Mycorrhiza (2000) 10:15–21

ORIGINAL PAPER

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Glomus claroideum forms an arbuscular mycorrhiza-like symbiosis with the hornwort *Anthoceros punctatus*

Accepted: 11 January 2000

Abstract Two isolates (BEG31 and BEG23) of *Glomus claroideum* (Schenck & Smith emend. Walker & Vestberg) were investigated for ability to form arbuscular mycorrhiza-like symbioses with the hornwort *Anthoceros punctatus* (L.). Spores were transferred to a cellulose acetate filter on water agar and a small portion of an *Anthoceros* thallus was placed directly upon the spores. Light-microscope observations 20 days after inoculation revealed branched hyphae growing within the thallus. After 45 days, arbuscules and vesicles were studied by light- and electron-microscopy. After 60 days in water agar culture, the colonised *Anthoceros* thalli were transferred to a low-nutrient medium agar. Hyphae spread in the agar and newly formed spores were observed 5 weeks after the transfer. After 4 months, about 1000 spores were formed in each Petri dish. This is the first report of an experimentally established arbuscular mycorrhiza-like symbiosis between an identified fungus belonging to the Glomales and a bryophyte.

Key words *Anthoceros punctatus* · Arbuscular mycorrhiza · Bryophytes · *Glomus* · Ultrastructure

Introduction

The arbuscular mycorrhiza (AM) symbiosis plays a significant ecological role and is important economically particularly for low-input agriculture. However, one of the major problems in investigations of the physiology and genetics of AM fungi (AMF), which belong to the Glomales (Morton and Benny 1990), is their obligate symbiotic life. These fungi are usually cultured in association with a whole plant in open systems ('pot cultures'; Gilmore 1968), which are difficult to maintain free from contaminant organisms. Closed pot cultures can be used to avoid contamination (Walker and Vestberg 1994) and there are alternative methods: aeroponic culture (Hung and Sylvia 1988), compartment systems (Jakobsen 1994), glass bead culture (Redecker et al. 1995), and the well-defined root organ culture system (Mugnier and Mosse 1987).

It has been known for a long time that liverworts and hornworts form AM-like associations (Stahl 1949). The "fine endophyte" *Glomus tenuis* was reported to colonise liverworts (Turnau et al. 1999) and mosses (Rabatin 1980) in their natural habitats. However, this species is not well defined. No other glomalean endophyte of bryophytes has yet been identified or cultured. The aim of the present study was to investigate whether a glomalean fungus, *Glomus claroideum*, known to form AM with vascular plants is also able to form an AM-like symbiosis with the bryophyte *Anthoceros punctatus*.

To simplify matters, in this article the term AM is used in a broad sense for all symbioses between plants and AMF, although (rootless) bryophyte associations do not represent a "mycorrhiza" in a narrower sense. A simple culture system for bryophyte AM would be useful in AM research, for example with respect to establishing single-spore isolates, maintaining monoxenic cultures, or studying the physiology and development of the symbiosis.

Material and methods

All cultures were grown at 20 °C, with a 14/10 h light/dark rhythm, and a photosynthetic photon flux density of 80 μmol · $m^{-2} \cdot s^{-1}$.

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Plant cultures

Thalli of *A. punctatus* were harvested from a natural habitat in the Spessart mountains (Germany) in October 1994 and cleaned by repeated subculturing of small peripheral thallus pieces. These cultures were completely free of fungal contamination and of AM but contained unidentified, very slow-growing bacteria. The thallus cultures were grown on Knop medium solidified with 2% Bacto-Agar (Difco Laboratories) and overlaid with a cellophane foil. This facilitated easy dissection and transfer of samples. Subcultures were performed every 6 months.

AM cultures

The *G. claroideum* isolates used were earlier named *G. fistulosum* (Skou & Jakobsen). Recently four *Glomus* species, including *G. fistulosum*, were synonymised with *G. claroideum*, since they lack morphological differences (Walker and Vestberg 1998). Spores of the isolates BEG23 and BEG31 were obtained from Dr. Chris Walker (Biological Research and Imaging Laboratory, New Milton, UK). Ten culture plates for each isolate were prepared by filling 0.6% water Bacto-Agar into 5-cm plastic Petri dishes and, after gelling, overlaying the agar with wet, autoclaved 0.45-µm cellulose acetate filters (Millipore, Eschborn, Germany). Ten spores, cleaned by repeated washing in sterile double-distilled water, were transferred with a pipette to the middle of each culture plate and a piece of an *Anthoceros* thallus (about 1 cm in diameter) was placed directly upon them.

