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In vitro axenic germination and cultivation of mixotrophic Pyroloideae (Ericaceae) and their post-germination ontogenetic development

Tomáš FIGURA[1,2*], Edita TYLOVÁ[1], Jan ŠOCH[1], Marc-André SELOSSE[2,3], Jan PONERT[1,4]

1 Department of Experimental Plant Biology, Faculty of Science, Charles University, Viničná 5, 128 44, Prague, Czech Republic

2 Institut de Systématique, Évolution, Biodiversité (ISYEB), Muséum national d'Histoire naturelle, CNRS, Sorbonne Université, EPHE, CP 39, 57 rue Cuvier, 75005 Paris, France

3 Department of Plant Taxonomy and Nature Conservation, University of Gdańsk, Wita Stwosza 59, 80-308 Gdańsk, Poland

4 Prague Botanical Garden, Trojská 800/196,171 00, Prague, Czech Republic

*corresponding author: E-mail: figura.tomas@gmail.com

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Abstract

Background and Aims. Pyroloids, forest subshrubs of the Ericaceae family, are an important model for their mixotrophic nutrition, which mixes carbon from photosynthesis and from their mycorrhizal fungi. They have medical uses but are difficult to cultivate *ex situ*, in particular their dust seeds contain undifferentiated, few-celled embryos, whose germination is normally

fully supported by fungal partners. Their germination and early ontogenesis thus remain elusive.

Methods. Optimised *in vitro* cultivation system of five representatives from the subfamily Pyroloideae, to study the strength of seed dormancy and the effect of different media and conditions (incl. light, gibberellins and soluble saccharides) on germination. The obtained plants were analysed for morphological, anatomical and histochemical development.

Key Results. Thanks to this novel cultivation method, which breaks dormancy and achieved up to 100% germination, leafy shoots were obtained *in vitro* for representatives of all pyroloid genera (*Moneses*, *Orthilia, Pyrola* and *Chimaphila*). In all cases, the first post-germination stage is an undifferentiated structure, from which a root meristem later emerges, well before formation of an adventive shoot.

Conclusions. This cultivation method can be used for further research or for *ex situ* conservation of pyroloid species. After strong seed dormancy is broken, the tiny globular embryo of pyroloids germinates into an intermediary zone, which is anatomically and functionally convergent with the protocorm of other plants with dust seeds such as orchids. Like the orchid protocorm, this intermediary zone produces a single meristem: however, unlike orchids, which produce a shoot meristem, pyroloids first generate a root meristem.

Keywords

Pyrola, *Monotropa*, Ericaceae, seed germination, *in vitro* culture, *Chimaphila*, protocorm, orchid, convergent evolution, seed dormancy, mixotrophy, *Moneses*

46 # Introduction

47 Mycorrhiza is worldwide symbiosis of most plants (Smith and Read, 2008). Usually,
48 plants provide photosynthates in exchange for fungal mineral nutrients (van der Heijden *et al*.,
49 2015). However, mycorrhizal exchange may be less reciprocal, and perhaps exploitative, in
50 mycoheterotrophic plants that derive carbon resources from their mycorrhizal fungi (Leake,
51 1994). Mycoheterotrophic plants evolved independently in various plant lineages (Merckx,
52 2013), and beyond full mycoheterotrophs, which are achlorophyllous, some green species
53 display a mixed strategy, obtaining carbon from both mycorrhizal fungi and their own
54 photosynthesis, so-called mixotrophy (Selosse and Roy, 2009). There are two non-exclusive
55 mixotrophic conditions (Merckx, 2013): some species are mixotrophic at adulthood, while
56 other species germinate in a mycoheterotrophic way, before switching to autotrophy or
57 mixotrophy during ontogeny (such as orchids; Dearnaley et al., 2016; Selosse *et al.*, 2016,
58 Těšitel *et al*. 2018).

59 Little is known about the biology of mycoheterotrophic and mixotrophic plants,
60 perhaps due to the difficulty of studying them (Merckx, 2013). The only cultivable group of
61 mixotrophic plants is the orchid family, although many of them have so far escaped
62 cultivation (Rasmussen, 1995). For this reason, orchids are the most explored mixotrophic
63 models, but unfortunately it is largely unknown which results obtained for them can be
64 generalised to other mixotrophic plants. Developing cultivation protocols for other
65 mycoheterotrophic or mixotrophic plants is therefore urgently needed to understand general
66 mechanisms connected with mycoheterotrophy.

67 Within Ericaceae, the subfamily Pyroloideae (hereafter pyroloids) comprises initial
68 mycoheterotrophs that develop, depending on the species, into auto- or mixotrophic adults
69 (Tedersoo *et al.*, 2007; Matsuda *et al.*, 2012; Hynson *et al.*, 2013a), with the exception of one

70 completely mycoheterotrophic species (Hynson and Bruns, 2009). This similarity with
71 orchids is an evolutionary convergence and it is therefore interesting to compare adaptations
72 of both groups to mycoheterotrophy. Pyroloids encompass about 40 subshrub species divided
73 into four genera, mostly distributed in northern temperate and boreal ecosystems (Takahashi,
74 1993; Liu *et al.*, 2010), and are of medicinal interest, mostly in Asia (e.g. Ma *et a*l., 2014;
75 Wang *et a*l., 2014).

76 The seeds of pyroloids consist of oval, central living tissue surrounded by a coat of
77 dead cells called a testa (Fürth, 1920; Christoph, 1921; Lück, 1941; Pyykkö, 1968; Takahashi,
78 1993). Despite the fact that the oval living part seems to be anatomically homogeneous, it is
79 composed of a one-layered endosperm and triploid nutritive tissue of 40 to 50 cells
80 surrounding a smaller embryo inside (Hofmeister, 1858; Fürth, 1920; Christoph, 1921;
81 Pyykkö, 1968). Similar seed structure was observed in the related, mycoheterotrophic genus
82 *Monotropa*, where the embryo consists of only two to three cells (Olson, 1993) to 5-9 cells
83 (Goebel, 1887) in addition to the endosperm, although minute seeds evolved independently in
84 this Ericaceae genus (Lallemand *et al.*, 2016). The embryo of pyroloids is larger, with 8 or 16
85 cells (Goebel 1887) to 30 cells (Christoph 1921). Similarly to *Monotropa*, the living part of
86 the seed contains limited reserves of lipids and proteins (Fürth, 1920; Christoph, 1921; Lück,
87 1940). Other mycotrophic plants exhibit a similar seed structure, but, for example, orchids
88 lack endosperm and the only living part of the seed is undifferentiated globular embryo
89 (Arditti and Ghani, 2000).

