Comparison of development of axenic cultures of five species of lichen-forming fungi

María del CARMEN MOLINA and Ana CRESPO

Departamento de Biología Vegetal II, Facultad de Farmacia, Universidad Complutense, Madrid 28040, Spain.

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Although several new techniques for isolating and culturing lichens have been reported in recent years, there is little information available concerning the variability in development of species from different taxonomic groups. A comparison of the early stages of the germination and growth of *Xanthoria parietina* (*Teloschistaceae*), *Parmelia saxatilis* (*Parmeliaceae*), *Physconia distorta* and two *Diplotomma* species (*Physciaceae*) was performed on two different media: one inorganic, and the other containing 4% glucose (LBM; Lilly-Barnett medium). Different germination and development rates, and isolation success rate (single- and multispore cultures) are reported. The new combination *Diplotomma rivasmartinezii* (Barreno & A. Crespo) Barreno & A. Crespo is made.

INTRODUCTION

The lichen-forming fungi constitute approximately 46% of all known ascomycetes, and about one fifth of all known fungi (Hawksworth 1988). Experimental attempts to culture lichen fungi have a long tradition of failure or limited success (Ahmadjian 1993, Ahmadjian *et al*. 1980). According to Crittenden *et al*. (1995), the reasons for the failure to obtain isolates from ascospores can be ranked as follows: failure of discharged ascospores to germinate (43%); non-discharge of ascospores (30%); germination of ascospores but subsequent growth not sustained (19%); and heavy fungal and/or bacterial contamination of discharged ascospores (8%). Moreover, compared with many free-living fungi, lichen-forming fungi grow more slowly and have complex nutrient requirements (Stocker-Wörgötten 1995).

In spite of these difficulties, the interest in culture of lichen fungi has increased in the last decade for a number of reasons. Lichen-forming fungi produce a wide range of secondary metabolites (Vicente 1991) that are involved in discrete metabolic processes (Mateos *et al*. 1993), many of which are unique to lichens. Furthermore, some of these compounds exhibit antimicrobial activity (Xavier Filho *et al*. 1990) or have other biological applications (Higuchi *et al*. 1993). Several of these lichen-derived substances may be obtained from axenic culture of the mycobiont (Hamada & Miyagawa 1995, Hamada *et al*. 1996), although the metabolites may differ from those found in the lichen itself (Hamada 1993).

Although significant numbers of lichen fungi have been successfully isolated and cultured (Crittenden *et al*. 1995), there is limited information concerning the developmental variability of species from different taxonomic groups (see Lallement 1985). Here we describe the development in axenic conditions of lichen fungi in three different families: *Teloschistaceae*, *Parmeliaceae* and *Physciaceae*.

MATERIAL AND METHODS

Lichen material

Details of the material, substrate, and area of collection are presented in Table 1. Thalli were used immediately after collection.