After 60 days on the cellulose acetate/water agar medium, the *Anthoceros* thalli were transferred to 9-cm Petri dishes on a low-nutrient medium solidified with 0.6% Bacto-Agar. The medium ("GM29") was composed of the macro-elements $Ca(NO_3)_2$, 0.2 mM; $MgSO_4$ and KCl each 0.1 mM; KH_2PO_4 and K_2HPO_4 each 0.01 mM; 2-[N-morpholino]ethanesulfonic acid (Mes), 1 mM and the trace-elements: $ZnSO_4$, 0.0077 µM; $MnCl_2$, 0.091 µM; H_3BO_3 1.6173 µM; $CoCl_2$, 0.0042 µM; Na_2MoO_4, 0.0082 µM; $CuSO_4$, 0.001 µM; $NaHSeO_3$, 0.001 µM; $NiCl_2$, 0.001 µM; Na_3VO_4, 0.001 µM; $K_2Cr_2O_7$, 0.0005 µM; $FeCl_3$, 1.165 µM; Na_2EDTA, 1.171 µM. Soil extract (0.1 ml/l) was added to the medium. This extract was prepared from a nutrient-poor soil taken from the natural habitat of *Anthoceros* by filling a 1-l flask with one third soil, then adding double-distilled water to a final volume of 0.5 l and boiling twice for 1 h within 24 h. The supernatant was decanted, paper-filtered twice and autoclaved (30 min) prior to storage at 4 °C. The final medium was titrated with KOH to a pH of 6.0 and autoclaved for 30 min.

Light microscopy

Thallus pieces were cut out of 20- and 45-day-old water agar cultures with a razor blade and cleared with 10% KOH in an autoclave at 121 °C for 10 min. They were then washed 3 times with water and incubated for 10 min in 3.7% HCl. The fungal structures were stained overnight with 0.05% trypanblue in lactophenol. After replacing the lactophenol by 50% lactic acid and incubating for 30 min, the roots were embedded in fresh 50% lactic acid and observed with a Zeiss IM35 inverted microscope.

Electron microscopy

Thallus pieces were cut out from 45-days-old cultures with a razor blade and fixed with 2.5% glutaraldehyde in 0.1 M HEPES buffer (pH 7.4) for 1 h. After rinsing, samples were postfixed with 2% OsO_4 in the same buffer for 1 h, dehydrated in an acetone series (10, 30, 50, 70, 90, 100, 100, 100%, each step 30 min), and infiltrated with a series of Spurr's resin (Spurr 1969). Samples were flat-embedded in small glass Petri dishes and polymerised at 60 °C. After removing the glass, infected parts of the thallus were identified microscopically, cut out and glued onto empty resin blocks. The samples were cut with a diamond knife on a Reichert-Jung Ultracut. Sections were post-stained with uranyl acetate and lead citrate and examined with a Philips CM 10 at 100 kV.

Results

Culture and spore production

After 60 days growth on water agar, plus 35 days growth on low nutrient agar, some spores were observed in the mycelium spreading within the agar (not shown). In 3-month-old cultures on low-nutrient agar, 800–1100 spores were formed in all the inoculated Petri dishes. The white spores were located in all planes of the agar, but less frequently close to the bryophyte thallus (Figs. 1, 2). Spores sometimes occurred in dense clusters (Fig. 1), but usually formed singly on the end of a hypha (Fig. 3). They were not different from those used for inoculation, except that the latter were more variable in size and for the most part slightly brownish in colour (Figs. 4, 5).

