1 **Dominance of *Paris*-type morphology on mycothallus of**
2 ***Lunularia cruciata* colonised by *Glomus proliferum***

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4 Henrique M.A.C. Fonseca [1*] · Joana I. L. Ferreira[2] · Ricardo L.L. Berbara[3] ·
5 Natalia P. Zatorre[3]

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8 [1*]Centre of Cellular Biology, Department of Biology, University of Aveiro, 3810-
9 193, Aveiro, Portugal.; [2]Faculty of Science, University of Porto, 4169-007, Porto,
10 Portugal.; [3]Universidade Federal Rural of Rio de Janeiro, Soil Department,
11 Seropédica, Itaguaí, RJ, CEP 23851-970, Brazil.

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13 *Corresponding Author. Centre of Cellular Biology, Department of Biology,
14 University of Aveiro, 3810-193, Aveiro, Portugal. E-mail: hfonseca@ua.pt;
15 Phone: +351-234370772; Fax: +351-234426408

16

17 ***Abstract***

18 Microscopic evidence confirms that *L. cruciata* hosting *G. proliferum* shows
19 major anatomical traits (arbuscules, coils, arbusculate coils and vesicles) generally
20 associated arbuscular mycorrhizal roots and the anatomical morphology of intra-
21 thalli mycelium is predominantly of the *Paris*-type. Colonised *L. cruciata* showed
22 a reduction of biomass when compared with axenic plants suggesting a drain of
23 resources towards the fungus and depletion of nutrients required for optimum

plant growth. The behaviour of mycothalli regarding available KH_2PO_4 indicates that the nutritional stress threshold for phosphorus (P) is above the residual amount of P already present in Phytagel™ and in plant inoculum. These raise the possibility that in certain circumstances the relationship between *L. cruciata* and *G. proliferum* be parasitic rather than symbiotic and open the door for future studies to ascertain the nature of liverwort-AM fungi relationships.

Key-words

Arbuscular mycorrhizal fungi; Phosphorus; *Arum*-type; Liverwort; Monoxenic cultures

Introduction

Arbuscular mycorrhizas (AM) are ubiquitous underground symbiotic associations between a wide diversity of plants and obligate symbiotic fungi of the phylum Glomeromycota (21). From this symbiosis plants generally obtain higher yields as they improve their capacity to acquire low mobile soil nutrients and increase resistance to biotic and abiotic stresses. Concomitantly the fungi are able to access the host photosynthate carbon pools and so to complete their life cycle (1, 15, 23). Most available information on the physiology and anatomy of mycorrhizae is related to the sporophyte of Tracheophyta. Conversely for non-vascular plants the knowledge is still scarce. Within these the liverworts are an important and extremely successful group found in all continents and environments. These plants, that are thought to be amongst the original colonisers of terrestrial habitats, appear to have remained relatively unchanged through time and probably hold the key to early terrestrial diversification of land plants (17, 18). Some complex

47 thalloid liverworts (Marchantiales) are known to form mycorrhiza-like
48 associations with AM fungi. Mycothallus (3) develop structures that are
49 analogous to those observed in AM roots, thus indicating possible functional
50 similarities (8, 10, 11, 13, 17, 19). In these plants, as in roots, the AM fungus
51 grows and connects two distinct environments: within the cells of the host body as
52 inter or intra-cellular mycelium; and externally in the soil or medium matrix, thus
53 extending the host plant capacity to access nutrients well beyond their body limits
54 into the soil matrix. In roots the internal mycelium has been shown to have one or
55 a combination of two different morphological types (2, 9, 22): *Arum* and *Paris*.
56 The *Arum*-type morphology, first described on *Arum maculatum*, shows
57 intercellular hyphae mainly growing longitudinally between cells with arbuscules
58 rising on short upright intra-cellar branches. The *Paris*-type, originally described
59 on *Paris quadrifolia*, is defined by cell-to-cell intracellular growth with the
60 formation of coils and arbusculate coils. Depending upon the host plant and
61 fungus these morph types can occur isolate or simultaneously within the same
62 plant forming a continuous mycorrhizal structure (5). In liverworts the
63 morphology of hyphae within mycothallus is not yet full characterised, however
64 most reports indicate that AM fungi generally progresses within the plant body
65 with patterns resembling the *Paris*-type (6, 11).

