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SHORT NOTE

Lunularia cruciata, a potential in vitro host for *Glomus proliferum* and *G. intraradices*

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Abstract A study was conducted to define culture conditions for in vitro growth arbuscular mycorrhizal fungi (AMF) with liverworts as hosts. *Lunularia cruciata* (L.) Dumortier ex. Lindberg developed *in vitro* monoxenic mycothalli with both *Glomus proliferum* Dalpé & Declerck (MUCL 41827) and *Glomus intraradices* Schenck & Smith (MUCL 43204). AMF inoculated plants were co-cultured in plastic Petri dishes with semi-solidified medium supplemented with sucrose and grown under filtered light. Mycothalli of *L. cruciata* produced external hyphae and spores in quantities equivalent to those obtained with Ri T-DNA transformed root systems.

Keywords Arbuscular mycorrhizal fungi · In vitro · Liverworts · *Lunularia cruciata* · Monoxenic culture

Introduction

Recent years have brought increasing interest in interactions between arbuscular mycorrhizal fungi (AMF) and bryophytes. This trend was particularly true after Schüßler (2000) described in vitro culture of *Glomus claroideum* with hornworts. Bryophytes are a paraphyletic group of nonvascular plants that include mosses, liverworts and hornworts. They consist of free-living gametophytes which are fixed to soil by rhizoids, as they do not have roots (Raven 2003). Hence, the terminology of "mycothalli" is preferred (Boullard 1988) instead of "mycorrhizae" to define their association with Glomeromycota. It is proposed that the mycorrhizal symbiosis coevolved from such primitive non-vascular plants during evolution (Redecker et al. 2000), but a functional link has never been established between AMF and liverworts (Read et al. 2000).

As AMF have yet to be cultivated axenically, it is becoming increasingly necessary to optimise in vitro culture conditions for mycorrhizal studies (Declerck et al. 2005). Cultures are generally produced with whole plants in soil at different degrees of sterility (Fonseca et al. 2001), or in vitro on monoxenic cultures of Ri T-DNA transformed roots (Becard and Fortin 1988). More recently, an attempt to culture in vitro monoxenic AMF with thalli of the hornwort *Anthoceros punctatus* was reported with the successful establishment of mycothalli, an external hyphal network and the production of newly formed spores (Schüßler 2000). Since then several studies with liverworts collected from nature have reported the presence of AMF within thalli (e.g. Duckett et al. 2004; Russell and Bulman 2005), indicative of the possible use of liverworts as in vitro AMF hosts.

The present work explores in vitro co-culture of two types. A *Glomus* species (*sensu* Schüßler et al. 2001) with two liverwort species of the phylum Marchantiopsida and proposes an alternative method of monoxenic culture for AMF. Two major challenges were solved: obtaining axenic cultures of the potential host plants and definition of appropriate monoxenic culture conditions for AMF growth.

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Materials and methods

Biological material

Glomus proliferum Dalpé & Declerck (MUCL 41827) and *Glomus intraradices* Schenck & Smith (MUCL 43204), acquired from GINCO (Mycotheque de l'Université Catholique de Louvain, Laboratoire de Mycologie, Belgique) were multiplied and maintained in monoxenic cultures with Ri T-DNA transformed roots of *Trifolium pratense* L., obtained as described by Berbara et al. (1995) on Strullu–Romand (MSR) medium (Declerck et al. 1998). Liverworts were collected in 2003 on the campus of the University of Aveiro, Portugal. *Lunularia cruciata* (L.) Dumortier ex. Lindberg was obtained from asexual propagules (gemmae) on splash cups and *Marchantia polymorpha* L. spores isolated from whole sporophytes cropped from archegoniophores. Gemmae and sporophytes were soaked for 15 s in 96% ethanol, immersed for 5 to 25 min in 7% commercial bleach (Domestos, Lever Portuguesa SA, Portugal), collected at approximately 5-min intervals and immediately rinsed four times for 5 min in autoclaved distilled water. Resulting isolates were separately placed in Petri dishes with Knop medium (Reski and Abel 1985) to promote plant growth. Axenic *M. polymorpha* sporangia were individually squeezed against the surface of semi-solidified medium to distribute spores evenly. Cultures of *L. cruciata* and *M. polymorpha* were maintained at 25°C in a Sanyo MLR–350H growth chamber with continuous light.