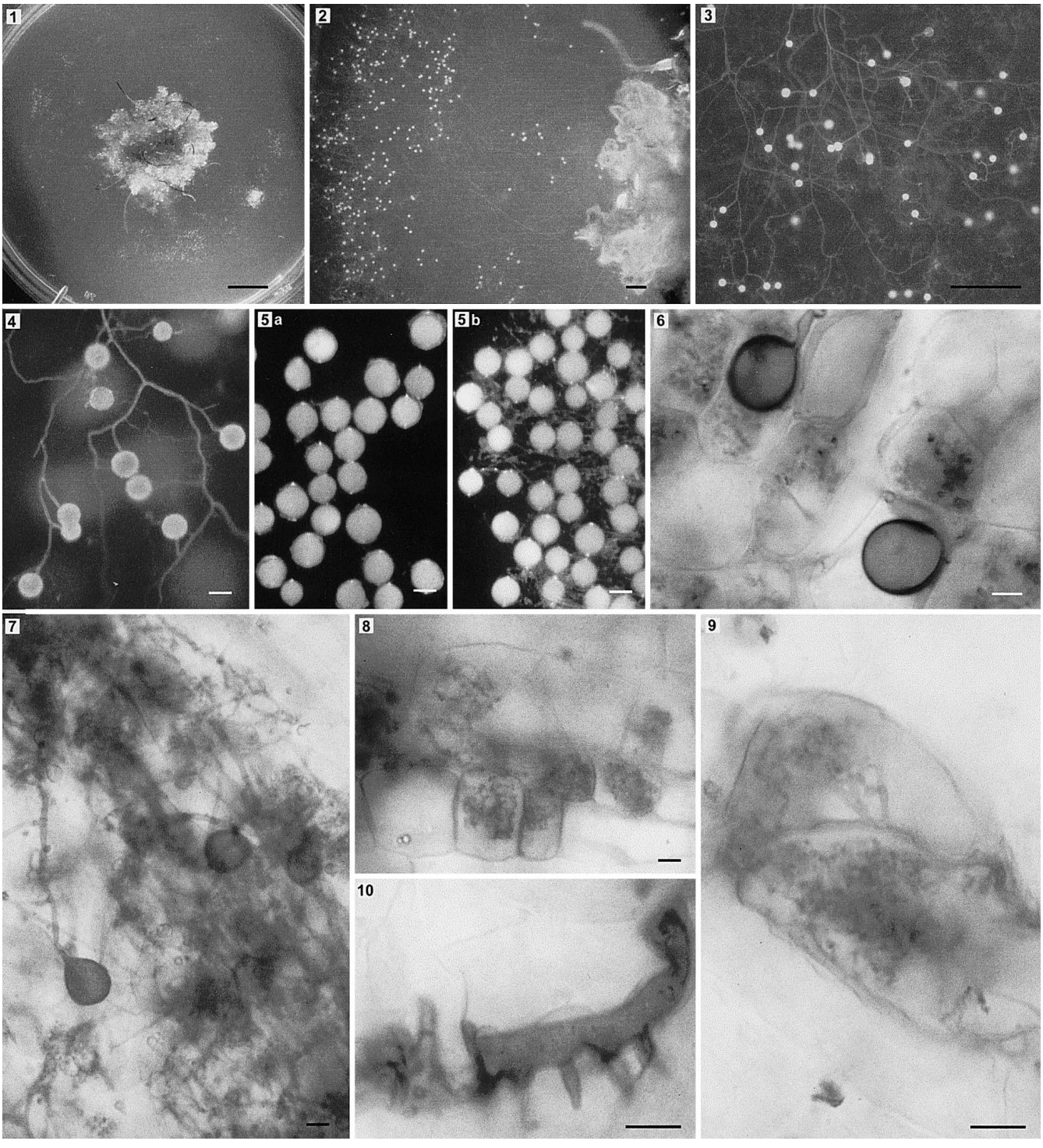
Trypan blue staining

Parts of the *A. punctatus* thalli harvested from the natural habitat showed an AM with vesicles and arbuscules within the thallus (Fig. 6). In laboratory cultures on agar medium described here, no differences were found between the AM formed by the BEG23 and BEG31 isolates. After 30 days growth on cellulose acetate/water agar, some branched hyphae were observed within the *Anthoceros* thalli (not shown). Fifteen days later, small parts of the thalli were colonised and arbuscules, vesicles and appressoria-like structures were observed (Figs. 7–10).