Isolation and culture of lichen mycobionts

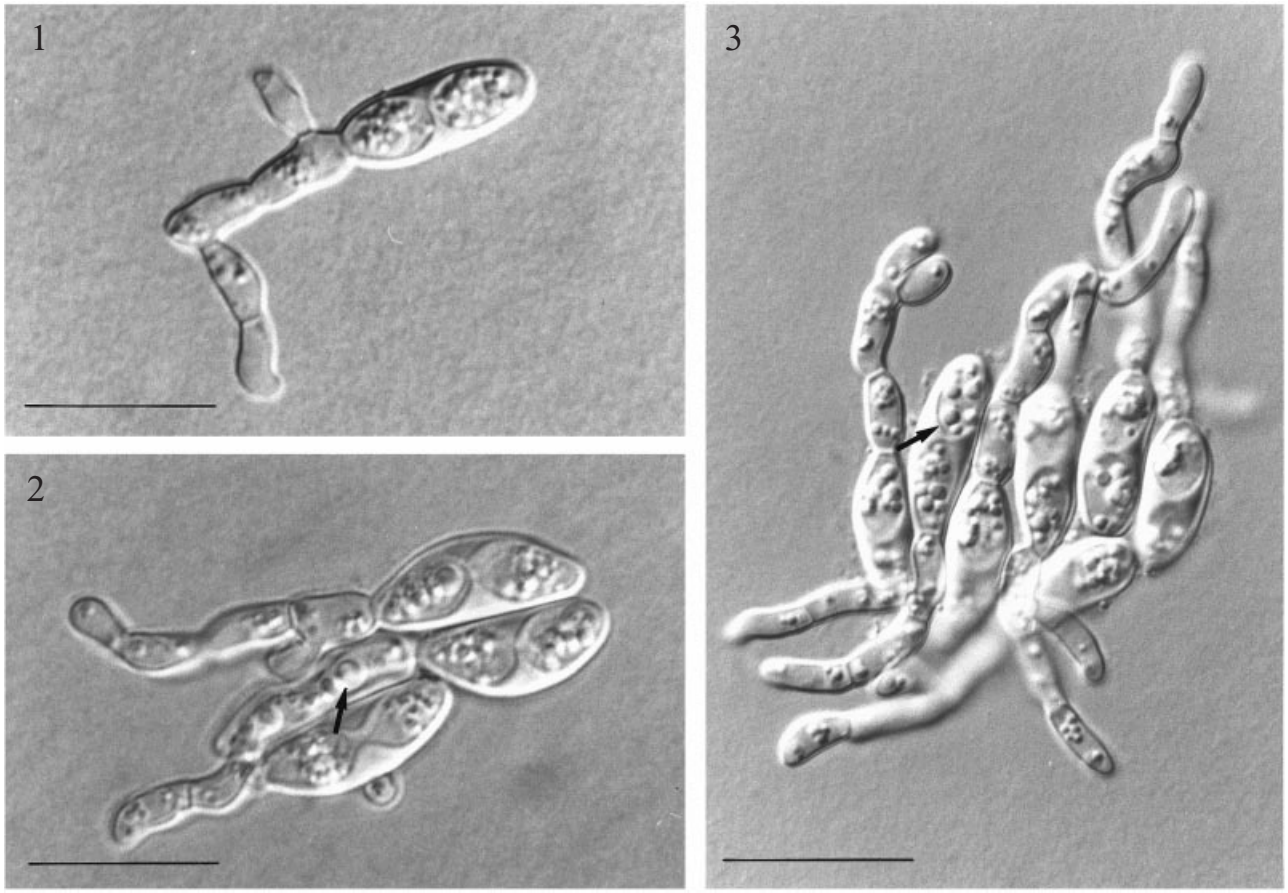
Isolated multispores were obtained from apothecia of the lichen-forming fungi. The fungi were grown from discharged spores following Ahmadjian (1993). Fruit-bodies were washed for 30 min in phosphate-buffered saline (PBS) including Tween 80 0.01% (v/v), according to the method used by Bubrick & Galun (1986). Apothecia were then soaked in double-distilled water for 1 h, changing the water several times during that period. Finally, they were carefully cleaned under a lens (Huber *et al*. 1994).

Clean ascomata were attached to the innerside of the lids of Petri dish with petroleum jelly. The other halves of the Petri dishes, containing Basal Bold Medium (BBM; Deason & Bold 1960) were then inverted over the lids and the ascospores allowed to discharge upwards onto the medium.

After germination, uncontaminated multispore or single spore isolates were transferred to 4% glucose LBM (Lilly &

Table 1. Plant material, substrate and area of collection

	Substrate	Area	Date
Xanthoria parietina	*Populus* sp.	Castillodel Olmo, Toledo	October 1998
Physconia distorta	*Quercus pyrenaica*	El Escorial, Madrid	November 1998
Diplotomma epipolium	Calciferous rock	Villalazán, Zamora	September 1998
Diplotomma rivasmartinezii	Gypsiferous rock	Morata, Madrid	February 1999
Parmelia saxatilis	Granite	Arganda del Rey, Madrid	April 1999



Figs 1–3. Germination of *Xanthoria parietina* grown in axenic conditions. **Fig. 1.** Bipolar, naked ascospore after germination. **Fig. 2.** Ascospore group contained lipid drops after germination (arrows). **Fig. 3.** Eight ascospore group after 5 d on inorganic medium (BBM). Bars = 15 μm.

Barnett 1951) modified by Lallemant (1985). Cultures were incubated at 18–20 °C in the dark. Periodically, mycobionts were examined using a Leitz DMR light microscope. For photography, an automatic ring flash system was fixed to the camera lens.