90 Post-germination development of such seeds in pyroloids with undifferentiated
91 embryo has attracted attention for a long time. First, researchers searched for seedlings in
92 nature (Irmish, 1855; Velenovský, 1892, 1905; Fürth, 1920), albeit with limited success. They
93 found only older seedlings, which revealed that a small root-like structure grows into an
94 extensively branched structure which is formed before the first shoot emerges (Irmish, 1855;

95 Velenovský, 1892; Fürth, 1920). By contrast, orchids, which also start mycoheterotrophic
96 development from an undifferentiated embryo, first form a specific structure called a
97 protocorm, from which shoots and roots later develop (Rasmussen, 1995; Dearnaley *et a*l.,
98 2016). This raised three questions: Are the structures observed in germinating pyroloids true
99 roots? How does this root-like structure develop from a tiny undifferentiated embryo? What is
100 its relationship to a protocorm?

101 Many attempts have been made to answer these questions, but with only ambiguous
102 results to the date. Velenovský (1892, 1905) believed this belowground structure is "neither a
103 root nor a stem" and called it "prokaulom," while Christoph (1921) suggested that this
104 structure is a root. Later, Lihnell (1942) and Copeland (1947) did the same anatomical
105 analyses and found a typical root anatomical structure, where the radial vascular bundle is
106 diarch, rarely triarch (i.e. has two or rarely three xylem strands). The middle layer of the root
107 called the cortex is made of three to four (or rarely more) cell layers from which the innermost
108 forms the endodermis (Lihnell, 1942; Copeland, 1947). The outermost root layer, the
109 epidermis, is one-layered and consists of isodiametric cells. Lateral branches grow from the
110 outer layer of the stele (Lihnell, 1942).

111 The method of burying seed packets at natural sites yields pyroloid seedlings easily,
112 but the anatomical structure of such seedlings has not been studied in detail. These root-like
113 structures, called “root-like structures” (Hashimoto *et al.*, 2012; Johansson and Eriksson,
114 2013), live heterotrophically below ground for months, if not years (Lihnell, 1942; Hynson *et*
115 *al.*, 2013a; Johansson *et al.*, 2017).

116 Other researchers tried to germinate pyroloid seeds *in vitro* axenically (Christoph,
117 1921; Lück, 1940; Lihnell, 1942) or symbiotically (Fürth, 1920; Lück, 1941; Lihnell, 1942).
118 Germination was achieved, although the results were "mostly uneven, not very
119 consistent"(Lihnell, 1942) and the seedlings usually stopped growing very soon after

120 germination (Christoph, 1921; Francke, 1934; Lück, 1940, 1941), sometimes being only a
121 "few tenths of a mm long" (Christoph, 1921). Similar tiny seedlings, which ceased growth,
122 were obtained for *Monotropa* (Francke, 1934). The best results were achieved by Lihnell
123 (1942) and yielded a few branched root-like seedlings of *Pyrola rotundifolia*.

124 In these studies, observing different pyroloids, a polarised cone-shaped structure
125 resembling a root with big epidermal cells grows from the tiny globular embryo (Christoph,
126 1921; Lück, 1940, 1941; Lihnell, 1942). At this stage, the seedling breaks the testa and forms
127 an approximately 1-mm long roll-shaped structure (Lihnell, 1942), whose central cells
128 undergo elongation (Lück, 1941). At this point growth usually ceases (Lück, 1940; Lihnell,
129 1942). Seedlings rarely grow further, producing a rod-shaped stage, where vessels start
130 differentiation (Lihnell, 1942), and, soon after, a root cap. Then, the seedling becomes darker,
131 possibly because of the accumulation of tannins (e.g. Holm, 1898; Christoph, 1921; Lück,
132 1940; Lihnell, 1942). Lihnell (1942) showed that such older seedlings have the structure of a
133 root and their illumination does not lead to the formation of chlorophyll. Shoots were never
134 produced *in vitro*.

135 Growth suddenly stopped in all *in vitro* experiments (Christoph, 1921; Lück, 1940,
136 1941; Lihnell, 1942), which raised the question of the ideal conditions for *in vitro* growth.
137 Germination usually started four to eight months after sowing (Lück, 1940, 1941; Lihnell,
138 1942) and it was difficult to keep the cultures moist for such a long period (Lihnell, 1942). It
139 is also hard to say if a specific experiment was really axenic, as the authors admitted
140 themselves (for example, Christoph, 1921, discussed whether worms were the reason for the
141 failure of cultivation), and in symbiotic cultures it was not clear whether the fungus really
142 formed symbiosis or not (Lihnell, 1942; indeed, sometimes "symbiotic" seedlings grew away
143 from the fungus). Moreover, *in vitro* cultivation media often contained substances of variable
144 composition, such as potato extract, yeast extract, malt, peptone, or even humus, soil extracts

145 (Christoph, 1921; Lück, 1941; Lihnell, 1942). Previous results from *in vitro* cultures are
146 therefore based on a few plants only and do not indicate cultivation conditions that are ideal
147 for more detailed observation.

148 Possible seed dormancy was also discussed. Christoph (1921) noticed that it is
149 difficult to soak pyroloid seeds in water, indicating impermeability of the seed coat. Seeds
150 disinfected with calcium hypochlorite solution showed the best germination after the longest
151 bleaching time (15 and 30 minutes; Lihnell, 1942). Lihnell (1942) and Lück (1941) suggested
152 that some "water-soluble substances" have an inhibitory effect on germination, and Harley
153 (1959) hypothesised that some substances could be removed by soaking in solution. Although
154 dormancy in minute seeds may seem unexpected, long bleaching of seeds enhances
155 germination in many orchids (e.g. Burgeff, 1936; Rasmussen, 1992; Rasmussen, 1995)
156 probably because hypochlorite solutions have high pH and strong oxidative effects on a wide
157 range of compounds, which could break impermeable seed coats (Arditti, 1967; Rasmussen,
158 1995; Zeng *et al.*, 2014). In some hardly germinating orchid species, pre-treatment with a
159 weak H_2SO_4 solution enhances germination, probably ensuring stronger degradation of seed
160 coats (e.g. Malmgren 1993; Ponert *et al.* 2013; Malmgren *et* Nyström 2019). However, the
161 mechanisms are unclear in orchids and strong differences exist between species. Dormancy
162 clearly requires further study in pyroloid seeds.