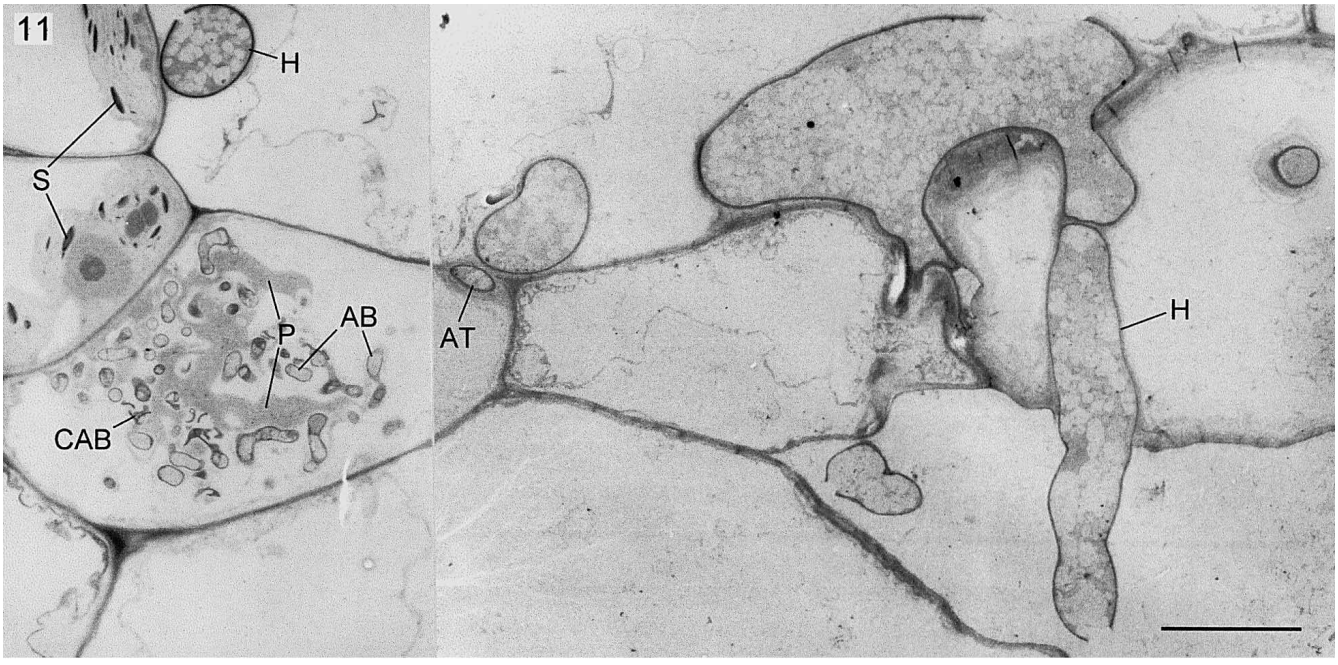
Electron microscopy

Two of the *G. claroideum* BEG23/*A. punctatus* cultures, grown for 45 days on cellulose acetate/water agar, were investigated by electron microscopy. In the flat-embedded specimens, infected parts of the thallus could be detected by the dark (osmiophilic) fungal structures. Hyphae, arbuscules and vesicles were identified in semi-thin sections. Intercellular hyphae growing within the thallus and hyphae penetrating *Anthoceros* cells, as well as an arbuscule, are shown in Fig. 11. The intercellular hyphae reached diameters of 10 µm, while branched arbuscule hyphae had diameters as low as 0.5 µm (Figs. 11, 12).

Numerous nuclei, electron-dense globules, mitochondria, and small vacuoles were present in the cytoplasm of hyphae growing within the *Anthoceros* thallus, as well as bacteria-like organisms (BLOs) which were not apparently surrounded by a host membrane (Figs. 12, 13).