Liverwort culture with AMF

Dual cultures were initiated through inoculation of *L. cruciata* and *M. polymorpha* with *G. proliferum* and *G intraradices* in non-compartmentalized 90-mm plastic Petri dishes (Steriling) containing 30 ml of SRV medium modified from MSR (Declerck et al. 1998): 3.0 mM $MgSO_4 \cdot 7H_2O$, 0.75 mM KNO_3, 0.87 mM KCl, 1.52 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.03 mM KH_2PO_4; 11 μM $MnSO_4 \cdot H2O$, 1 μM $ZnSO_4 \cdot 7H_2O$, 30 μM H_3BO_3, 0.96 μM $CuSO_4 \cdot 5H_2O$, 0.02 μM NaFe·EDTA, 0.03 μM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.01 μM $Na_2MoO_4 \cdot 2H_2O$, 0.49 μm V_2O_5, 0.85 μM $CoSO_4 \cdot 7H_2O$; 5 g l^{-1} Phytagel (Sigma); pH 5.5 before autoclaving (120°C for 25 min).

Growth factors/vitamins were added by filtration through 0.2 μm mesh filter (Ministart-Sartorius): 0.004 μM biotin, 0.29 μM cyanocobalamin, 1.88 μM panthotenate-Ca, 4.38 μM pyridoxine-HCl, 2.96 μM thiamine-HCl, 8.1 μM nicotinic acid. Cubes approximately 4 mm wide, containing AMF spores and a few hyphae, were collected from monoxenic cultures with *T. pratense* on MSR (Declerck et al. 1998) and inserted into wells of approximately the same size in new SRV, with and without 29.2 mM added sucrose in a 2×2×2 factorial design with four replicates per treatment.

The fungal inocula were covered with axenic liverwort thalli and grown in a Sanyo MLR–350H chamber for 120 days, kept at 25°C, with a 10/14 h light/dark photoperiod. Light from three fluorescent lamps (Sanyo FL40SS W/37) filtered by one layer of *Smooth Tracing Paper* (STP; Calque Satin Canson, ref. 17–119, 90 g m^{-2}) generated an average light intensity of 68.3±6.4 μmol s^{-1} m^{-2} measured at eight different positions per shelf with a Li-COR photometer (Model Li-250) equipped with a spherical sensor. Inocula viability was assayed on SRV with 29.2 mM sucrose by replacing the thalli with five 1-cm long, root segments of axenic Ri T-DNA transformed *T. pratense* and cultured as described above, with four replicates, but without light.

Subculture of mycothalli of *L. cruciata* was repeatedly done on SRV medium with 29.2 mM sucrose, by introducing green mycothalli segments of approximately 0.5 cm of length, obtained from between 40 and 120 days old mature cultures, showing uniform production of external hyphae and new spores throughout the Petri dish.

Spores obtained from sub-cultivation of mycothalli were assessed for viability and infectivity through the inoculation of clusters of at least 50 spores onto axenic cultures of Ri T-DNA transformed roots of *T. pratense* and axenic thalli of *L. cruciata*. Resulting monoxenic cultures (ten replicates each) were grown on SRV medium with 29.2 mM sucrose for 40 days as described above. Viable and infective spore clusters are defined as those capable of producing mycorrhiza or mycothalli with external hyphae and new spores.

Light microscopy To observe fungal distribution within *L. cruciata*, pieces of mycothalli and adjacent medium were cropped from 40- and 120-day-old cultures, fixed in Bouin's fluid and cleared with 10% KOH, at 80°C for 20 min. Samples were washed in distilled water, acidified in 1 N HCl before being dehydrated and embedded in paraffin wax. Sections of about 10 μm were cut with a microtome (Leitz model 1512), mounted on microscope slides and stained overnight in 0.05% trypan blue (Phillips and Hayman 1970). Images were digitally acquired with a Carl Zeiss Axiocam HR apparatus fitted into a simple and compound microscope and processed with Axiovision software v3.1.2. Number of spores and hyphal length were measured under a stereomicroscope, with a 6×6 square hairline graticule of 20.25 mm^2, regularly placed at 0.5-cm intervals over the surface of an inverted Petri dish, following the method of McGonigle et al. (1990). Data were evaluated for significance ($P<0.05$) using multivariate analysis of variance (ANOVA/MANOVA) and post hoc Scheffé's test, Statistica software for Windows (Statsoft Inc.).