66 Phosphorus (P) is an important macronutrient involved in key structural and
67 metabolic functions of all organisms. Moreover, it is widely accepted that in many
68 plants AM fungi plays a main role in the resistance to biotic and abiotic stresses
69 by improving the host capacity to uptake major nutrients from soil, ex., inorganic
70 phosphate (23). Although this is true for Tracheophyta the available information
71 regarding nonvascular plants and particular those cultured *in vitro* is scarce or

72 nonexistent (7). Plus the nutrient requirements of liverworts vary considerably
73 from those of most vascular plants.

74 The present work aims to address (i) the physiological effect of phosphorus on
75 biomass production of *L. cruciata* colonisation by AM fungi. (ii) To survey the
76 morph types of *G. proliferum* mycelium within *L. cruciata* thallus.

77 *Materials and methods*

78 Biological material and growth conditions

79 *Glomus proliferum* Dalpé & Declerck (MUCL 41827), acquired from GINCO
80 (Mycotheque de l'Université Catholique de Louvain, Laboratoire de Mycologie,
81 Belgique) was multiplied and maintained in monoxenic cultures of *Lunularia*
82 *cruciata* (L.) Dumortier ex. Lindberg. Plants and fungi were kept throughout the
83 experiments at 25 ºC with a 10/14 hours light/dark photoperiod in a Sanyo MLR–
84 350H chamber with light of an average intensity of 68.3 ± 6.4 µmol $s^{-1}m^{-2}$ as
85 described by Fonseca *et al*. (8).

86 Inocula preparation

87 Inocula were obtained from axenic and monoxenic thallus of *L. cruciata* cultured
88 for 100 days on SRV (8) with 29.2 mM sucrose, monoxenic cultures used showed
89 profusion production of external hyphae and spores of *G. proliferum* (Fig. 1).

90 Light microscopy

91 To study the pattern of colonisation of *L. cruciata* by *G. proliferum* 0.5 cm thallus
92 segments were cultured for 49 days on SRV medium as described by Fonseca *et*
93 *al*. (8). The growth length of 44 mycothallus apices were measured weekly along

94 an imaginary line through the thallus midrib (Fig. 2a). Measured segments were
95 then cropped, fixed in Bouin's fluid and cleared with 10% KOH, at 80°C for 20
96 min. Samples were washed in distilled water, acidified in 1 N HCl before being
97 dehydrated and embedded in paraffin wax. Sections of about 10 µm were cut with
98 a microtome (Leitz model 1512), mounted on microscope slides and stained
99 overnight in 0.05% trypan blue (16). Images were digitally acquired with a Carl
100 Zeiss Axiocam HR apparatus.

101 **Phosphorus experiment**

102 Discs of thallus (10.7 ± 1.7 mm^2 made by Ø3.27 mm cork-borers) from axenic
103 and monoxenic *L. cruciata* were cultured for 70 days on 30 ml of SRV (8) with
104 29.2 mM sucrose and three levels of added phosphorus. A 2×4 factorial design
105 was setup with the fungal treatment consisted of the presence *G. proliferum* and
106 absence of fungus. The phosphorus treatment consisted of SRV media with three
107 levels of added KH_2PO_4 (123.0, 61.5 and 30.7 µg KH_2PO_4) and SRV medium
108 without added KH_2PO_4. There were ten replicate Petri dishes per treatment.

109 **Data collection**

110 Thallus length was estimated by engraving the contour of each thallus apex, under
111 stereoscope microscope, on the lower side of the plastic Petri dish. At the end of
112 the experiment the lines drawn along the thallus midrib were measured. Plant
113 biomass was estimated as dry weight after oven-drying (60°C) to a constant
114 weight. Number of spores and hyphal length were measured under a
115 stereomicroscope with a 6×6 square hairline graticule of 20.25 mm^2 regularly
116 placed at 0.5 cm intervals over the surface of inverted Petri dishes and following

117 the method by McGonigle *et al.* (12). Data were evaluated for significance
118 (P<0.05) using multivariate analysis of variance (ANOVA/MANOVA) and post
119 hoc Least Significant Differences test with Statistica software for Windows v4.5
120 (Statsoft Inc.).