163 To summarize, no reliable protocol for *in vitro* germination of pyroloids exists and
164 their early ontogenetic development remains elusive. We therefore sought to develop an
165 efficient protocol for *in vitro* culture. We successfully report early steps of the post-
166 germination development from undifferentiated embryo to leafy plant, in terms of storage
167 compounds, and morphological and anatomical development, which enabled us to clearly
168 answer the long-standing abovementioned questions about the nature of root-like structures of

169 pyroloid seedlings, their development from a tiny undifferentiated embryo and their
170 relationship to a protocorm.

171

172 # Materials and methods

173

174 Plant material

175 We selected five European species as representatives of all four genera of the
176 subfamily Pyroloideae (*Pyrola media, Pyrola minor*, *Orthilia secunda*, *Moneses uniflora*,
177 *Chimaphilla umbellata*; Table S1). To compare with another subfamily of Ericaceae where
178 minute seeds and mycoheterotrophic germination independently evolved (Freudenstein *et al*.,
179 2016; Lallemand *et al*., 2016), we used *Monotropa uniflora* from the Monotropoideae (Table
180 S1). Ripe capsules were collected, dried at room temperature, and extracted seeds were stored
181 in the dark and dry conditions at +4°C (Table S1).

182

183 Cultivation media

184 To find a suitable cultivation medium for germination and growth, we tested nine
185 media originally designed for orchid *in vitro* culture (Table S2). All media 0.7% agar (w/v,
186 Sigma-Aldrich) and 1 – 3 % sucrose (Table S2). After pH was adjusted to 5.8 using NaOH,
187 media were autoclaved at 144 kPa, 121°C (Tuttnauer 2540 EK-N) for 20 min and poured into
188 5 cm plastic Petri dishes, unless otherwise indicated. Medium Knudson C with activated
189 charcoal (Sigma-Aldrich) was used for all subsequent experiments, unless otherwise
190 indicated. Activated charcoal is used to improve germination and growth of orchids and slow-
191 growing tissue cultures generally, perhaps due to its ability to adsorb toxic products of plant

192 metabolism (van Waes, 1985; Thomas, 2008). To test the effect of different soluble
193 saccharides that could mimic the carbon provided by the fungi in natural situations, sucrose
194 was excluded or replaced with the monosaccharide 100 mM glucose or the disaccharide
195 50 mM sucrose or trehalose. To test the effect of gibberellins, 0.01, 0.1 or 1 mg/L of GA_3
196 (Sigma-Aldrich) was added to the medium before autoclaving.

197

198 Seed disinfection and sowing

199 Seeds were disinfected in 5 mL syringes and sown as a suspension in sterile deionised
200 water as described previously (Ponert *et al.*, 2011, 2013), but the application times of
201 disinfection solutions of H_2SO_4 and $Ca(OCl)_2$ were modified in a fully factorial design to find
202 proper seed treatment. All treatments of disinfection were pre-incubated in 70% ethanol for 5
203 minutes, washed three times with deionised water (< 0.2 $\mu m.cm^{-1}$), treated with 2% H_2SO_4 for
204 10 minutes or not, treated with $Ca(OCl)_2$ solution for 5, 10 or 15 minutes, and finally washed
205 three times with sterile deionised water. $Ca(OCl)_2$ solution was prepared by dissolving 20 g of
206 chlorinated lime (Kittfort, Czech Republic) in 100 mL of deionised water, filtering through
207 filter paper and adding a drop of Tween 20. For all subsequent experiments, we selected the
208 most efficient treatment: 70% ethanol for 5 minutes, washed three times with deionised water,
209 2% H_2SO_4 for 10 minutes, $Ca(OCl)_2$ solution for 10 minutes, washed three times with sterile
210 deionised water. Seven Petri dishes sealed with air permeable foil (Parafilm M) were prepared
211 for each experimental treatment.

212

213 Cultivation conditions

214 Because seeds ripen late in the season, all cultures were incubated in the dark at 4°C
215 for three months after sowing to simulate winter, and then transferred to the dark at 20°C,

216 except for experiments where the effect of light or cold stratification period was tested (see
217 below). Cultures were observed every two weeks using a Krüss, MSZ 5400 Stereo Zoom
218 Microscope (magnification 40x) and an Olympus Provis AX70 microscope for higher
219 magnification. Germination rate was counted three times after end of cold stratification, at 30,
220 60 and 90 days. The last count (3rd; after 90 days at 20°C) was used to count total germination
221 rate, because there no further germination was observed. To count the germination rate, seeds
222 without embryo or with obviously undeveloped embryo were excluded and well-developed
223 seeds with broken testa were regarded as germinated seeds (Fig. S1). To estimate seedling size
224 in selected experiments, the total length of all branches of each seedling was measured from
225 pictures taken with a Nikon D7000 + Micro Nikkor 55/2.8 using ImageJ 1.6.0_24 software.

226 To study the establishment of shoots in detail, we used approximately 3-5 mm long
227 seedlings of *P. minor* pre-cultivated on Knudson C medium (Table S2) for 60 days after
228 transfer to temperature 20°C. Each seedling (n=22) was transplanted to an individual nine cm
229 Petri dish with BM-1 medium (Table S2). Pictures of plants were taken every 10 days. Plants
230 with established shoots were continuously collected for anatomical analyses.

231 Plants with growing etiolated shoots were transferred to light (16 h light / 8 h dark)
232 and cultivated for the next month to produce green leafy shoots. Plants with green shoots were
233 deflasked, washed with water and potted in a mixture of coarse expanded perlite, fine pumice
234 gravel, fine pine bark and loamy soil (1:1:1:2) in clay pots. Pots were sealed in polyethylene
235 bags to keep air humidity high and were kept on a windowsill at 25°C in moderate light.

236

237 Anatomical analysis

238 Plant material (seeds, seedlings of *P. minor*) was fixed in 4% formaldehyde in
239 phosphate buffer (0.1 M, pH 7.1). Selected samples were embedded in paraplast after

240 dehydration using an ethanol-butanol series (for details, see Soukup and Tylová, 2014).
241 Sections (10 µm) were prepared using a Leica 2155 microtome and collected on microscopic
242 slides coated with alum gelatine adhesive. Cryosections were prepared on a Shandon
243 cryomicrotome. Hand sections were prepared on a Leica hand-microtome. For whole-mount
244 preparations, samples were gradually equilibrated in 65% glycerol and mounted in NaI-based
245 clearing solution of high refractive index (Soukup and Tylová 2014). Histochemical tests
246 involved staining with safranin O (2-h incubation) and Fast Green FCF (2 min). Lipids were
247 detected with Sudan Red 7B (1 h) according to Brundrett *et a*l. (1991). Proteins were stained
248 with Ponceau 2R in 2% acetic acid (10 min) and Azur II (10 s) according to Gutmann *et al.*
249 (1996). Detection of starch involved staining with Lugol solution. Observations were made
250 with an Olympus BX51 microscope (Olympus Corp., Tokyo, Japan) equipped with an Apogee
251 U4000 digital camera (Apogee Imaging Systems, Inc., Roseville, CA, USA) or with a Zeiss
252 LSM 880 confocal microscope.