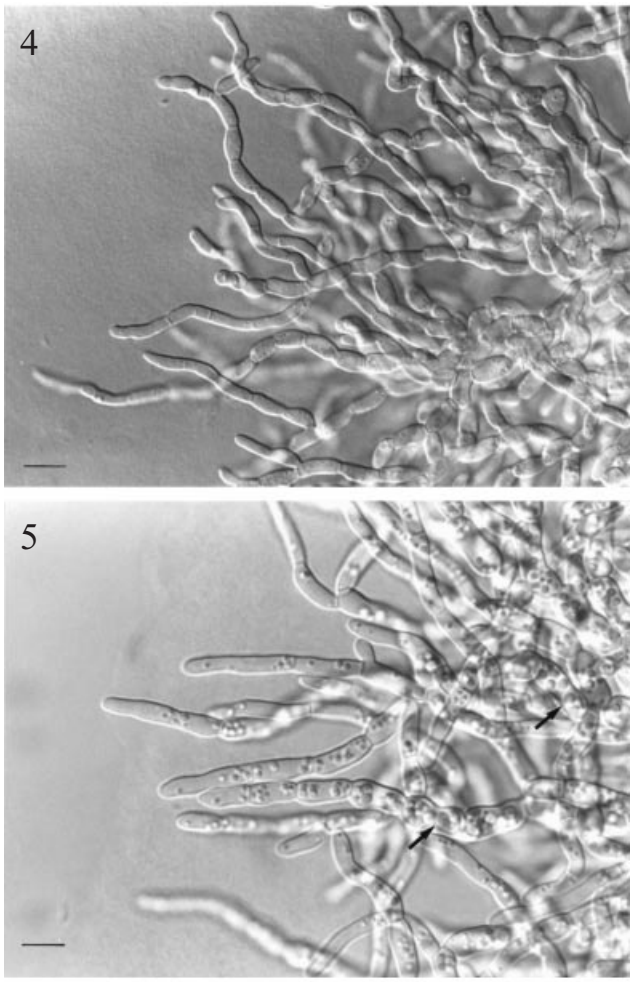
RESULTS

Xanthoria parietina

This species produced a large quantity of bipolar, naked ascospores (Figs 1–3), which were ejected upwards onto the surface of the medium during the first 8 h. After 3 days germination was observed, generally in groups of eight ascospores. These spores contained lipid drops that were visible during the initial developmental stages (Figs 2–3). However, a weak and superficial radial mycelium developed if the fungus was maintained on the inorganic medium for 1 mo (Fig. 4). When the fungus was transferred to 4% glucose LBM 15 days after germination, the diameter of the hyphae was greater than that of those grown on inorganic medium (Fig. 5). Moreover, an increase in lipid concentration was observed. In this case, the radial development rate was 8.3 mm mo^{-1}. The anatomical structure after three months was according to Lallemant's (1985) observations, but the development rate was slower in our cultures.

Physconia distorta

Apothecia ejected subglobular and uniseptate spores upwards; these had a brown external ornamented capsule (Fig. 6) that was eliminated after germination. Occasionally, the capsules were observed broken on the mycelium (Figs 7 and 10). In general, meiotic products that appeared after sporulation in BBM were detected as groups of 2–3 spores or as isolated



Figs 4–5. Development stages of *Xanthoria parietina*. **Fig. 4.** Detail of fungus grown on BBM for 1 mo. **Fig. 5.** On 4% glucose LBM 15 d after germination. Arrows show lipid drops. Bars = 15 µm.

spores. Degenerated or poorly developed spores were frequently found together with germinated spores (Fig. 8).

Ejection occurred during the first 12 h. Germination could be observed after 6–7 days, after which a radial mycelium was generated. After one month, a poor mycelium with long intersepta and few lipid drops had grown on the surface of the inorganic medium (Fig. 9). However, if the fungus was transferred to 4% glucose LBM 15 days after germination, considerable differences were observed: fattened and globular intersepta, numerous lipid drops, and a considerable synthesis of secondary metabolites, which gave rise to the dark thallus-like colonies that subsequently developed (Figs 10–11).

After two months, 57% (32 of 56) of the single spores had successfully yielded isolates. All plurisporic isolates were successful in yielding isolates (61 of 61). The development rate of this kind of culture was 5 mm mo^{-1}.

