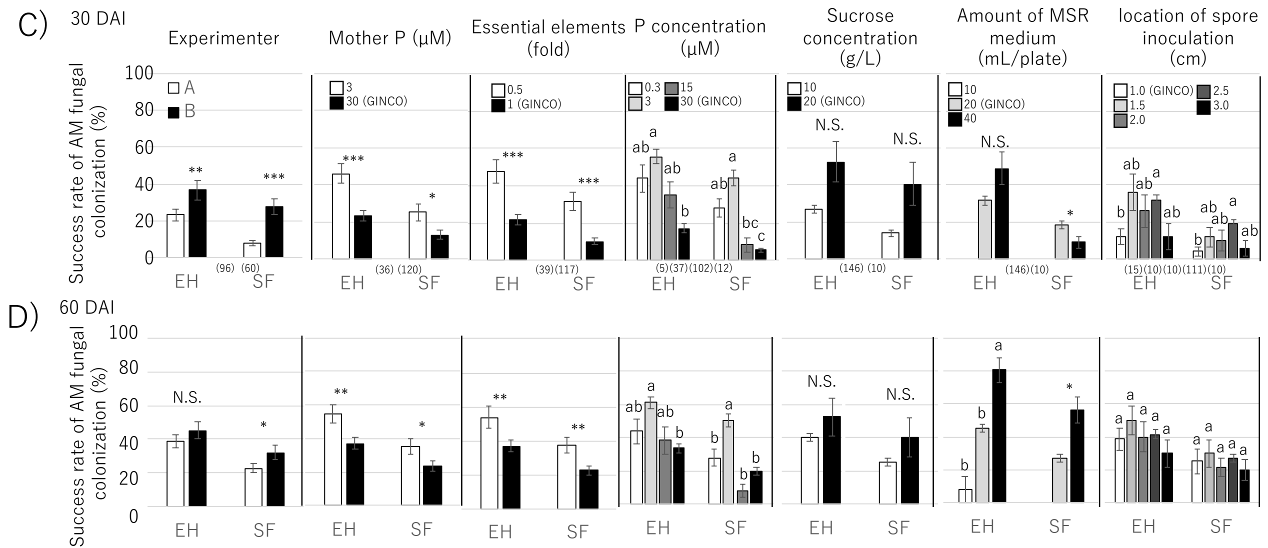


517

518 **Figure 1** | Evaluation of growth of hairy roots derived from different plant species.
519 Gross morphology (A) and dry weight (B) from hairy root of carrot (*Daucus carota*
520 strain carrotDC2), flax (*Linum* sp. strain flax NM), and chicory (*Cichorium intybus*
521 strain ChicoryA4NH) at 4 weeks after incubation are shown. Error bars and CV
522 indicate the standard error and coefficient of variance, respectively (n=12). Different
523 letters indicate significant differences as determined by Tukey's honestly significant
524 difference test (P <0.05).

525





526 **Figure 2** | Evaluation of the success rate of AM fungal colonization in different culture conditions.

527 Elongation of extraradical hyphae (EH) from hairy roots (HR) of Flax (*Linum* sp. MUCLflax NM, A), and spore formation (SF, B).

528 White bars indicate 500 μm. Success rate of arbuscular mycorrhizal (AM) fungal colonization, calculated by elongation of

529 extraradical hyphae (EH) and spore formation (SF) of each plate, for each treatment at 30 (C) and 60 (D) days after inoculation

530 (DAI). Error bars indicate standard errors. Different letters indicate significant differences as determined by the Games-Howell

531 test (P <0.05). Asterisks indicate significant differences by Welch's t-test (***, P < 0.001; **, P < 0.01; *, P < 0.05).