121 ## *Results*

122 **Effect of phosphorus**. The quantity of phosphorus present in Strullu-Romand (4)
123 and SRV (8) media is suitable for both axenic and monoxenic Ri T-DNA
124 transformed roots cultures colonised by AM fungi and for mycothallic *L. cruciata*.
125 However, when levels of P is reduced by 50 or 75% from SRV levels any
126 significant responses were observed on mycothallic *L. cruciata* dry weight and
127 AM fungus hyphae and spore production (Table 1). Only when any KH_2PO_4 was
128 added to the medium did plants show a decrease in biomass and the fungus a
129 reduction in spore production, compared to the treatment with 123 μg KH_2PO_4
130 (Table 1). Alternatively, if one analyses the data in terms of presence/absence of
131 AM fungus colonisation the results shows that liverwort growth was negatively
132 affected ($P< 0.05$) in monoxenic conditions. Colonised plants showed a reduction
133 of 54.7% in dry weight, compared with axenic *L. cruciata* (Table 1).

134 **Thallus growth rate**. When cultured in 30 ml of SRV (with 123 μg of added
135 KH_2PO_4) mycothallus of *L. cruciata* showed during the first 35 days a steady
136 growth rate of 0.382 $mm{\cdot}day^{-1}$ (r^2= 0.999). From then on (the last 14 days of
137 culture) the growth average rate decreased about 13.1% at the end of the
138 experiment (Fig 2b) giving an overall average growth rate of 0.358 ± 0.025
139 $mm{\cdot}day^{-1}$ for 49 days culture.

140 **Anatomical characterisation of symbiosis**. *L. cruciata* is a complex thalloid
141 liverwort with an internal differentiated anatomy (Fig. 2c). Intercellular hyphae
142 were conspicuous throughout the mycothalli with few exceptions: hyphae were
143 scarcely present among the small chlorophyllous cells within the photosynthetic
144 layer; no fungus was observed in the meristematic zones on thallus apices; and, as
145 reported by Fonseca *et al*. (8), no hyphae colonised rhizoid cells. Conversely, *G.*
146 *proliferum* was profusely present within the highly vacuolated parenchyma cells
147 with special prevalence in the central midrib area where trypan blue stain revealed
148 a distinct fungal layer (Fig. 2c). Within this layer a denser network of internal
149 hyphae could be seen connecting arbuscules, coils and arbusculate coils, as well
150 as, scattered vesicles. Hyphae and arbuscules were also present in oil cells (Fig.
151 2d). The morphological type of *L. cruciata* colonisation by *G. proliferum* was
152 predominantly of the *Paris*-type (9, 22) (Fig. 2e), however in places the hyphae
153 and arbuscules denoted a pattern closest related to the *Arum*-type morphology
154 (Fig. 2f). Here the hyphae appear to grow close to the plant cell wall with
155 arbuscules rising on short upright intra-cellar branches.

156 *Discussion*

157 The cultures used as inoculum for the experiments had their origin in 2003 (8).
158 Since then they have been regularly sub-cultured producing a reliable means to
159 maintain and multiply *G. proliferum*. In addition, the use of *L. cruciata* as host for
160 AM fungi has the advantage over the Ri T-DNA transformed root systems as they
161 allow easy manipulation of the external mycelium. With these cultures the plants
162 usually occupy less than half the Petri dish area and sometimes leaving

163 undisturbed 3/4 of available medium surface with no apparent adverse effect on
164 external mycelium growth (Fig. 1).

