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Experimental Microbiology of Lichens: Mycelia Fragmentation, A Novel Growth Chamber, and the Origins of Thallus Differentiation

DANIELE ARMALEO
Department of Botany, Duke University, Durham, NC 27706, USA
Tel. 919-6843868, Fax 919 684 5412

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Abstract

Two methods are described to simplify and improve the laboratory manipulations of lichens and of their isolated fungal symbionts (mycobionts). The first allows fragmentation of cultured mycobiont mycelia to generate colony-forming units. Microbiological procedures requiring large numbers of single colonies (e.g. mutagenesis, mutant selection, genetic transformation) can thus be applied to isolated lichen fungi, which do not produce vegetative or sexual spores in culture. The second method involves a simple lichen growth chamber which readily permits control of nutrient, moisture, and air supply to the thallus on an artificial substrate. The observations on *Cladonia grayi* soredia developing in this chamber are the basis for a working hypothesis on lichen morphogenesis. Early lichen development is schematized as the biological sum of the growth patterns of the isolated fungus and the isolated alga. The region of direct and intimate contact between a fungal hypha and an algal cell is thought to generate the differentiation into medulla, algal layer and cortex typical of most lichens.

Keywords: lichen culture, mycobiont culture, *Cladonia grayi*, morphogenesis

1. Introduction

The routine manipulation of the lichen life cycle in the laboratory continues to be a challenge, despite major recent advances in culturing methods (reviews: Ahmadjian, 1987; Bubrick, 1988). To help elucidate the molecular interactions

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guiding the development of the symbiotic thallus, improved culture methods are needed, both for the individual symbionts and for the lichen as a whole. In combination with the techniques of molecular biology and genetics, which are now entering this field (Ahmadjian et al., 1987; Culberson et al., 1988; Kardish et al., 1990; Armaleo and Clerc, 1991), "tissue culture" will increase our understanding of lichens just as it has done with animals and plants. Lichen mycobionts grow slowly and do not form vegetative or sexual spores in culture. Therefore, procedures commonly used for mutagenesis and genetic analysis in other fungi need to be modified to be applicable to lichen mycobionts.

The first part of this paper describes how small mycelial fragments can be generated from mycobiont cultures and used as "starter units" for mutagenesis and colony formation, in place of conidia or ascospores. The second part describes a lichen growth chamber in which micro-environmental conditions can be simply controlled and changed without compromising sterility. The versatility of the chamber overcomes many of the limitations of present lichen culture methods, with which ready control of substrate, moisture, and nutrients is complicated at best. The first observations made on soredia of *Cladonia grayi* cultured in the chamber suggest a simple scheme for early lichen morphogenesis, with both alga and fungus playing major roles.

2. Materials and Methods

Mycobiont fragmentation

The mycobiont is a single-spore isolate of *C. grayi* (C.F. Culberson's culture number GR30-197#8). To generate small fragments, mycobiont cultures are grown in tubes containing liquid medium, typically Lilly and Barnett (1951), and 1 g/ml glass beads (Thomas Scientific, 0.5 mm diam., cat. #5663–R50). The glass beads are acid-washed in bulk before use (after being soaked in 1 N HCl for about 30 min and occasionally stirred with a glass rod, the beads are rinsed with tap water for 1 hr, washed 5 times with distilled water, and oven dried). Weighted aliquots are either sterilized in glass culture tubes together with the liquid medium or, when sterile plastic tubes are used, added to the culture after separate sterilization.

To start a liquid culture from a compact colony grown on solid medium, the colony is first disrupted with a homogenizer or ground with a glass rod in the tube containing the glass beads. Subsequently, each culture is fragmented every 2–4 weeks by agitation for 5 sec on a vortex mixer at maximum setting. Fragmenting too often or for much more than 10 sec may kill the culture. The optimum combination of agitation time vs frequency varies with different

mycobionts. A fraction of the total fragments generated is nonviable due to irreversible cell damage. When quantitative values are needed, the fraction of viable to nonviable fragments in a culture can be estimated by comparing the total number of fragments (counted with a hemacytometer) with the number of colonies obtained (after 2–3 weeks) by plating known aliquots of the liquid culture. In the *C. grayi* cultures, the fraction of viable fragments is 10–20%. The percentage of actual living biomass is much higher because the dead fragments are the smallest ones.