532

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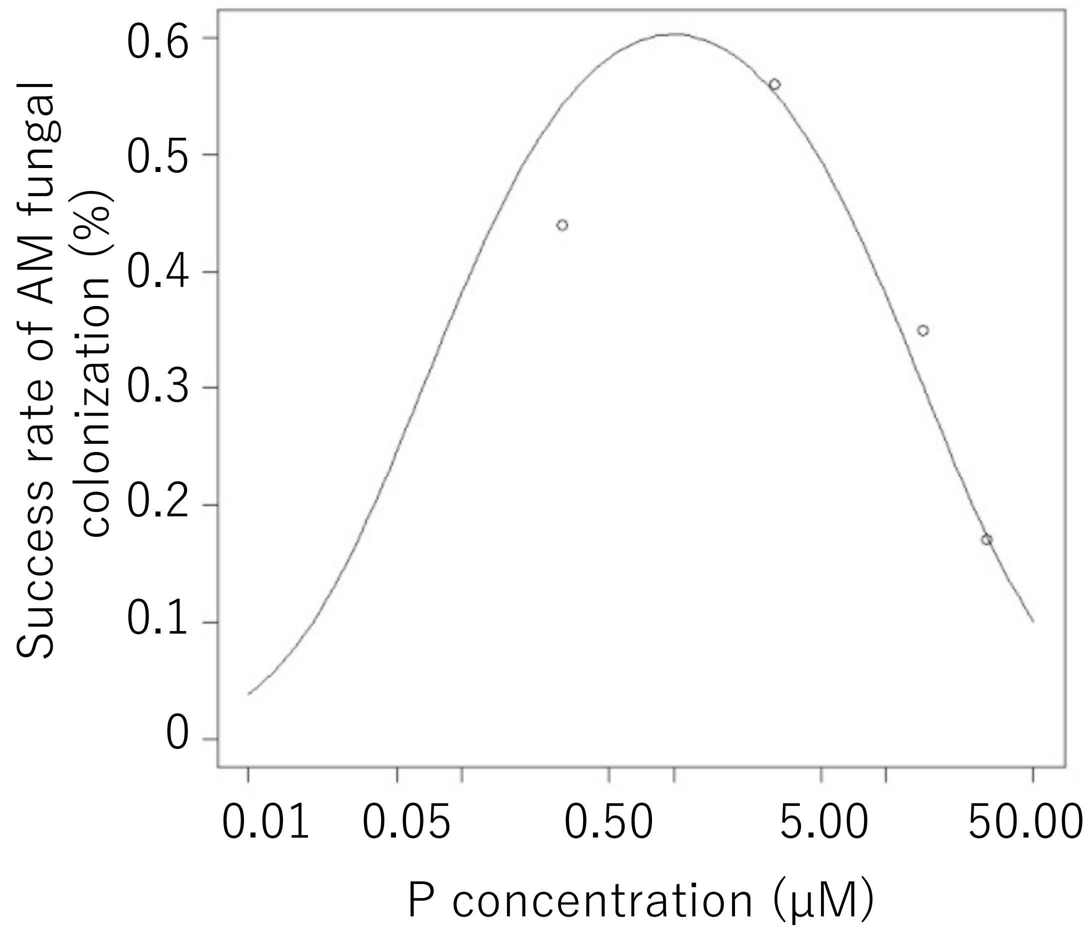

533

534 **Figure 3** | -log $(x)^2$ model calculated from success rate of arbuscular mycorrhizal
535 (AM) fungal colonization of *Rhizophagus irregularis* DAOM197198, calculated by
536 elongation of extraradical hyphae (EH) for 0.3, 3, 15, and 30 μM P at 30 days after
537 inoculation in Experiment 2.