165 *In vitro* cultured *L. cruciata* with and without *G. proliferum* behaved indifferently
166 to changes in medium added P. Both plant dry weight and AM fungi growth
167 (number of spores and external hyphae length) could not resolve significant
168 changes with these used levels of KH_2PO_4 that is in accordance with the capacity
169 of liverworts to exhibit normal development on wide range of media (7). A
170 different plant and fungus behaviour was observed when any KH_2PO_4 was added
171 to the SRV medium. For these plants there was trace amounts of P derived from
172 residual P brought by the initial plant inoculum (thallus discs) and by medium
173 Phytagel™ component (14). As differences in plant and fungal growth were
174 observed between P treatments we may speculate that the limiting threshold for P
175 to induce differences in plant and AM fungal behaviour lay above residual P
176 present in the experiment. Moreover, in the present culture conditions, data on
177 plant overall growth pattern strongly suggests that the colonisation of *L. cruciata*
178 represents a heavy burden for the plant growth thus implying that the colonisation
179 of *L. cruciata* by *G. proliferum* is not a mutualistic symbiosis but rather a parasitic
180 one. However, the anatomical traits observed in this association are consistent
181 with those present in mycorrhizae colonised by *G. proliferum*. The liverwort may
182 gain from the association only if important nutrients, such as phosphorus, are
183 directly unavailable to the plant or if available they are below the optimum
184 threshold for maximal mycothallus growth.

185 Considerations about the morph type of *G. proliferum* growth within *L. cruciata*
186 thallus were already raised by Fonseca *et al*. (8). At the time it was suggested that

the architecture *G. proliferum* was more consistent with the *Paris*-type. Our extensive survey of mycothallus of *L. cruciata* agrees with this assertion. The fungus colonises almost all parts of the thallus except those close to the meristematic zones. Our study also confirms findings reported by Fonseca *et al.* (8) that *G. proliferum* hyphae and arbuscules were present in oil cells and absent from rhizoids. Although the opposite was described for other liverworts (11, 19). The persistent absence of hyphae in rhizoids from *in vitro* studies of liverworts (8) and hornworts (20) may indicate that *in vitro* the diffuse presence of light within the medium and all over the plant excludes the rhizoids from being the principal source of plant colonisation. Within the thallus midrib, trypan blue staining revealed a layer of intense marked cells where colonisation by *G. proliferum* formed a zone rich in arbuscules, coils, arbusculate coils and other AM anatomical traits. The predominant morphs are consistent with the *Paris*-type as the hyphae were observed to progress from cell-to-cell and, particularly within the midrib fungal layer, numerous arbuscules and coils were present. However scattered within the AM fungal zone of the parenchymatous layer one sporadically observed clusters of cells where the fungi appear to progress in a mode more consistent with the *Arum*-type.

Conclusions

From the observations in this study and from those of an earlier study on mycothallus of *L. cruciata* (8) we propose that the colonisation by *G. proliferum* not only has the major mycorrhizal traits generally associated with the colonisation of roots by AM fungi (arbuscules, coils, arbusculate coils and vesicles), but it also shows that the internal hyphae colonises the thallus

211 predominantly with a *Paris* morphotype. Furthermore, the colonisation of *L.*
212 *cruciata* by *G. proliferum* resulted in a reduction of host biomass compared with
213 axenic plants suggesting a bypass of resources towards the fungus. Hence the
214 relationship between *L. cruciata* and *G. proliferum* may not always be, if ever,
215 symbiotic in nature. Finally, the present study shows that, despite any response
216 from *L. cruciata* to changes in medium above 30.7 μg KH_2PO_4, the P stress
217 observed in mycothallus when any P was added indicates that the threshold for
218 optimum growth is above the residual amounts of P brought by contaminants in
219 Phytagel^{TM} and P in the plant inoculum. This finding opens the door for future
220 nutritional studies to ascertain the nature of liverwort-AM fungi relationships.