253

254 Endogenous starch HPLC analysis

255 To confirm the presence of starch in seedlings, we characterised the endogenous
256 saccharide spectrum of selected pyroloids. Six-month-old (including period of cold
257 stratification) seedlings of *Mone. uniflora*, *O. secunda* and *P. minor* (n=3 for each species)
258 cultivated on BM-1 medium were collected in liquid nitrogen. Soluble carbohydrates were
259 extracted following the protocol of Kubeš *et al*. (2014).

260 The pellets left after soluble saccharide extraction were carefully washed with Milli-Q
261 ultrapure water (sonicated in 1 mL of water for 15 min, centrifuged at 14000 rpm for 15 min,
262 supernatant removed) and used for starch analysis. Starch was enzymatically degraded by α-
263 amylase (Sigma-Aldrich) and amyloglucosidase (Sigma-Aldrich) following the protocol of

264 Steinbachova-Vojtiskova *et al.* (2006) and glucose content was quantified with the HPLC

265 system described above, except for the use of an IEX Ca form 8 µm column in this case.

266

267 Data analysis

268 Statistical analyses were performed using statistical software R 3.2.3 (R Core Team

269 2015). The normality of data was tested using the Shapiro-Wilk test (Shapiro and Wilk, 1965)

270 and homogeneity of variances was tested using the Bartlett test (Bartlett, 1937). Differences

271 between the measurements were statistically tested with ANOVA, followed by the Tukey–

272 Kramer test (Kramer, 1956) for data with a normal distribution and the Kruskal–Wallis test

273 (Kruskal and Wallis, 1952), followed by pairwise comparisons using Wilcoxon's rank-sum

274 test for data that did not have normal distribution. To compare the effects of different

275 disinfectants on seed germination, we used two-way ANOVA, followed by a Tukey–Kramer

276 test.

277

278 Results

279

280 Effect of seed disinfection

281 It turned out to be impossible to sow non-disinfected seeds *in vitro* because of

282 overwhelming contamination (data not shown). The highest germination rate of all tested

283 species was reached after H_2SO_4 treatment and the effect of H_2SO_4 was significant in all

284 tested species (*P. minor*: $F_{[1,24]}=193.96$; $p=5.4x10^{-13}$; *M. uniflora*: $F_{[1,40]}=8.23$; $p=0.0065$; *O.*

285 *secunda*: $F_{[1,23]}=24.32$; $p=5.5x10^{-5}$) except for *C. umbellata*; $F_{[1,28]}=0.06$; $p=0.8$; Fig 1). The

286 effect of $Ca(OCl)_2$; was significant in *P. minor* ($F_{[2,24]}=57.42$; $p=7.1x10^{-10}$) and *O. secunda*

287 ($F_{[1,23]}$=3.79; p=0.038), but the optimal length of disinfection differed between these taxa.

288 Longer incubation in $Ca(OCl)_2$ strongly promoted germination of *P. minor*, but slightly

289 inhibited germination of *O. secunda* (Fig. 1). *Mone. uniflora* germination was also higher

290 after longer incubation in $Ca(OCl)_2$, especially after the pre-treatment with H_2SO_4 (Fig. 1B),

291 but the effect was not significant ($F_{[2,40]}$=2.13; p=0.13). The interaction between the effects of

292 H_2SO_4 and $Ca(OCl)_2$ was significant for *P. minor* ($F_{[2,24]}$=35.97; p=6.0x10^{-8}) and for *O.*

293 *secunda* ($F_{[1,23]}$=4.05; p=0.031) only.

294

295 Effect of different media

296 Optimal media differed between species (Fig. 2A-C). Generally, the highest

297 germination rates were observed on the media BM-1 (with or without activated charcoal), DS

298 and MoN (Fig 2; Fig. S2). Media 1/4-2, Knudson C and MS allowed slightly lower

299 germination generally (Fig 2; Fig. S2). Surprisingly, few seedlings of *C. umbellata*

300 germinated on Knudson C medium ($\chi^2_{(7)}$ = 8.45; p= 0.29; Fig. S3B). The best medium for

301 germination of *P. minor* was BM-1 (with or without activated charcoal), with a germination

302 rate of almost 100% ($\chi^2_{(8)}$ = 32.59; p= 7.29x10^{-5}; Fig. 2A). For *Mone. uniflora*, the highest

303 germination rates were achieved on media BM-1 and DS ($\chi^2_{(5)}$ = 7.34; p= 0.20; Fig. 2B).

304 *Orthilia secunda* germinated on all media tested, however, after longer incubation the highest

305 germination rate was achieved on BM-1 medium without activated charcoal ($F_{[7,41]}$=3.21;

306 p=0.0083; Fig. 2C).

307 Media also differed in their suitability for seedling growth in a more or less similar

308 way (Fig. 2D-F). We were unable to compare the effects of the different media on *C.*

309 *umbellata* growth because few seedlings developed on medium Knudson C. The biggest

310 seedlings of *P. minor* developed on medium BM-1 (with or without activated charcoal) and

311 on DS medium, although the differences between treatments were not significant

312 ($F_{[7,16]}$=0.984; p=0.48; Fig. 2D). The largest seedlings of *Mone. uniflora* developed on media

313 DS and BM-1 without activated charcoal ($F_{[6,15]}$=21.76; p =1.32x10^{-6}; Fig. 2E). The largest

314 seedlings of *O. secunda* developed on MoN medium followed by BM-1 medium with

315 activated charcoal ($F_{[7,17]}$=14.54; p=1.19x10^{-5}; Fig. 2F).

316

317 Effect of light

318 The germination of both tested species (*P. minor* and *O. secunda*) was inhibited by

319 light (16/8 h photoperiod; Fig. S4A,B). For *O. secunda*, this difference was significant (Fig.

320 S4B; $\chi^2_{(1)}$ = 5.4; p= 0.02 in 2nd month; only one plate in the 3rd month because light cultures

321 became contaminated). For *P. minor*, a similarly small proportion of seeds germinated after

322 the 1st month in both treatments and further germination was only slightly lower on light

323 (differences not significant; $F_{[1,8]}$=0.058; p=0.82; Fig. S4A). However, after another 6 months

324 of cultivation, seedlings on light did not grow, while the seedlings in the dark grew, produced

325 many branches and even some buds and first shoots (data not shown). Interestingly, seedlings

326 cultivated on light displayed a reddish colour (Fig. 3B).