Lichen growth chamber

Components: Fig. 1 shows a photograph and diagram of the chamber. Its

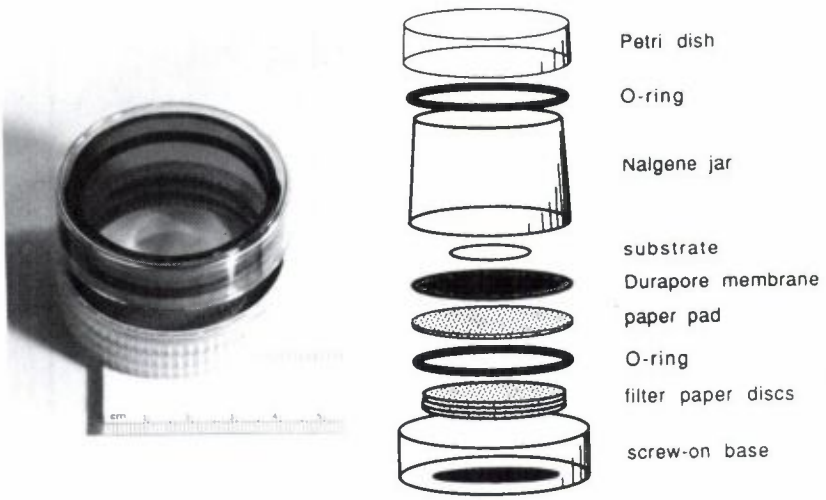

Figure 1. Lichen growth chamber. Assembled unit on left, components on right. Details in text.

main body is derived from a 60 ml Nalgene jar (Nalge Company, cat. #2119–0060) modified as follows: A 3.5 cm hole is made in the center of the original screw-on lid, and the base of the original jar is cut off. The screw-on lid becomes the base of the growth chamber, whose upper opening is covered with the bottom of a 5 cm sterile plastic Petri dish. Additional components: two 2.5 mm thick O-rings (outer diameter 4.7 cm), a variable number of filter paper discs (Whatman #1, diam. 4.25 cm), one thick paper pad (Millipore Corp., cat. #AP10 047 SO), one Durapore 0.22 μm filter (Millipore

Corp, cat. #GVWP 04700), growth substrate (typically, a 2.5 cm Millipore HAWP 02500 nitrocellulose filter, but see below for other substrates). All components, except the plastic Petri dish, can be autoclaved. It is advisable to rinse with deionized water and then dry all components and filters before assembly to remove traces of contaminating substances (e.g. detergents) which may be left-over from the manufacturing process.

Assembly: (1) A variable number (between 0 and 6, see below) of filter paper discs is placed inside the base. (2) One O-ring is placed inside the base. (3) One paper pad is placed over the O-ring. (4) One Durapore filter is placed onto the paper pad. (5) The Durapore filter and the underlying pad are precisely centered onto the O-ring by running a thin instrument (e.g. forceps) around their edges. (6) The body of the container is screwed onto the assembled bottom with care not to displace the Durapore membrane, the pad, and the O-ring, from their centered position. Displacement may result in contamination. (7) The second O-ring is placed on the outside of the chamber, about 0.5 cm below the top edge. (8) The substrate (optionally resting on a Whatman filter) is placed onto the Durapore surface. (9) The chamber is wrapped in aluminum foil and autoclaved for 15 min. (10) After cooling, the chamber is covered in a sterile hood with the bottom of a sterile 5 cm Petri dish.

Soredia culture and microscopy

About 20 freshly harvested *C. grayi* podetia (thallus appendages, cup-shaped in *C. grayi*) were rinsed in a stream of distilled water for 15 min, and soredia (globular clusters of algal cells enveloped by hyphae) were collected in sterile distilled water from the inside surface of the washed podetia. A 1 ml soredial suspension was centrifuged for 30 sec in two sterile microcentrifuge tubes. After discarding the supernatants, the resuspension and centrifugation in sterile distilled water was repeated three times. Finally, all soredia were pooled in 100 μl of sterile deionized water, and 10 μl aliquots were delivered onto the growth substrates placed in sterile chambers (each containing 6 filter paper disks). The resulting soredial density was approximately 1000/cm^2. After seeding, the chambers were wetted once with Bold's mineral medium as modified by Ahmadjian and Jacobs (1981) and every 2 weeks thereafter with double-distilled water. The concentration of the medium was one-tenth that used by Ahmadjian because the salt solution becomes concentrated over time at the growth surface. Wetting was achieved by placing the chambers in a shallow container filled to approximately 1 cm depth with water or nutrient

solution. When the lichen substrate became moist by capillarity (about 10–30 sec), the chamber was withdrawn from the liquid. Routinely, the incubation was at 15°C under fluorescent light (50 $\mu E/m^2/s$ PAR) for a 12 hr light/dark cycle.

The substrates tested for their ability to support growth of *C. grayi* soredia in the chamber were nylon, powdered vermiculite, powdered silica, glass fiber, filter paper, and nitrocellulose filters. The data reported refer to the nitrocellulose cultures. After 3–6 months in the chamber, the filter with the culture of interest was removed with forceps and placed over a paper pad (Millipore Corp., cat #AP10 047 SO) moistened with sterile water to prevent drying during microscopy. The paper pad was fastened to a mechanical arm to allow observation at different angles under the dissecting microscope. For higher magnification, the moist pad with the filter was placed horizontally under a higher power microscope and observed at 100×. Illumination was always from the sides, with one or two dissecting microscope lights. The pad was rewetted during microscopy if necessary. After microscopy the cultures were returned to the chambers. No obvious contamination was noted, even after repeated filter transfers.