221 ***Acknowledgements***

222 The authors would like to thank Dr. Lourdes Pereira and Dr. Manuel Santos for
223 the use of their Laboratory facilities; acknowledge the financial support of
224 Fundação para a Ciência e a Tecnologia (SFRH/BSAB/740/2007), Portugal and a
225 fellowship grant from Inter-American Institute for Global Change Research (IAI)
226 CRN II/14 which is supported by the US National Science Foundation (Grant
227 GEO-04523250)

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Predomínio da morfologia tipo-*Paris* em talos de *Lunularia cruciata* colonizados por *Glomus proliferum*

Resumo

Observações de microscopia ótica confirmam que *L. cruciata* colonizada por *G. proliferum* apresenta caracteres anatômicos (arbúsculos, hifas, arbúsculos enovelados e vesículas) geralmente associadas a raízes micorrízicas arbusculares em que o micélio intra-tálico apresenta uma anatomia predominantemente do tipo *Paris*. *L. cruciata* colonizada apresentou redução de biomassa quando comparada com plantas axénicas, sugerindo dreno de recursos para o fungo e conseqüente redução de nutrientes necessários para o ótimo crescimento da planta. O comportamento do talo-colonizado em relação à disponibilidade de KH_2PO_4 no meio indica que o limiar de stress nutricional para fósforo se encontra acima do somatório das quantidades residuais deste elemento presentes no Phytagel™ e no inoculo. Os resultados sugerem a possibilidade, de em certas circunstâncias, a relação entre *L. cruciata* e *G. proliferum* ter características de parasitismo e não de simbiose, abrindo novas perspectivas para futuros estudos na determinação da natureza da relação hepática - fungo arbuscular.

Palavras chave

Fungos micorrízicos arbusculares; Fósforo; Tipo-*Arum*; Hepáticas; Culturas monoxénicas

315 ***Table 1***

316 Table 1 – Biomass, number of fungal spores and hyphae length production of *Lunularia cruciata*, cultured for 70 days in 30 ml of SRV
317 medium with 29.2 mM sucrose, with and without *Glomus proliferum* and with different levels of added KH_2PO_4 plus without added
318 phosphorus. *Yes* – plants colonised by *G. proliferum*; *No* – axenic *L. cruciata*.

G. proliferum	KH_2PO_4 (µg)	Dry weight (g)	Number of spores	Hyphae length (mm)
Yes	0.0	0.066 ± 0.017 a	11038 ± 9232 a	69805 ± 41445 a
Yes	30.8	0.082 ± 0.010 ab	12766 ± 6966 a	75937 ± 38726 a
Yes	61.5	0.074 ± 0.032 ab	25940 ± 15216 ab	72975 ± 48888 a
Yes	123.0	0.091 ± 0.023 b	54749 ± 34710 b	135278 ± 73912 a
No	0.0	0.131 ± 0.030 c	---	---
No	30.8	0.159 ± 0.025 c	---	---
No	61.5	0.129 ± 0.016 c	---	---
No	123.0	0.153 ± 0.027 c	---	---