Figs. 1–5 Light microscopy, dark-field illumination, *bars* Fig. 1 10 mm, Figs. 2, 3 1 mm, Figs. 4, 5 100 μm. **Fig. 1** *Anthoceros punctatus* thallus grown with *Glomus claroideum* BEG23 in a Petri dish for 5 months. About 1000 *G. claroideum* spores were formed within the agar. **Fig. 2** *A. punctatus*/*G. claroideum* BEG31 symbiosis, grown for 3 months, showing branched hyphae within the agar and a large number of fungal spores. **Fig. 3** Higher magnification of *G. claroideum* BEG31 spores in a 3-months-old culture. Fine, branched hyphae are also visible. **Fig. 4** *G. claroideum* BEG31 spores in a 5-months-old culture. **Fig. 5** *G. claroideum* BEG31 spores, oblique illumination: **a** used for inoculation, **b** harvested from an *A. punctatus*/*G. claroideum* agar culture. **Figs. 6–10.** Light microscopy, trypan blue-stained samples in transmitted light; *bars* 10 μm. **Fig. 6** AM in a field-collected *A. punctatus* thallus, showing vesicles and arbuscules. **Fig. 7** AM formed by *A. punctatus* and *G. claroideum* BEG31, showing vesicles and arbuscules. **Fig. 8** Arbuscules formed by *G. claroideum* BEG31 within the *A. punctatus* thallus. **Fig. 9** High magnification of a *G. claroideum* BEG31 arbuscule in *A. punctatus*. **Fig. 10** An appressorium formed by *G. claroideum* BEG23 in the *A. punctatus* AM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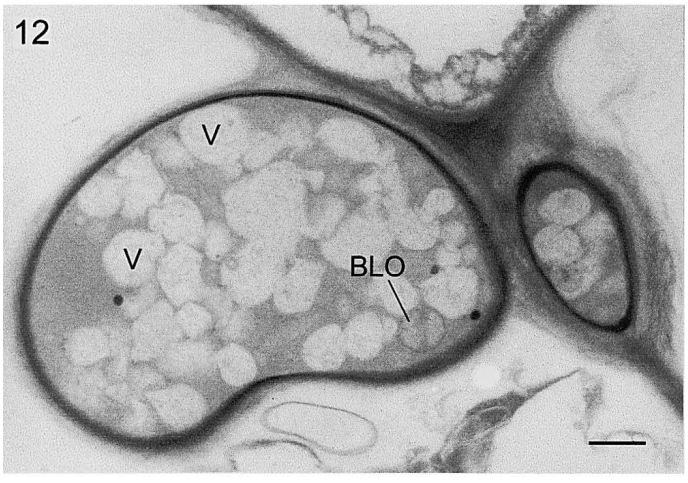
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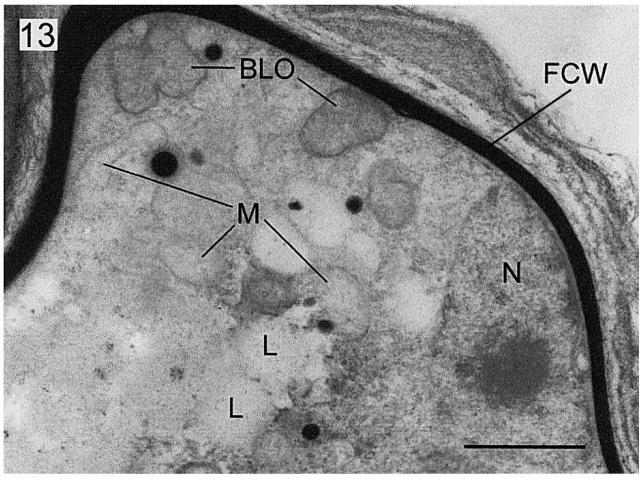
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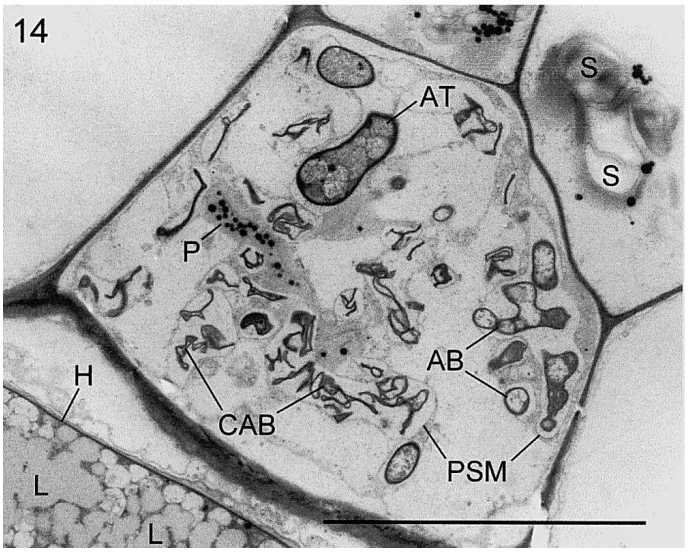
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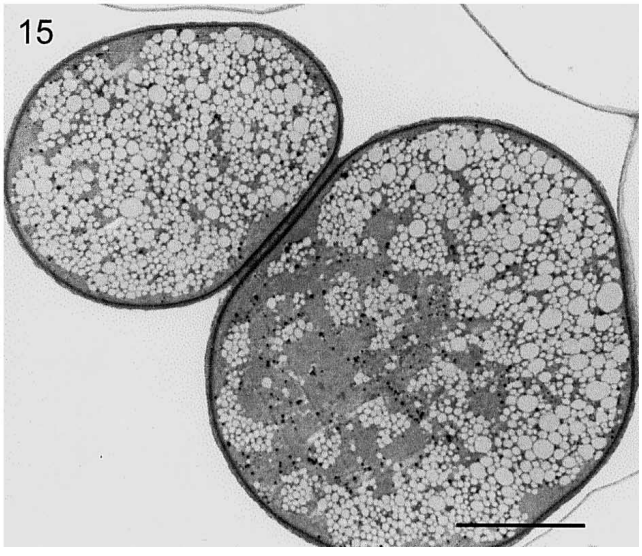
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Figs. 11–15 Electron microscopy of *A. punctatus*/*G. claroideum* BEG23 AM in 45-days-old water agar cultures; *bars* Figs. 11, 14, 15 10 μm, Figs. 12, 13 1 μm