Table 1 Spore production and hyphal length of *Glomus intraradices* and *G. proliferum* cultured for 120 days with *Lunularia cruciata* or *Trifolium pratense* on SRV medium with or without 29.2 mM of sucrose

Plant/Fungus	Sucrose (mM)	Number of spores (spores cm^{-3})	Hyphal length (mm cm^{-3})
L. cruciata			
G. intraradices	29.2	408±155 b	394.8±113.7 b
	0.0	0±0 a	0.4±0.3 a
G. proliferum	29.2	935±583 b	525.4±56.3 b
	0.0	0±0 a	0.5±0.4 a
T. pratense			
G. intraradices	29.2	288±40 b	598.1±38.9 c
G. proliferum	29.2	573±197 b	504.4±19.3 b

Values are the means of four replicates ±standard deviation; when followed by the same letter, within fungal species and parameters, they are not significantly different ($P<0.05$)

Results

Monoxenic in vitro cultures of *L. cruciata* with *G. proliferum* and *G. intraradices* were obtained under the experimental conditions described. These cultures have been subcultured and maintained for more than 2 years and have become a primary source of inocula for *G. proliferum* and *G. intraradices*. For *L. cruciata*, mycothalli, external hyphae and new spores were only obtained when light was filtered with STP. Direct light was found to inhibit spore germination and fungal growth.

Only *L. cruciata* was able to sustain growth of the tested AMF species. The production of external hyphae and new spores was dependent on the presence of sucrose in the medium (Table 1). Cultures with 29.2 mM sucrose showed vigorous AMF growth with most plants attaining maximal thallus expansion within the first 40 days of culture [Fig. 1 (1–2)]. At the end of this period plants were still predominantly green and had an extensive external hyphal network with new spores at different stages of development [Fig. 1(3)]. New spore production increased until approximately 120 days of culture. By then cultures showed extensive production of spores, with no significant difference between hosts (Table 1). Old thalli showed increased browning as a result of progressive loss of green photosynthetic tissue [Fig. 1(4)], necessitating subculture before 120 days.

For *M. polymorpha* no new external hyphae or spores of *G. proliferum* and *G. intraradices* were observed under any conditions. However, spores used in these cultures were found to be viable when inoculated with Ri T-DNA-transformed roots of *T. pratense*, as they produced mycorrhizal roots.

All mature spore clusters obtained from mycothalli cultures of both AMF species were shown to be viable and infective when tested with both axenic cultures of Ri T-DNA-transformed roots of *T. pratense* and on axenic thalli of *L. cruciata*.