Diplotomma species

Apothecia produced long, dark-coloured ellipsoid spores (Figs 12 and 15). Both *D. epipolium* and *D. rivasmartinezii*[1] ejected 7- or 8-spored groups (Figs 14–16). This, together with the small size of the spores and some bacterial contamination explains the failure to isolate single-spore cultures. Both species had an external layer which shed progressively without loss it after germination (Figs 13–14, 16–17).

The morphology and dimensions of the spores were moderately divergent in the *Diplotomma* species (Barreno & Crespo 1977): diaspore productivity differed markedly: 16.0 spore groups per apothecium from *D. epipolium* spores, and 9.4 from *D. rivasmartinezii*. *D. epipolium* germinated 10 days after sporulation (Fig. 13), while those of *D. rivasmartinezii* took 5 d longer to do so (Figs 16–17). Moreover, all spores from the plurisporic isolates of *D. epipolium* germinated in the early stages (Fig. 13), whilst only one or two spores germinated from *D. rivasmartinezii* over the same period (Fig. 17). Surprisingly, in the latter case, a mycelium with a 95% (123 of 130) success rate was observed 5 mo after initial germination on inorganic medium.

Finally, *D. epipolium* had a grey mycelium with a development rate of 0.4 mm mo^{-1} when the initial plurisporic isolates were transferred to the organic medium (LBM) shortly after germination. In this case, the success rate was 100% (23 of 23). However, those of *D. rivasmartinezii* almost always died.