319 # Figures

320 ## Legend Figure 1

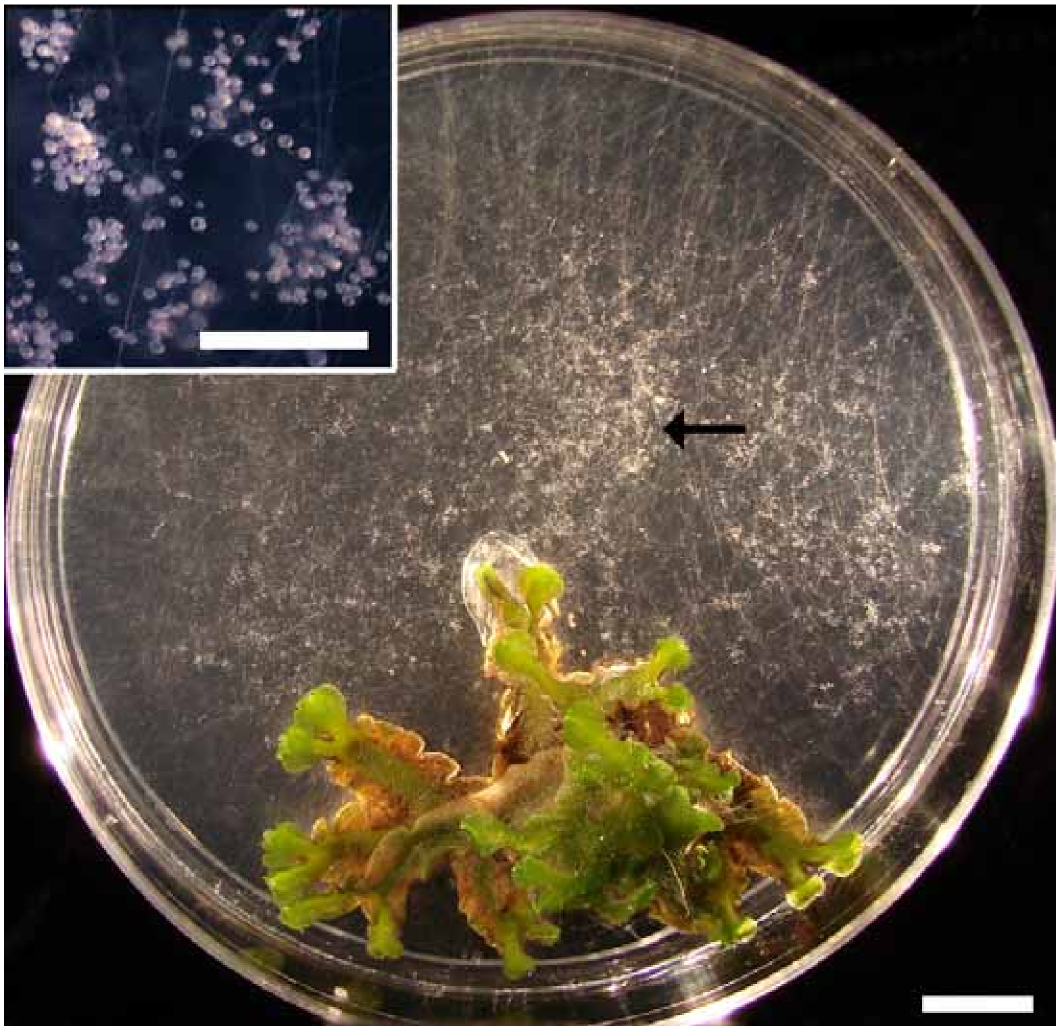
321 Fig. 1 – Maintenance culture of *Lunularia cruciata* with *Glomus proliferum*
322 grown for 100 days in 30 ml SRV medium with 29.2 mM of sucrose and used as
323 inocula source for the experiments. Plant discs sowed asymmetrically in Petri
324 dishes allowed the fungus to grow undisturbed on more than half of dish area.
325 (*arrow*) Indicates high concentration of hyphae and spore clusters with the *inset*
326 showing external mycelium and spores imbedded in the medium. Bars 10 mm;
327 inset, 1 mm.

328 ## Legend Figure 2

329 Fig. 2 – *Lunularia cruciata* colonised by *Glomus proliferum* grown for 49 days
330 on SRV medium with 29.2 mM of sucrose. (***a***) Mycothallus apex showing midrib
331 imagine *line arrow* used for the last measurement of thallus length. (*open arrows*)
332 Spore clusters imbedded in the medium. (***b***) Mycothallic averages and standard
333 deviations (vertical bars) of growth length of 44 apices measured at 7 days
334 intervals. Fitted line and equation describes the lengthening pattern of
335 mycothallus during the first 35 days of growth. Equation: y, Length (mm); x, Time
336 (days); r^2, R-squared value. (***c*** to ***f***) Light microscopy of trypan blue-stained
337 samples: (***c***) Anatomic section of mycothallus showing (1) photosynthetic layer
338 under an upper epidermis; (2, fungal layer) thallus' midrib parenchyma with high
339 concentration of arbuscules, vesicles and oil cells; (3) the lower epidermis with
340 (4) rhizoids and (5) scales; (***d***) Arbuscules within (6) oil cells located in the
341 thallus' midrib parenchyma. These cells show their oily content only partially