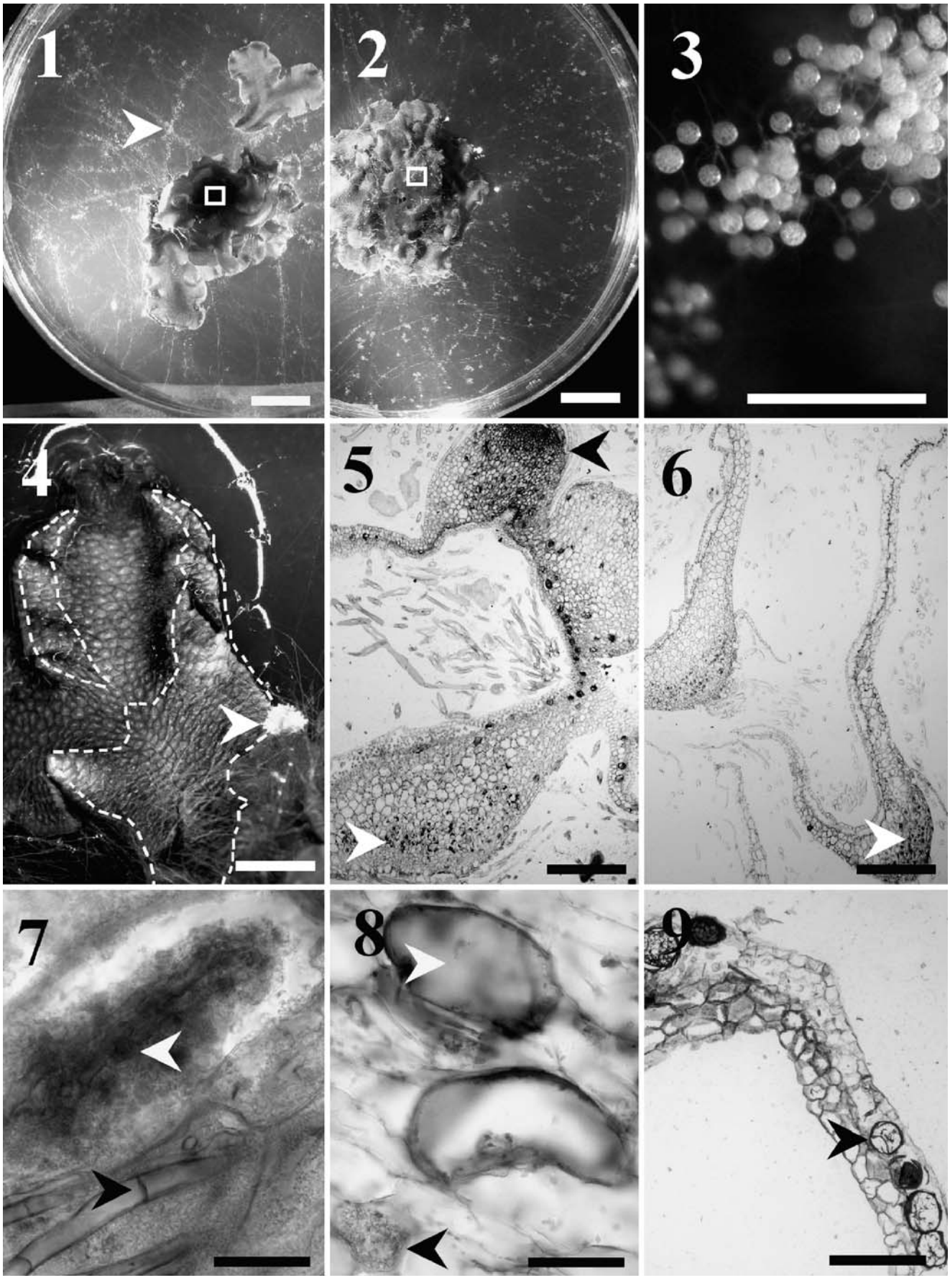
Morphology of mycothalli

Mycothalli of *L. cruciata* with *G. intraradices* and *G. proliferum* showed a similar morphology [Fig. 1(1–2)]. The fungi were conspicuous throughout the mycothalli, with the exception of meristematic zones at the apices [Fig. 1(5)] where no AMF structures were observed. Conversely, within the highly vacuolated parenchyma cells of the central midrib [Fig. 1(5, 6)] the fungi could be observed forming intracellular arbusculate structures [Fig. 1(7)], and vesicles [Fig. 1(8)]. Thallus zones with oil cells frequently show septate hyphae [Fig. 1(7)]. Spores were present within the media [Fig. 1 (3)] and also within the thallus, especially between the upper epidermis and the upper photosynthetic layer [Fig. 1(9)].

Discussion

The present study presents a method to maintain in vitro stock cultures of both *G. proliferum* and *G. intraradices*, using axenic *L. cruciata* plants (Marchantiales, Hepatophyta) as host. A similar methodology using an Anthocerotophyta plant was previously proposed by Schüßler (2000), but a major difficulty was met in the failure to obtain axenic in vitro cultures of *Anthoceros punctatus*. The use of liverworts as hosts for AMF necessitated (1) obtaining axenic cultures of potential host plants and (2) defining the appropriate culture conditions for AMF to grow in association with liverwort tissues. In vitro culture was more readily achieved for *M. polymorpha* than for *L. cruciata*, as the former was obtained from spores contained within intact sporangia, whereas the latter came from gemmae taken from open splash cups. To devise appropriate in vitro culture conditions, the apparently different requirements of the autotrophic liverworts and AMF had to be taken into account, as preliminary work had shown that differences in light intensity affect AMF spore germination, external hyphal growth and liverwort morphology. Thus, STP filter was used.