327

328 Effect of gibberellic acid and selected soluble saccharides

329 We found little effect of GA_3 on germination of the tested species, namely *P. minor*

330 (Fig. S4C,D) and *O. secunda* (Fig. S4E,F). For *O. secunda*, there was no difference in

331 germination rate between GA_3 concentrations on both media tested (Fig. S4E,F), except after

332 the 1st month on Knudson C (higher germination on the highest GA_3 concentration;

333 $F_{[3,15]}$=7.94; p=0.002; Fig. S4F). For *P. minor*, there was a weak stimulatory effect after the 1st

334 month on BM-1 medium at the highest (1 mg/L) GA_3 concentration ($\chi^2_{(3)}$ = 4.719; p= 0.002;

335 Fig. S4C).

336 Tested saccharides stimulated germination of seeds of both tested species. *Pyrola*

337 *minor* was stimulated by all saccharides, with sucrose being the best ($\chi^2_{(3)}$ = 20.57, p=

338 0.0001, Fig. 4A), and the seeds cultivated without soluble saccharides germinated very rarely.

339 *O. secunda* reached higher germination rates only on sucrose ($\chi^2_{(3)}$ = 16.34, p= 0.001, Fig.

340 4B), while glucose and trehalose hardly enhanced germination. Seedling size was similarly

341 affected by saccharides, with all saccharides acting on *P. minor* (Fig. S5A, Fig. S6A,

342 $F_{[3,14]}$=27.11; p=4.32x10^{-6}) and sucrose only on *O. secunda* (Fig. S5B, Fig. S6B; $F_{[3,16]}$=9.57;

343 p =0.00074). After three additional months of cultivation, seedlings from all saccharide-

344 supplemented treatments produced at least a few long roots, while those from saccharide-free

345 cultures did not, indicating that trehalose and glucose were also partially utilised. Moreover,

346 after cultivation for one year, the individual seedlings on media with glucose and trehalose

347 showed significant growth (Fig. S7).

348

349 Ontogenesis of seedlings

350 All species tested produced elongated seedlings of similar morphology (Fig. 3),

351 irrespective of the medium. We used *P. minor* as a model species to analyse seedling

352 development in detail. Like other pyroloids, *P. minor* has a minute seed with few-celled

353 embryo surrounded by a cellular endosperm enclosed in single-layered transparent testa with

354 pitted cell walls (Fig. 5A). Histochemical test indicates storage of lipids (Fig. S8C,E) and

355 proteins (Fig. S8B,D) but not starch (Fig. S8A) in mature seeds. When germinated aseptically

356 on media supplemented with soluble carbohydrate, germination starts with enlargement of the

357 embryo at the micropylar pole (Fig. 5B) and with the formation of an apical meristematic area

358 (in seedlings < 0.5 mm long; Fig. 5F) that later passes into root meristem (Fig. 5G). The
359 transition from embryo into growing root is fast, but gradual. Less organised tissue is
360 produced during a short initial phase to form an "intermediary zone" (Fig. 5C-H) that exhibits
361 a heterogeneous surface cell pattern (Fig. 5D) and differentiates vascular tissues in its central
362 part (Fig. 5G,H). In seedlings above 0.5 mm long, typical root organisation is achieved within
363 the growing apex (Fig. 5G). The root produced includes radial vascular bundle devoid of pith
364 (mostly diarch), exarch xylem with protoxylem close to the pericycle, suberised endodermis,
365 and a root cap (Fig. 5I,J, Fig. S8I,L).

366 Seedling establishment on sucrose-amended media is accompanied by gradual
367 disappearance of lipids (Fig. S8C,F,J) and accumulation of starch (Fig. S8F,H,K). Starch
368 grains are located in cortical cells (Fig. S8H-I,K-L). High starch content was confirmed by
369 HPLC analyses (Table S3.). Conversely, germination on media lacking carbohydrates
370 terminated in the very initial phase, with very limited enlargement of embryo even after 11
371 months of cultivation (Fig. S8G).

372 The emerging root started to branch very early and produced an extensive root system
373 (Fig. 6A), which initiates the first shoot bud much later (Fig. 6B). To observe the timing and
374 positioning of the emergence of the first bud, we transferred seedlings individually (n=16) to
375 new Petri dishes soon after germination on sucrose-amended BM-1 media. Seedlings
376 produced their first buds at different times, starting 35 days after transplantation, and 7 out of
377 16 plants did not sprout after six months. The first shoot always sprouted a few mm away
378 from the original position of the embryo, often at the nearest root branching (8 out of 9 plants;
379 Fig. 6C-E). Very often additional buds appeared approximately one to two months after the
380 first one, emerging along the root axis at sites of branching (Fig. 6D). One individual
381 displayed 7 sprouts after six months. The buds grew quickly into leafy shoots which became
382 green when transferred to the light (Fig. 6F,G). Seedlings left in a small Petri dish with other

383 seedlings grew much slower and did not form buds till the end of the experiment. We also
384 successfully germinated branching *Mono. uniflora* (n=3; Fig. S9A on BM-1 with activated
385 charcoal; Fig. S3A) from which one plant produced multiple buds within a dense nest-like
386 root cluster after one year of cultivation (Fig. S9B).

387 In summary, germination was successful to the stage of green leaves in *P. minor* (Fig.
388 6F-G) and also in *Ch. umbellata, O. secunda, Mone. uniflora* and *P. media* (not shown). The
389 seedlings of *P.minor* were successfully transferred to *ex-vitro* conditions, and cultivated for
390 the next six months (not shown).

391

392 Discussion

393

394 We developed an efficient novel protocol for *in vitro* axenic sowing of pyroloids,
395 which enable us, for the first time, to successfully germinate representatives of all pyroloid
396 genera and a related mycoheterotroph *Monotropa uniflora*. This protocol allowed us to
397 overcome problems with seed germination and seedling grow cessation that were previously
398 reported. Germination is probably driven mostly by physical dormancy because gibberellins
399 had little effect on germination, while intensive pre-treatment (chemical scarification) of
400 seeds greatly improved germination. This new, reliable protocol allowed us to produce
401 seedlings in great number and to describe ontogenesis clearly, from early germination to leafy
402 plantlet. In all cases, a tiny undifferentiated embryo produces an intermediary zone, which
403 subsequently establishes a first root. Adventitious shoots grow later from this and secondary
404 roots (Fig. 7, on the right). Our results suggest convergence in mixotrophic plants with dust-
405 like seeds in regressive evolution leading to a single meristem that builds mycorrhizal tissue
406 at germination.