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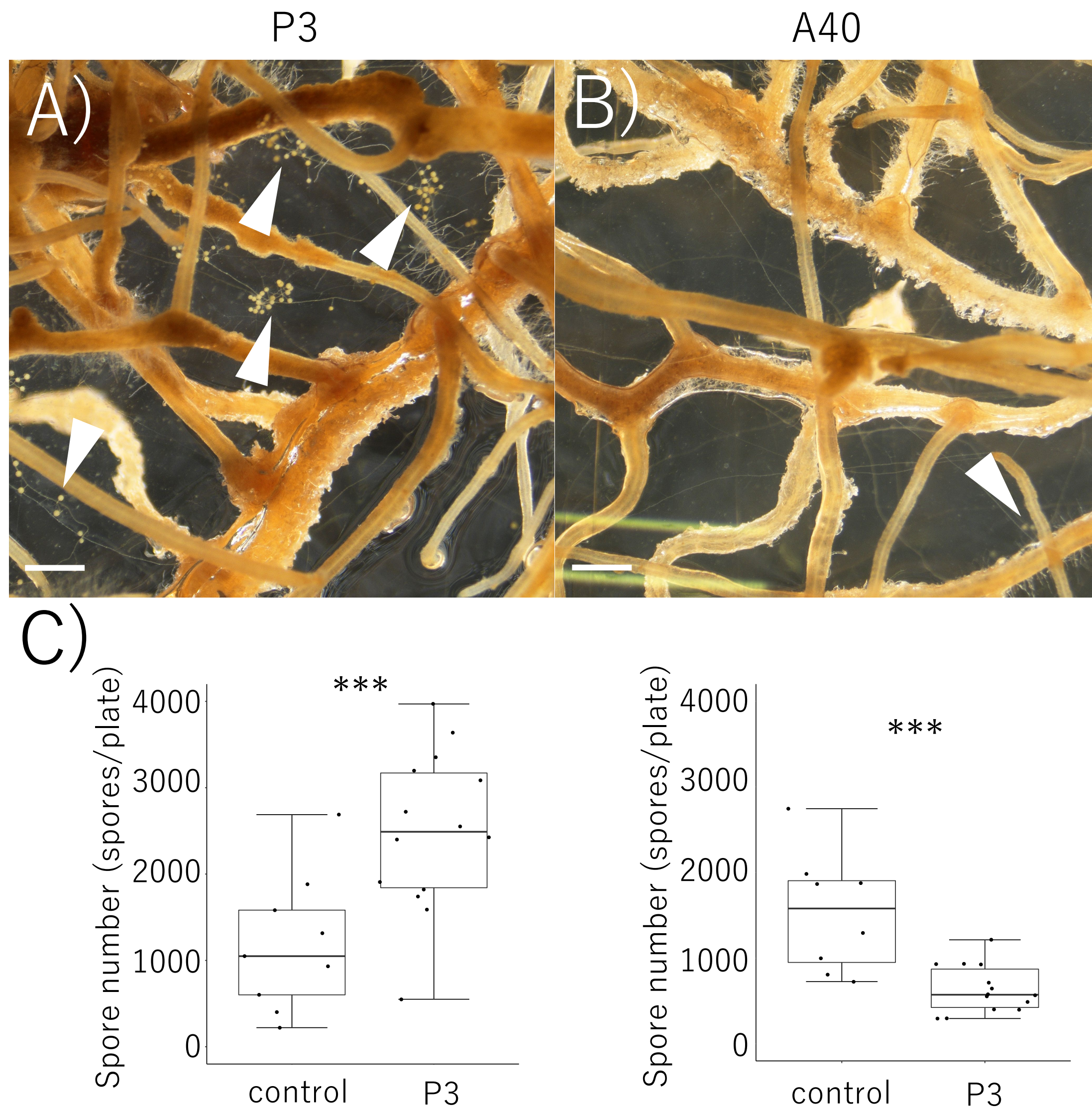

538

539 **Figure 4** | Evaluation of the spore formation in different culture conditions.

540 Spore formation *Rhizophagus irregularis* DAOM197198 P3 (A) and 40 mL (B)

541 treatments in Experiment 2, and number of spores of both treatments (C) at 11

542 months. Error bars indicate standard errors. White bars indicate 500 μm, and white

543 arrows indicate spores formed in the modified Strullu-Romand medium. Asterisks

544 indicate significant differences by Welch's *t*-test (***, $P < 0.001$).

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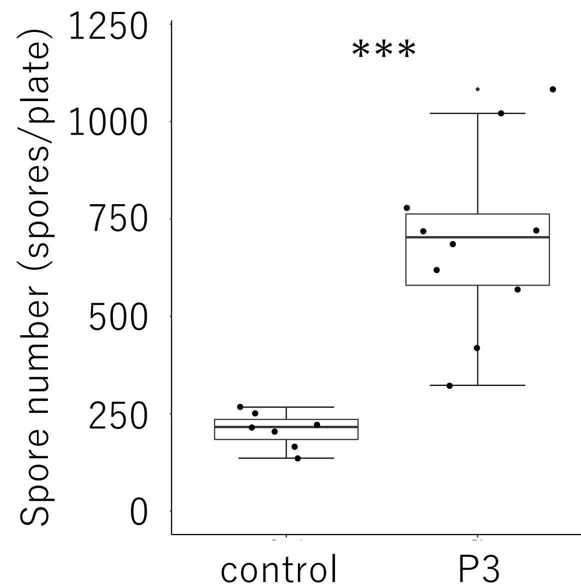

545
546 **Figure 5 |** Evaluation of the spore formation in the optimized culture conditions.
547 Number of spores of *Rhizophagus clarus* MAFF 520086 in control (30 μM P) and P3
548 treatments in Experiment 3 at 90 days after inoculation. Error bars indicate
549 standard errors. Asterisks indicate significant differences by Welch's *t*-test ($p<0.001$
550 =***).