Both liverworts could be grown in vitro in the absence of added sucrose in the medium. However, as also found by Duckett et al. (2004), irradiance is much lower in the growth cabinet than in nature and gives rise to suboptimal growth. Thus, a sucrose supplement was required to compensate for inefficient photosynthesis. This effect is particularly evident for *L. cruciata*, even when plants are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◀ **Fig. 1** *1–9*: *Lunularia cruciata* cultured in 90-mm Petri dishes with AMF on SRV medium with 29.2 mM of sucrose. *1–2*: Forty-day-old cultures with *Glomus proliferum* and *G. intraradices*, respectively. *Squares* indicate location of the inoculum wells. *Arrow* points a portion of hyphae network with new formed spores imbedded in the medium. *3*: High magnification of *G. proliferum* spores. *4*: Mycothallus of *L. cruciata* cultured for 120 days with *G. proliferum* showing increase loss of photosynthetic surface and spread of brown patches (*hatched line*). *Arrow* points to a cluster of spores formed over the upper epidermis. *5–9*: Light microscopy, trypan blue-stained samples in transmitted light; *bars* in *1* and *2*: 10 mm; *3–6*: 500 μm; *7–9*: 50 μm. *5*, *6*: Anatomic sections of 120-day-old thalli colonised with *G. intraradices* and *G. proliferum*, respectively. *Black arrow* points to a meristematic apex. *White arrows* show arbuscules within thallus' midrib parenchyma of mycothallus. *7*: Detail of a zone of thallus with high concentration of oil cells containing septate hyphae (*black arrow*) and collapse arbuscule of *G. proliferum* (*white arrow*). *8*: Details of mycothallus of *L. cruciata* showing an oil cell (*black arrow*). *White arrow* points to a vesicle of *G. proliferum*. *9*: New spore of *G. proliferum* formed within the air chambers under the upper epidermis and above the photosynthetic layer (*arrow*)

grown at higher irradiance than that used in this study (data not shown). As *L. cruciata* mycothallus formation, external hyphal growth and spore production are demanding on plant resources, this further justified a sugar supplement in the culture medium. Composition of nutrient media is still a matter of debate for most liverwort species (Duckett et al. 2004). The SRV medium composition was designed to be as close as possible to MSR (Declerck et al. 1998) used for in vitro AMF cultures with transgenic roots, but the oligoelement composition was adjusted to take into account the requirements of the liverworts (Basile and Basile 1988). However, improvements to the medium may be possible as the optimal nutrient requirements for mycothalli of *L. cruciata* may be different from those in SRV. It is also necessary to evaluate the feasibility of the present method for other AMF.

Liverworts are complex thalloid plants with an internal anatomy that includes a photosynthetic layer under the upper epidermis in air chambers that open dorsally by air pores, an inner parenchymatous region rich in starch and a ventral zone with scales and numerous rhizoids. *G. intraradices* and *G. proliferum* widely colonised thallus tissue of *L. cruciata*, the exception of the meristematic apices where no fungal structures were observed. This is in agreement with observations in other liverworts (Carafa et al. 2003; Selosse 2005). Hyphae were also absent from rhizoids, as observed by Schüßler (2000) in *A. punctatus*. Conversely, other authors working with thalli collected directly from nature reported that rhizoids were the main site of colonisation by AMF (Carafa et al. 2003; Read et al. 2000; Russell and Bulman 2005; Turnau et al. 2005). This apparent difference in behaviour may be in part explained by the in vitro growth conditions of *L. cruciata* where plant and fungal tissues are uniformly exposed to light.

AMF colonisation of liverworts under natural conditions develops mainly through rhizoids in the soil. A high-frequency concentration of hyphae and arbuscules was found to be located in a zone close to the lower-epidermis (rhizoids) within the thallus' midrib parenchyma of *L. cruciata*. The AMF structures were described to occur in the central midrib in a zone close to the photosynthetic layer in *M. foliaceae* (Russell and Bulman 2005). Colonisation of *L. cruciata* by *G. intraradices* and *G. proliferum* appears to be intracellular, with hyphae crossing from cell to cell and branching into arbuscules or occasionally forming vesicles. This pattern could be consistent with a *Paris* type AMF colonisation (Gallaud 1905; Russell and Bulman 2005; van Aarle et al. 2005). In parenchyma zones where cell contents were frequently rich in oil bodies, septate hyphae and collapsed arbuscules were frequently observed, suggesting that there is an adverse environment for AMF development in these cells, or that arbuscule turnover is more rapid.

The profusion and ubiquity of sporulation in *L. cruciata* cultures suggest an advantage for inoculum production. Also, spores forming on the surface of the medium or over the thallus may be easily cropped and used as axenic inoculum for further studies.

The present work provides an approach to perform studies on the similarities of arbuscular mycorrhizae between liverworts and root-bearing plants, hence allowing identification of shared traits that have evolved within Embryophyta. It also provides possibilities to better understand the nature of the interaction between AMF and liverworts and to ascertain if these intimate associations are symbiotic, parasitic or saprophytic, a topic which today is still a matter of debate (Read et al. 2000). The phylogenetic distance of liverworts from tracheophytes (Selosse 2005) makes them a potential tool to probe mechanisms that allow AMF to establish symbiosis with plants, their comparative evolution and the nature of these relationships.

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