407

408 **Germination conditions**

409 Gibberellic acid (GA_3), which greatly stimulates germination of many plant species
410 (Shu *et al.*, 2016), had little effect on pyroloid germination. A strikingly similar situation
411 occurs in orchid minute seeds, where gibberellins usually have little effect on seed
412 germination (Arditti, 1967; Rasmussen, 1995), despite occasional reports of inhibition (Van
413 Waes and Debergh, 1986) or stimulation (Pedroza-Manrique *et al.*, 2005; Pierce and
414 Cerabolini, 2011). Low sensitivity to gibberellins may be a general feature of dust-like seeds.
415 The situation in orchids is sometimes explained by the absence of endosperm (Arditti and
416 Ghani, 2000; Yeung, 2017), because the stimulation of germination by gibberellins is mostly
417 connected to the induction of the expression of genes encoding enzymes hydrolysing the
418 endosperm (Groot and Karssen, 1987; Groot *et al.*, 1988; Schuurink *et al.*, 1992; Leubner-
419 Metzger *et al.*, 1996). Yet, since pyroloid seeds have a one-layered endosperm (Hofmeister,
420 1858; Fürth, 1920; Christoph, 1921; Pyykkö, 1968), the low sensitivity to gibberellins of dust-
421 like seeds may not be associated with the absence of endosperm but with other characteristics
422 yet to be clarified.

423 The strong inhibitory effect of light on germination could serve as a protection against
424 germination on the soil surface, where mycorrhizal fungi may not be present. A similar
425 mechanism of light inhibition is well known in many terrestrial species of orchids (Arditti,
426 1967, 2008; Rasmussen, 1995). We also found a strong effect of disinfection on seed
427 germination. More intensive seed disinfection stimulated germination and longer incubation
428 in calcium hypochlorite solution after sulphuric acid treatment was often most effective.
429 Similarly, Lihnell (1942) achieved the highest germination rate after the longest incubation in
430 calcium hypochlorite solution, which he was unable to explain. Since these solutions also act
431 corrosively to destroy the impermeable coats of dust-like seeds (e.g. Arditti, 1967;

432 Rasmussen, 1995; Zeng *et al.*, 2014), we propose that strong physical dormancy due to the
433 testa explains the long pre-germination period and the low germination rates observed *in situ*
434 (Hashimoto *et al.*, 2012; Johansson and Eriksson, 2013; Hynson *et al.*, 2013b; Johansson *et*
435 *al.*, 2017). After proper chemical scarification, we reached 100% and 99% germination for *P.*
436 *minor* and *O. secunda* seeds, respectively, which is much higher than observed *in situ*
437 (Hashimoto *et al*. 2012; Hynson *et al.*, 2013b) or *in vitro* (Lück, 1940; Lihnell, 1942) in
438 different species of the same genera. It is hard to discuss possible actions of disinfection
439 agent's on seeds in detail, because chemical composition of pyroloid seeds is unknown. In
440 orchids, seed coats seems to consist of lignin, lipids and other uncharacterized compounds
441 (Barsberg et al., 2013; 2018). Ethanol is a good solvent for many lipids and wax compounds
442 and it usually makes seeds less hydrophobic and more accessible for subsequent treatment
443 with aqueous solutions. Lignin is highly sensitive to oxidative effect of hypochlorite
444 solutions, so we could expect disintegrating of seed coat during hypochlorite treatment. The
445 effect of H_2SO_4 is hard to guess, but we could expect existence of some other compound
446 sensitive to acid hydrolysis, but not the alkaline one.

447 *Chimaphila umbellata* and *Mone. uniflora* germinated at significantly lower rates
448 comparing with *P. minor* and *O. secunda* in our experiments. Since these two genera form a
449 clade separate from that encompassing *Pyrola* and *Orthilia* (Freudenstein *et al.*, 2016;
450 Lallemand *et al.*, 2016), some additional dormancy mechanism may occur in this clade, which
451 remains to be identified. In soils, dormancy, added to photoinhibition, may increase the ability
452 of the plant to wait for suitable fungi required for germination.

453 Our *in vitro* methods provided seedlings in great number and shed light on the early
454 development of pyroloids, with replicates for dissection to an extent that was never reached
455 before, allowing a clear discussion of early ontogenesis of pyroloids – assuming, of course,
456 that our artificial, non-symbiotic conditions do not alter ontogeny, as supported below.

457 **Cultivation protocol**

458 Based on our results, we propose a cultivation protocol that would allow to germinate all
459 pyroloids generally. This procedure uniquely combines different techniques used in orchid
460 culture previously to satisfy needs of pyroloids. Seed disinfection implies: 70% ethanol for 5
461 minutes, washed out three-times with a deionised water, then 2% H_2SO_4 for 10 minutes,
462 followed by $Ca(OCl)_2$ solution for 10 minutes, washed three times with sterile deionised
463 water. The best cultivation medium is BM-1, but DS and MoN work also well. For
464 cultivation conditions, we recommend 4°C for 3 months after sowing, and subsequently
465 20°C, in dark. Seedlings can be transferred to the new medium when they are approximately 5
466 mm long. When shoots will start to grow, seedlings should be transferred to the light and, at
467 that time, it is better to put them separately into 100 ml cultivation jars.

468

469 **Development of pyroloid seedlings**

470 The dust seed structure with an undifferentiated globular embryo is typical for many
471 mycoheterotrophic plants (Leake, 1994) and plants with mycoheterotrophic early
472 development (Dearnaley *et al.,* 2016). Such dust seeds have limited reserves, the storage
473 compounds of which are mostly unknown. In pyroloids, previous studies found storage lipids
474 and proteins in mature seeds (Fürth 1920; Christoph 1921; Lück 1940). Similarly, ripe seeds
475 of orchids usually contain lipids and proteins (Harrison 1977; Manning and Van Staden 1987;
476 Rasmussen 1990; Richardson *et al*. 1992; Yam *et al*. 2002; Li *et al*. 2016), and few orchid
477 species display starch in mature embryos (Tian and Wang 1985; Guo and Xu 1990; Yeung
478 and Law 1992). Our histochemical tests confirmed storage lipids and proteins, but absence of
479 starch in *P. minor* seeds, thus further amplifying the convergence with orchids.