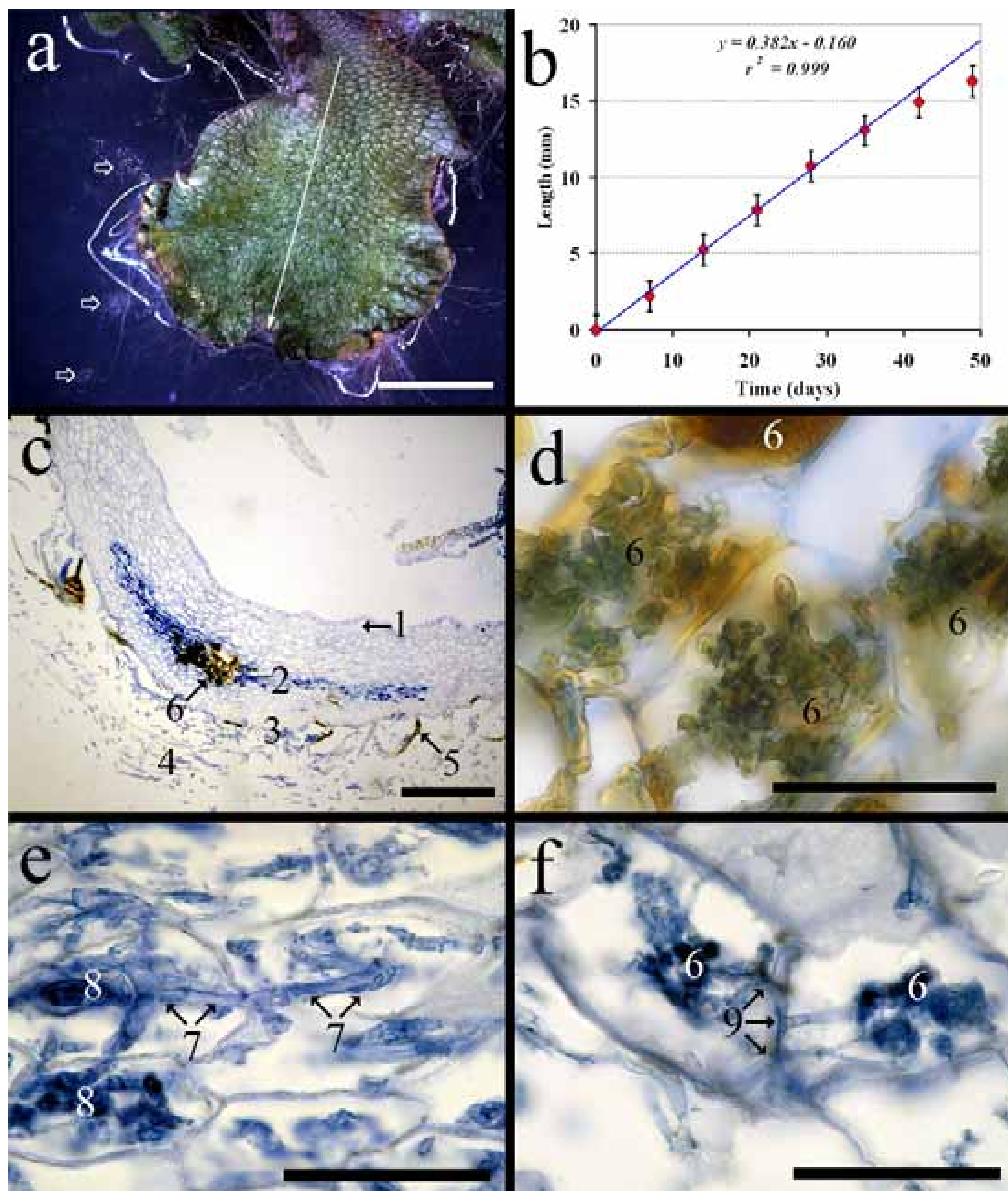
342 removed by the method for microscopy. (***e***) Small section of mycothallus midrib
343 anatomy exemplifies a common morphological pattern showing (7) hyphae
344 crossing cell-to-cell in a pattern characteristic of the *Paris*-type. (8) Arbusculate
345 coils. (***f***) The less frequent morphology of *Arum*-type was also present in some
346 cluster of cells within the thallus midrib showing several (6) overlapping
347 arbuscules connected to (9) hyphae progressing close to liverwort cell wall. Bars:
348 (a) 5 mm; (c) 200 µm; (d) 50 µm; (e, f) 20 µm.

349 **Figura 1**



350

351 **Figura 2**



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