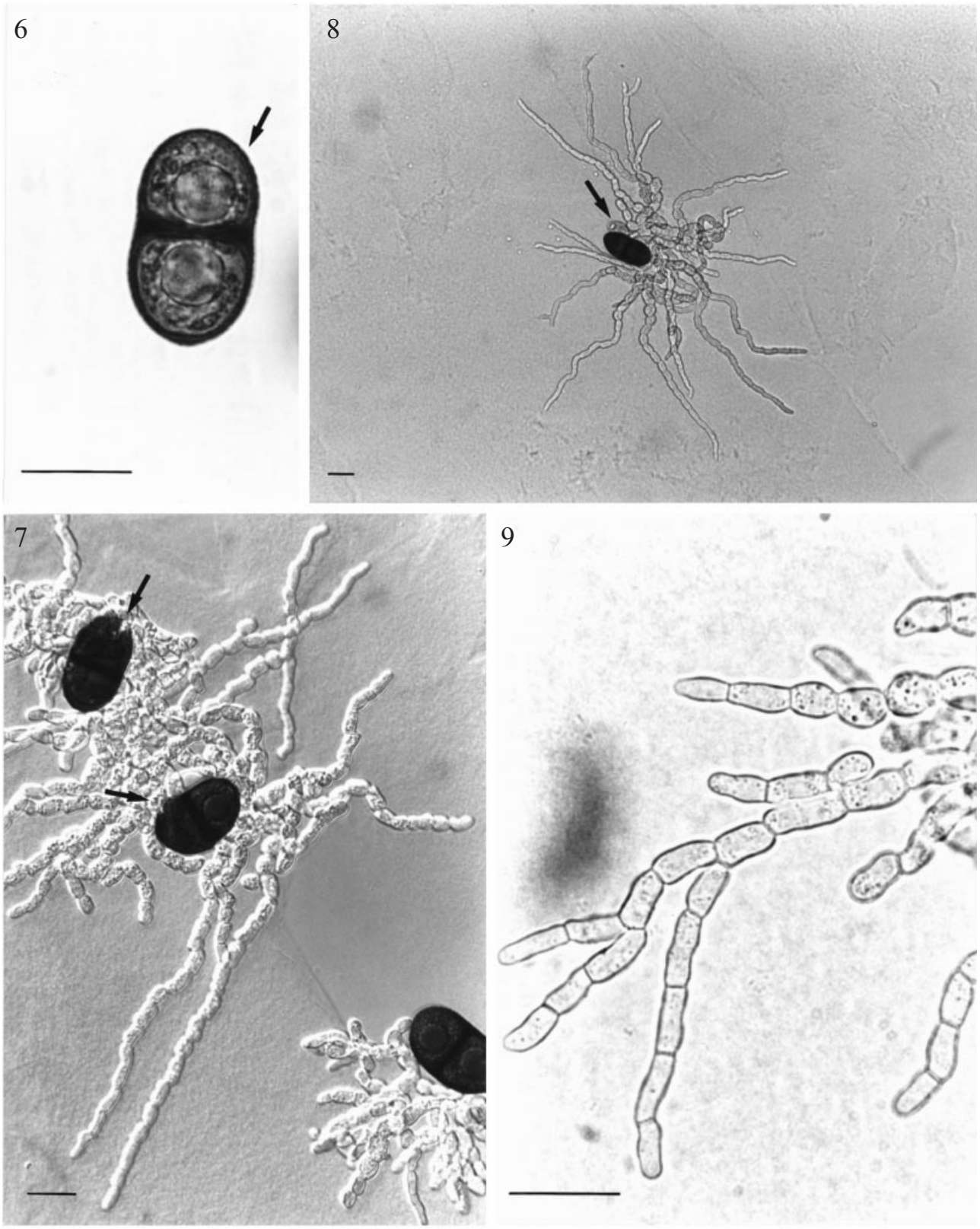
Parmelia saxatilis

This species had naked, globular spores with thickened walls (Fig. 18). They took 15–20 d to germinate and the first hyphae sometimes appeared on both sides of the spore (Fig. 19). Ascospores were observed as groups of four and eight spores, giving rise to a poor, superficial mycelium with long intersepta (Figs 20, 21). When transferred to 4% glucose LBM, monosporic isolates had a success rate of 47.8% (11 of 23). Multisporic cultures developed slowly (less than 1 mm diam in 3 mo), but isolates were obtained in 84% (26 of 31) of the cases.

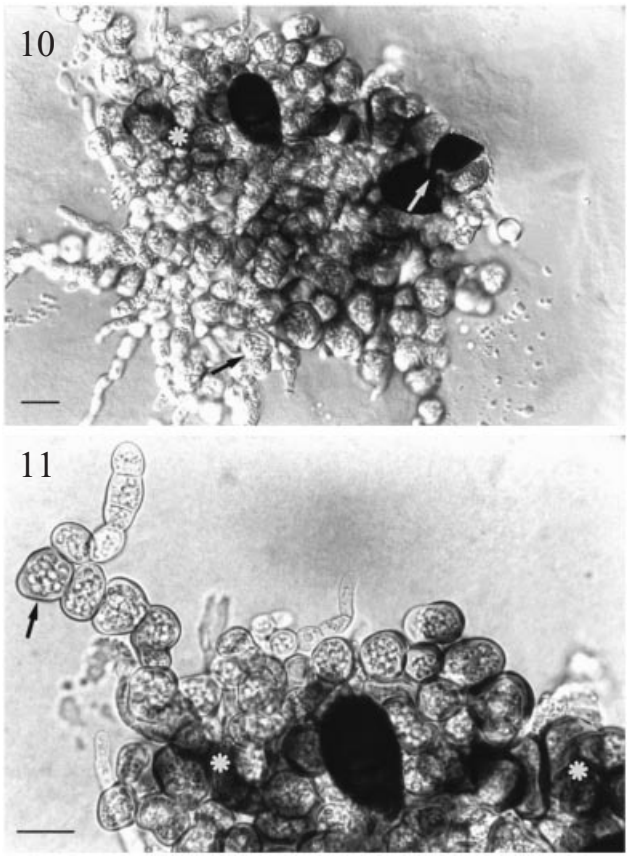
DISCUSSION

Each spore was initially as described (Figs 1, 6, 12, 15 and 18) in Clauzade & Roux (1985) and Purvis *et al.* (1992). Moreover, on nutrient media, all lichen fungi investigated formed cartilaginous, thallus-like colonies with filamentous cells at the periphery, and swollen, almost globose cells with very compact central parts (Figs 5, 9 and 21), as described previously by Honneger *et al.* (1993). There is little information available concerning the ontogenetic variability of species from different taxonomic groups, although the ontogenic development of species differs greatly. On the whole, it appears that the fungal partner requires a carbon source in order to generate a three dimensional structure (Hamada & Miyagawa 1995, Huber *et al.* 1994, Molina *et al.* 1997*a*). Nevertheless, *D. rivasmartinezii* had a mycelium with a 95% success rate 5 mo after initial germination on BBM, whereas in most cases it died during the first month on enriched medium; this might explain why this fungus requires a special culture medium.

[1] **Diplotomma rivasmartinezii** (Barreno & A. Crespo) Barreno & A. Crespo **comb. nov.** Basionym: *Buellia rivasmartinezii* Barreno & A. Crespo, in Follmann & Crespo, *Philippia* **2**: 283 (1975).