480 During germination, lipids and storage proteins were utilised and seedlings shifted to
481 use starch as the main storage compound, as confirmed by histochemistry and HPLC. This
482 switch to starch was already observed in several pyroloids (Christoph, 1921; Lück, 1940;
483 Lihnell, 1942) and is common among orchids (Manning and Van Staden, 1987; Rasmussen,
484 1990; Richardson *et al.*, 1992). The transition from lipid and protein reserves in seeds to
485 starch in seedlings therefore also convergently characterizes mycoheterotrophic germination.

486 Mycoheterotrophic pyroloid seedlings grow *in vitro* as branching roots for a long time
487 before the first green shoot emerges. Such non-green seedlings grow below ground in nature
488 very slowly, for an even longer time (Hashimoto *et al.*, 2012; Johansson and Eriksson, 2013;
489 Hynson *et al.*, 2013b; Johansson *et al.*, 2017). Seedling growth was also promoted by more
490 complex cultivation media (e.g. BM-1, compared to Knudson C or MS), which could indicate
491 dependency on some other organic compounds provided in nature by fungus. Complex media
492 are beneficial also for many mixotrophic orchids (Rasmussen, 1995; Arditti, 2008).

493 Post-germination development differs from orchids despite some parallels. Orchid
494 germination forms a protocorm whose enlargement establishes a shoot meristem on its
495 anterior pole (Burgeff, 1936; Leroux *et al.*, 1997; Yeung, 2017). In pyroloids, germination
496 starts with enlargement of the embryo at the suspensor pole, which develops into a small
497 elongated structure growing apically-the 'cone stadium' of Lück (1941) and Lihnell (1942).
498 Soon after, the apical region establishes a root meristem and grows into a typical root.
499 Although the transition is pretty fast, a short region (app. 0.5 mm) close to the former embryo
500 exhibits a slightly different internal structure (Fig. 7), which is why we call this part the
501 "intermediary zone" (see discussion below).

502 The first root grows and establishes lateral roots to develop an extensively branched
503 root system as also observed *in situ* (Irmish, 1855; Velenovsky, 1892, 1905; Bobrov, 2009;
504 Hashimoto *et al.*, 2012). For a long time, it was unclear whether these structures are true roots

505 (see Goebel, 1900; Lihnell, 1942). Velenovský (1892, 1905) suggested these are "neither root
506 nor stem". We clearly show a typical root organisation (incl. root cap, endogenous origin of
507 lateral roots and radial vascular bundle; Fig. 5 H,I,J), as concluded in earlier anatomical
508 studies (Lihnell, 1942; Copeland, 1947). The abovementioned discussions were probably
509 caused by insufficient anatomical examination and confusion between the intermediary zone
510 and the true roots. As in the observations of Velenovský (1905) and Lihnell (1942) and adult
511 roots (Hynson, 2009), the roots observed had no root hairs. Our data clearly show that the first
512 shoot sprouts from a small section between the original position of embryo and the first root
513 branching site. Shortly after, other adventitious shoots usually emerge from other places of the
514 root system. Such root sprouting also exists in adult pyroloids in natural conditions
515 (Copeland, 1947; Klimešová, 2007).

516

517 **Is the intermediary zone a protocorm?**

518 Typical plant embryos develop from both radicle (basal pole establishing the primary
519 root) and plumule (apical pole establishing the primary shoot) poles, while in pyroloids or
520 orchids one embryo pole does not grow: respectively, the primary shoot or the primary root is
521 completely missing (Fig. 7). Other mycoheterotrophic species with dust seeds that have been
522 investigated to the date, *Afrothismia hydra* (Burmanniaceae; Imhof and Sainge, 2008) and the
523 genus *Voyria* (Gentianaceae; Imhof, 2010; Imhof *et al.*, 2013), also germinate by a single,
524 basal (radicle) embryo pole. This independent loss of one meristematic embryo pole in plants
525 with dust-like seeds indicates some general selection pressure in mycoheterotrophic
526 germinations perhaps for several non-exclusive reasons: (i) the limited resources available
527 cannot support two growth sites (Imhof and Sainge, 2008) and /or (2) the absence of
528 requirement for shoots and roots in mycoheterotrophy, since a single organ for interaction
529 with the fungus is sufficient, and/or (3) the later mycorrhizal organ makes one embryo pole

530 unable to differentiate into a meristem. We therefore propose that transformation of one
531 embryo pole into mycorrhizal tissue is a necessary evolutionary step enabling reduction of
532 seed reserves to a minimum of the production of a true dust-like seed structure.

533 It could be argued that plants cultivated *in vitro* exhibit different development from
534 plants in nature. However, the seedlings observed by us fully fit these observed *in situ*
535 (Irmish, 1855; Velenovsky, 1892, 1905; Hashimoto *et al.*, 2012; Johansson and Eriksson,
536 2013; Hynson *et al.*, 2013a). In particular, the drawings and photographs *in vitro* by Christoph
537 (1921), Lück (1940, 1941) and Lihnell (1942) reveal similar seedlings.

538 How to classify the structure that develops between the undifferentiated globular
539 embryo and the typical root? It starts with polarised growth at suspensor pole of the embryo
540 and continues with cell divisions in an emerging meristematic area at its apical part. Later, it
541 becomes thick, with extremely heterogeneous large epidermal cells and a broad central area of
542 vascular tissues, but without a typical root structure. We therefore called it here the
543 intermediary zone. In previous studies, it was named a “root-like structure” (Hashimoto *et al*.,
544 2012), a “procaulom” (Velenovsky, 1892, 1905) or a “protosoma” Bobrov (2004, 2009,
545 2014). We believe that previous studies did not explore the anatomical structure in detail (an
546 analysis now allowed by the number of seedlings provided by our cultivation methods) and
547 therefore did not distinguish between the intermediary zone and the true roots. Moreover,
548 previous studies analysed seedlings that were already much larger.

549 Orchids also display an intermediary transition stage between the globular embryo and
550 the first shoot meristem that is larger than the pyroloid intermediary zone and is called a
551 protocorm (Rasmussen, 1995; Yeung, 2017), because it precedes formation of the typical
552 plant cormus (i.e. the first shoot or root). Since the intermediary zone of pyroloids also
553 precedes the cormus (here, the first root), we also suggest use of the term protocorm for the
554 intermediary zone of pyroloids. The term protocorm was first used for post-embryonic stages

555 of clubmosses (Treub, 1890), which also form a mycoheterotrophic structure appearing before
556 formation of the first shoot and root. This term was later transferred to orchids (Bernard,
557 1909) and it is thus historically not orchid-limited, but really naming a convergently evolved
558 structure. Recently, protocorm was even used in the obligately parasitic Rafflesiaceae, for an
559 endophytic structure developing from proembryonic endophytic tissue before the formation of
560 the flower-bearing shoot (Nikolov *et al,*. 2014; Nikolov and Davis, 2017). Interestingly,
561 Harley (1959) also suggested the term protocorm for seedlings of *Monotropa*, which are
562 closely related to pyroloids. The pyroloid protocorm is significantly smaller and grows on the
563 opposite embryo pole compared to orchids, but this transitory structure precedes the typical
564 cormus in both mycoheterotrophic developments. Investigations of the early ontogenetic
565 development of other unrelated mycoheterotrophic plants with dust seeds (e.g Burmaniaceae,
566 Gentianaceae, Triuridaceae; Eriksson and Kainulainen, 2011) are pending. Scarce studies
567 have revealed that *Afrothismia hydra* (Burmanniaceae; Imhof and Sainge, 2008) and *Voyria*
568 spp. (Gentianaceae; Imhof, 2010, 2013) produce a root as the first organ, but nothing is
569 known about initiation of the root from the globular embryo in these plants.