Fig. 11 Hyphae (*H*) growing within the *A. punctatus* thallus. One *Anthoceros* cell containing an arbuscule is seen at the left. Plastids (*P*) within non-colonised cells contain starch grains (*S*), in contrast to plastids within cells containing arbuscular branches (*AB*) and collapsed arbuscular branches (*CAB*). The plant cell wall is loosened where penetrated by the arbuscular trunk (*AT*)

Fig. 12 Higher magnification of the region shown in the middle of Fig. 11. The hypha contains a bacterium-like organism (*BLO*) and small vacuoles (*V*)

Fig. 13 Part of a fungal hypha growing between *Anthoceros* cells. BLOs, a nucleus (*N*), lipid droplets (*L*), and fungal mitochondria (*M*) are visible. The fungal cell wall (*FCW*) appears dark (osmiophilic)

Fig. 14 *Anthoceros* cell, containing a partly disintegrated arbuscule. Arbuscular trunk (*AT*), arbuscular branches (*AB*), and collapsed arbuscular branches (*CAB*) are seen. The plastid (*P*) within the colonised cell contains many plastoglobuli but no starch. Plastids in the neighbouring cells contain large starch granules (*S*). The arbuscular branches are enclosed by a perisymbiotic membrane (*PSM*). Note also the thick hypha (*H*) outside the cell, containing many lipid droplets (*L*)

Fig. 15 Two young vesicles formed within the *Anthoceros* thallus

Figure 14 shows a partly disintegrated arbuscule with a large trunk and cell-wall remnants of collapsed hyphae. Fungal structures were enclosed by a perisymbiotic host membrane. Cells containing arbuscules showed a fragmented vacuole and plastids with plastoglobuli but without large starch grains. In contrast, cells not colonised by the fungus had a large central vacuole and contain plastids with large starch grains (Figs. 11, 14). Young vesicles, shown in Fig. 15, contained many lipid droplets and nuclei, and were 30–40 μm in diameter. No fungal structures were observed within the *Anthoceros* rhizoids.

Discussion

Most ferns (Gemma et al. 1992) and some lycopods (Duckett and Ligrone 1992; Schmid and Oberwinkler 1993) form AM. In bryophytes, AM have been reported for hepatics (Duckett and Read 1991; Ligrone and Lopes 1989; Pocock and Duckett 1984, 1985; Stahl 1949; Turnau et al. 1999), hornworts (Ligrone 1988), and mosses (Parke and Linderman 1980; Rabatin 1980). However, it is not always clear whether the fungus participating in the symbiosis indeed belongs to the Glomales, e.g. the fungi symbiotic in *Lycopodium clavatum* and in the achlorophyllous gametophyte of the fern *Psilotum nudum* form relatively small vesicles and no arbuscules (Peterson et al. 1981; Schmid and Oberwinkler 1993). For the AM formed by mosses, it was considered that the fungus probably colonises senescent plant material and that mutualism is unlikely (Ligrone 1988).