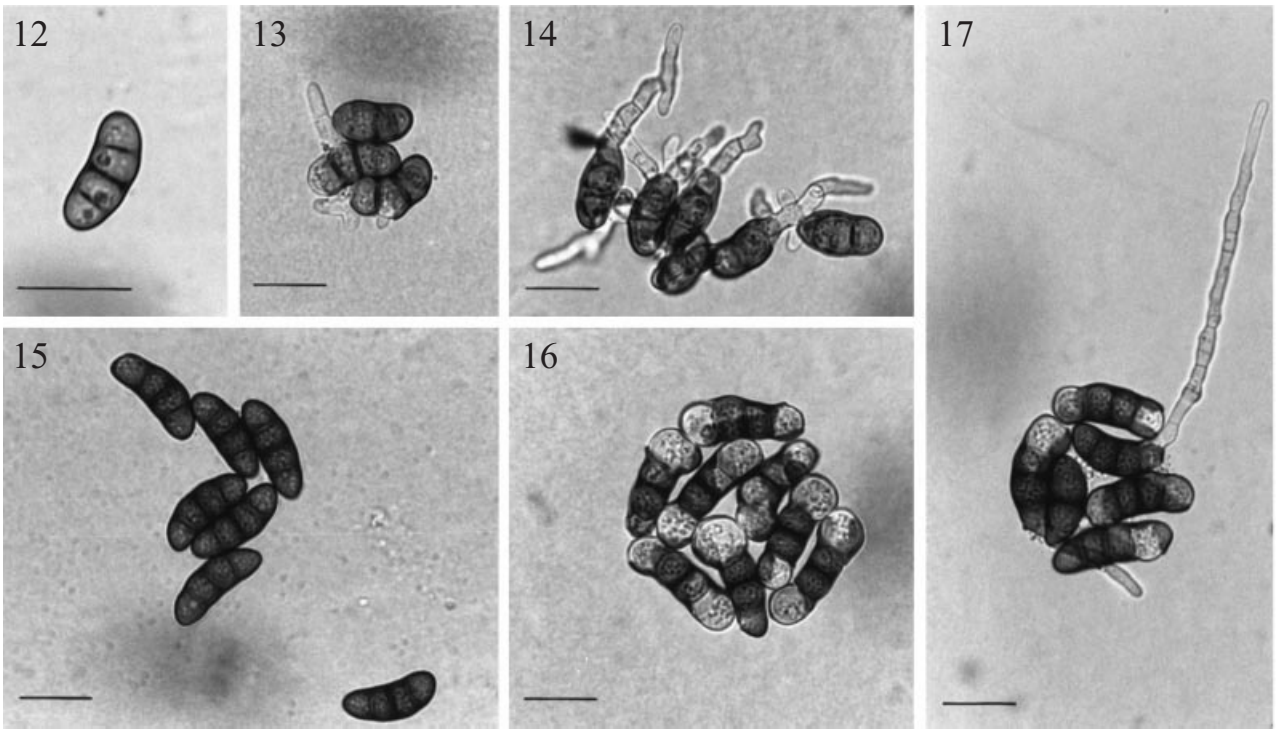
Figs 6–9. Development stages of *Physconia distorta* grown in axenic conditions. **Fig. 6.** Subglobular and uniseptate ascospore with external ornamented capsule (arrow). **Fig. 7.** Poor superficial mycelium after 1 mo on inorganic medium (BBM); arrows show broken external capsules. **Fig. 8.** Degenerated or poorly developed spores (arrow). **Fig. 9.** Mycelium with long intersepta and few lipid drops. Bars = 15 μm.