570

571 **Conclusion**

572 We used unique combination of cultivation techniques which, for the first time
573 allowed us to develop an efficient cultivation protocol to germinate and grow leafy plants of
574 representatives from all pyroloid genera. Our unique methods for pyroloid cultivation may be
575 used for conservation purposes and also for physiological investigations, since pyroloid
576 mixotrophic nutrition at adulthood is currently of considerable interest (e.g., Lallemand *et al.*,
577 2017). The cultures obtained allow fine analyses of germination, with the transition from lipid
578 and protein storage in seeds to starch accumulation, based on saccharides from the
579 environment. Seedlings form first roots, before adventitious sprouting. The first root emerges

580 from an intermediary zone (Fig. 7), which we suggest should be called a protocorm, due to its
581 functional and developmental similarity with protocorms in other plants. Our data further
582 support the many convergent traits in mycoheterotrophic germination and early development
583 (especially the existence of a single meristem) in plants with dust seeds.

584

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598

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831

832 Figure captions

833 Fig. 1. Effect of different disinfection treatments on seed germination of (A) *Pyrola minor,*
834 (B) *Moneses uniflora*, (C) *Chimaphila umbellata*, (D) *Orthilia secunda*. *In vitro* cultivation on
835 medium Knudson C was evaluated after three months after the cold stratification. Median
836 values are given (n=7). Different letters indicate significant difference according to the
837 ANOVA followed by Tukey HSD post-hoc test for multiple comparison.

838

839 Fig. 2. Effect of different media on germination of (A) *Pyrola minor,* (B) *Moneses uniflora*,
840 (C) *Orthilia secunda* and growth of (D) *Pyrola minor,* (E) *Moneses uniflora*, (F) *Orthilia*
841 *secunda*. Both germination and total length of seedlings were measured 3 months after cold
842 stratification. Different letters indicate significant difference. Results of Tukey HSD post hoc
843 test for multiple comparisons are indicated by capital letters and results of Pairwise Wilcoxon
844 Rank Sum test by lowercase letters.

845

846 Fig. 3. Representatives of *in vitro* grown seedlings of species used in this study. A) *Pyrola*
847 *minor* cultivated in dark, B) *Pyrola minor* cultivated on light and C) *Moneses uniflora*, D)
848 *Orthilia secunda*, E) *Chimaphila umbellata* and F) *Monotropa uniflora* cultivated in dark.
849 Scale bars: 0,5 mm.

850

851 Fig. 4. Effect of selected soluble saccharides on germination of *Pyrola minor* (A) and *Orthilia*
852 *secunda* (B). *In vitro* cultivation on Knudson C medium where sucrose was replaced by
853 saccharide indicated. Different letters indicate significant difference. Results of Tukey HSD
854 post hoc test for multiple comparisons indicated by capital letters and results of Pairwise
855 Wilcoxon Rank Sum test by lowercase letters; bars indicate 1st and 3rd quantiles. Pairwise
856 comparisons were calculated separately for each month.

857

858 Fig. 5. Germination and early development of *Pyrola minor*. (a) mature seed with globular
859 embryo. (b) germination starts with enlargement of embryo (arrows indicate the former
860 embryo). (c) seedling develops further into root through less organized intermediary zone
861 with very heterogenic surface cell pattern. (d) detail of the surface cell pattern of intermediary
862 zone. (e) seedling of 1-1.5 mm size with already established root meristem. (f) meristematic
863 zone is established very early after germination. (g) seedling of less than 1 mm size with
864 established meristem and differentiated vascular tissues in central part of intermediary zone
865 close to the former embryo. (h) seedling of *ca*. 3 mm size with already established lateral root
866 primordium (white arrow) and differentiated vascular tissues (in detail). (i) root structure is
867 obvious at 1.5 cm from the position of former embryo in 3 cm long seedling. Abbreviations:
868 en, endodermis; x, protoxylem vessels of radial vascular bundle; c, cortex; rh, rhizodermis. (j)
869 root tip of 3 cm long seedling. Abbreviations: lrc, lateral root cap; p, protoderm; qc, quiescent
870 center. a, b, e, j, whole-mounts, DIC; c, whole-mount, confocal image; d, whole-mount, UV;
871 f-h, paraplast sections, Safranin O + Fast Green FCF; I, hand section, UV; j, paraplast
872 section, Lugol + Orange G. Black arrows indicate position of former embryo; scale bars 50
873 µm.

874

875 Fig. 6. Transition to shoot formation in *P. minor* seedlings. (a) Nine-months old seedling with
876 established root system prior the onset of shoot growth. (b) Nine-months old seedling with
877 emerging shoot (arrow). (c) shoot bud emerges at first root branching site close to the position
878 of former embryo (r, root; sh, shoot bud; iz, intermediary zone connecting the former embryo;
879 x, xylem), section stained with Safranin O and Fast Green FCF. (d) later, additional shoot
880 buds emerge along root axis (arrows). (e) detail of first shoot bud, whole-mount preparation.
881 (f) fully-established *in vitro* plant. (g) adventitious roots emerging at stem node (sh, shoot).
882 Scale bars 0.5 cm, except c, e: 200 µm.

883

884 Fig. 7. Comparison of germination and subsequent development of orchids (left) and
885 pyroloids (right). Embryo is highlighted in red, protocorm in blue, shoots in green and roots in
886 brown. Abbreviations: a, adventive root; br, branching roots; e, embryo; end, endosperm; h,
887 hilum; iz, intermediary zone; p, protocorm; ram, root meristem; sh, shoot; sam, shoot
888 meristem; t, testa; x, xylem. Thin bar is 100µm, dotted bar 1mm and thick bar 0,5 cm.

889