With this *A. punctatus*/*G. claroideum* co-culture, an AM between an AMF known to form symbioses with higher plants and a bryophyte is described for the first time. Although not investigated in detail, the time-course of the *A. punctatus*/*G. claroideum* AM establishment seems to be similar to AM formation in vascular plants. When inoculated with 10 spores, some hyphae could be seen within the *Anthoceros* thallus after 30 days. After 45 days, parts of the thalli were colonised and arbuscules as well as vesicles were present. It was possible to establish a complete life cycle of *G. claroideum* in a simple Petri dish agar culture, resulting in the formation of a large number of spores within 3 months. They were located in all planes of the agar and sometimes formed dense clusters, indicating that light did not inhibit spore formation. The mature spores looked similar to those used for inoculation, except that the latter were slightly brownish in colour and more irregular in size. Both features are probably due to the different substrates.

Electron microscope investigations show that the hyphae colonise the thallus by growing through intercellular spaces or by penetrating dead *Anthoceros* cells. The death of these cells is probably not caused by the fungus, since parts of non-growing *Anthoceros* thallus regions are often senescent (Ligrone 1988). Hyphae also penetrate living plant cells to form arbuscules. Degenerating arbuscules with collapsed hyphae were found in the 45-days-old cultures, indicating a short lifetime for these structures. The low amount of starch in the plastids of cells containing arbuscules also reflects the situation of vascular plant AM (Bonfante 1984) and of the lycopod and bryophyte AM cited above. Vesicles were not abundant after 45 days culture and the vesicles investigated electron microscopically were relatively young.

BLOs with a diameter of 0.5–0.7 μm were present in the *G. claroideum* hyphae. They have the same ultrastructural features as BLOs described in numerous AMF (Scannerini and Bonfante 1992) and in undetermined fungi forming AM with hepatics (Ligrone and Lopes 1989), hornworts (Ligrone 1988), and *Lycopodium clavatum* (Schmid and Oberwinkler 1993). The BLOs in *G. claroideum* did not seem to be enclosed by a host membrane, although BLOs enclosed by a host membrane and belonging to the group II pseudomonads (Bianciotto et al. 1996) have been reported in *Gigaspora margarita*, investigated with high-pressure freezing methods.

The bryophyte AM is also interesting with respect to the hypothesis that symbiotic association with AMF was a primary event during the evolution of land plants (Pirozynski and Malloch 1975). This is supported by fossil reports (Remy et al. 1994) and DNA-sequence analyses (Gehrig et al. 1996; Redecker et al. 1999; Schüßler 1999; Simon et al. 1993). The spectrum of photoautotrophic organisms forming symbioses with glomalean fungi is wide, ranging from vascular plants

to hornworts and even to cyanobacteria in the case of *Geosiphon pyriforme* (Schüßler and Kluge 1999; Schüßler et al. 1994).

Flavonoids and isoflavonoids have been shown to influence AM formation in several studies (e.g., Chabot et al. 1992) and have, therefore, been considered to play a signalling role, as in the *Rhizobium*-legume symbiosis. However maize mutants deficient in chalcone synthase activity (and thus in flavonoid biosynthesis) form a normal AM, suggesting that flavonoids are not crucial to AM formation (Bécard et al. 1995). In this context, it is interesting to note that no flavonoids have been found in hornworts, in contrast to other bryophytes (Becker 1994; Becker, personal communication).

Bryophyte AM could be an alternative to the well-defined root organ cultures (Bécard and Piché 1992; Diop et al. 1994; Mugnier and Mosse 1987). However, we have not yet established monoxenic cultures and further experiments are needed to show whether the *A. punctatus*/*G. claroideum* AM develops without the bacteria present in the cultures. If this is the case, then the bryophyte AM could be a useful tool for investigating the biology of AM symbioses and for establishing in vitro spore cultures.

Acknowledgements I am grateful to Dr. Chris Walker for providing the BEG23 and BEG31 cultures and to the Deutsche Forschungsgemeinschaft for supporting part of this work (SFB199).

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