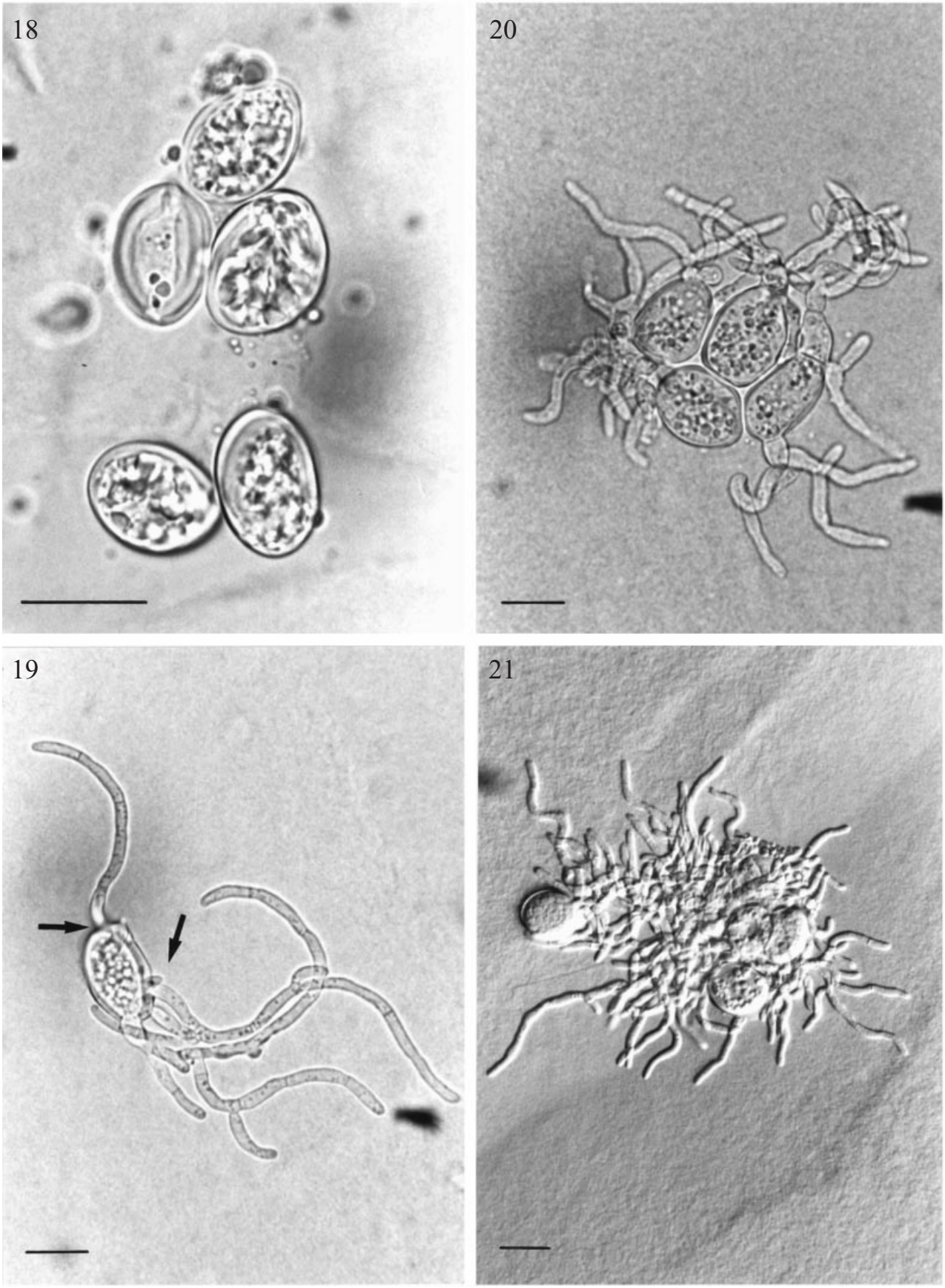
Figs 10–11. Development stages of *Physconia distorta* grown on 4% glucose LBM. **Fig. 10.** Plurisporic culture with secondary metabolites (white arrow) and broken ascospores (black arrow). **Fig. 11.** Detail of single ascospore culture. Arrows show lipid drops. Bars = 15 μm.

Additionally, the growth on organic medium appears to be necessary to increase the production of secondary metabolites (Hamada 1996, Hamada *et al*. 1996) and polyols (Honneger *et al*. 1993). This circumstance might explain the synthesis of secondary metabolites in the first stages of *Physconia distorta* cultures on the enriched medium (Figs 4–5). Secondary metabolites were not detected in fungal partners at the same growth stage when cultured on inorganic medium.

Likewise, *Xanthoria parietina* produces parietin on enriched media (Honneger & Kutasi 1990). Even when the fungal partner was incubated on 4% glucose LBM it produced this anthraquinone over a long period (Pereyra *et al*. 1996). Although ribitol has been recommended as the carbon source to be used in the medium because it is the polyol transferred as carbohydrate in the majority of lichens containing a green alga (Galun 1988, Honneger *et al*. 1993), *X. parietina* and *P. distorta* grew successfully on the glucose medium (a more economical medium). However, these results cannot be considered to be generally applicable since *Diplotomma*, which belongs to the same family as *P. distorta* (i.e. *Physciaceae*), had a slower ontogenetic development, especially in *D. rivas-martinezii*. Interestingly, the related *Buellia stellulata* has been successfully cultured in axenic conditions using media containing a large amount of added sucrose or ethylene glycol (Hamada 1996). On the other hand, some authors have synonymized these *Diplotomma* species on morphological features (Clauzade & Roux 1985). However, the behaviour of *D. epipolium* (Figs 12–14) and *D. rivasmartinezii* (Figs 15–17) growing on the same organic medium is completely different. For this reason, others studies will be necessary to support the view that both names could be considered as belonging to a single species.



Figs 12–17. Ejection and germination of ellipsoid ascospores from *D. epipolium* (**Figs 12–14**) and *D. rivasmartinezii* (**Figs 15–17**). **Figs 12** and **15.** Ascospores recently ejected. **Figs 13** and **16.** Ascospores after 10 d on BBM. **Figs 14** and **17.** Ascospores after 15 d on BBM. Bars = 15 μm.



Figs 18–21. Ejection and germination of globular, naked ascospores from *Parmelia saxatilis*. **Fig. 18.** Ascospores recently discharged. **Fig. 19.** Germination 15–20 d after discharge; arrows show as the first hyphae appear on both sides of spore. **Fig. 20.** Group of ascospores. **Fig. 21.** Poor and superficial mycelium with long intersepta. Bars = 15 μm.

In contrast, the success rate for single-spore isolations in *Xanthoria parietina* was 100%. This fungus developed faster (8.3 mm mo^{-1}). However, representatives of the *Telochistaceae* had a 39% success rate in the study of Crittender *et al*. (1995), measured as the number of species successfully isolated as a proportion of the total number of species attempted.

The family *Parmeliaceae* yielded 47% of successful isolates in Crittenden *et al*. (1995). The most frequent reason for unsuccessful isolations in that programme was the failure of the ascospores to germinate on an agar medium. *Parmelia omphalodes* was an example of this. However, *Parmelia acetabulum* germinated within 10 days when grown on BBM, and also grew on glucose and malt extract medium (Honneger *et al*. 1993), rather as we found with *P. saxatilis*. This may have been because the germination medium contained only agar, and no mineral compounds, vitamins, or other additives. Nevertheless, the growth of this fungus was slow recalling the *Diplotomma*'s, probably because it has more complex nutrient requirements.

The developmental rate of the species can be ranked in decreasing order as follows: *X. parietina* (8.3 mm mo^{-1}) > *P. distorta* (5 mm mo^{-1}) > *D. epipolium* (0.4 mm mo^{-1}) > *P. saxatilis* (0.16 mm mo^{-1}) > *D. rivasmartinezii* (0 mm mo^{-1}). Likewise, multisporic culture success rates are: *X. parietina* (100%) = *P. distorta* (100%) > *D. epipolium* (95%) > *P. saxatilis* (84%) > *D. rivasmartinezii* (0%). With regard to single spore cultures, the ranking was: *X. parietina* (100%) > *P. distorta* (57%) > *P. saxatilis* (47%). These differences in development in aposymbiotic conditions of fungi from different groups are not surprising since a great variety of fungi are involved in lichen symbioses and the polyols released by green algae may be different (Stocker-Wörgötten 1995). On the other hand, environmental factors such as water availability (Crittenden *et al*. 1995) and the physiological state of the lichen at the time of collection, for example the degree of apothecium maturation (Molina *et al*. 1997a), may determine the viability of spores.

It generally seems to be the case that successful monosporic isolates are more difficult than plurisporic ones, as ejection in the plurisporic group ensures the growth of at least one meiotic product. Moreover, since the mycelium seems to develop from several ascospores, it is possible that thalli are formed from different genotypes. This hypothesis might explain the great variability observed at the morphological (Clauzade & Roux 1985), physiological (Molina *et al*. 1997b), and genetical (Fahselt 1987, Hageman & Fahselt 1990, Reyes *et al*. 1996) levels in lichen-forming fungi.

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Corresponding Editor: *D. L. Hawksworth*