Histological sections were prepared from soredia and squamules which had been individually removed from the substrate with forceps under a dissecting microscope. The samples were fixed in formalin-acetic acid-alcohol, dehydrated with t-butyl alcohol and embedded in a hard paraffin mixture. The sections (10μ in thickness) were stained with iron hematoxilin, safranin, and fast green (Johansen, 1940).

3. Results

Mycobiont fragmentation

The mycelial disruption method is being used in conjunction with mutagenesis to obtain faster-growing mutants or mutants resistant to the fungicide benomyl, as well as other mutations necessary to begin genetic analysis of the mycobiont (in preparation). It is also used routinely to grow and expand mycobiont cultures since it increases the rate of biomass production, presumably through the generation of new apical growth. Figure 2 illustrates the kind of single-colony growth obtained upon plating a diluted suspension of mycelial fragments from culture GR30-197#8. The variabiliity in size, color and texture apparent upon close inspection in this population of presumably genetically identical colonies is a common but not well understood phenomenon in fungi (Rayner and Coates, 1987). In this case, the heterogeneity may be a part due to the size variation of the mycelial fragments used as starter-units.

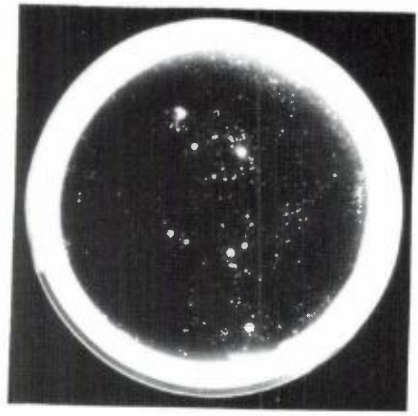

Figure 2. Colonies derived from a mycelial culture disrupted with glass beads and plated on LB agar medium in a 10 cm Petri dish. Details in text.

Lichen growth chamber

The chamber's versatility is due to the following features: (1) The Durapore filter is a permeable base which maintains sterility while allowing free and rapid access of media and air to the thallus. Growth conditions can therefore be changed at will without opening the chamber. (2) The filter paper discs located in variable number under the Durapore membrane constitute a water reservoir whose capacity determines the rate at which the chamber dries out. The wet/dry cycling can be easily controlled: without filters, for instance, the chamber dries in 12–24 hr; with 6 filters, in 3–4 days. (3) Different growth substrates can be tested under controlled conditions. (4) The cultured lichen can be observed *in situ* through a dissecting microscope, or the substrate filter can be easily removed for closer observation or manipulation. Repeated, careful handling of lichen cultures has not led to significant contamination. (5) Many chambers can be watered at the same time by placing them for a few seconds in a tray of the wetting solution.

Among the substrates tested for their ability to support growth of *C. grayi* soredia in the chamber, the best results were obtained with nitrocellulose membranes, and are reported here. The developing culture progresses to the squamule stage in 3–4 months. It does not progress much further for several months and, after about 1 year, most squamules begin to degenerate,i.e. turn brown or white. Some, however, remain green. Most morphologies described were observed between 3 and 6 months after seeding. Soredia were not followed individually during development, and stages are here described as sequential

on the assumption that the ones closer to the original soredia represent ontogenetically earlier stages, and the more "lichen-like" later stages. The majority of the seeded soredia contained a core of less than 10 algae surrounded by a mycelial shell, but larger ones were also present.

At early stages, polygonal bodies develop in which 5–6 spherical algal packets are located at the vertices and mycelium in the interior (Fig. 3A). This radial symmetry becomes axial as subsequent growth leads from a body spread out on the substrate to a finger-like squamule projecting upward (Fig. 3B,C). Cross-sections of these finger-like squamules reveal the preservation of symmetry, with hyphae radiating from a central axis and algal packets at the periphery (Fig. 3D). Each squamule can develop from a single soredium but can also fuse with neighboring squamules (Fig. 3D) to generate larger "mosaic" squamules. As squamules develop further, radial symmetry gives way to dorsiventral differentiation, a process leading to the formation of a cortex, an algal region, and a medulla (Fig. 3E,F). This differentiation involves flattening of the finger squamule. The regions closer to the substrate appear to experience partial degeneration and eventually become the medulla. The dorsal areas develop a well-defined cortex, and the algal packets progressively coalesce into a uniform layer (Fig. 3F). Throughout this process, the algal packets visible in the histological sections produce the lumpy appearance of the squamule's dorsal surface (Fig. 4, left panel) and are most evident in the spherical protrusions at the growing edges of the squamules (Fig. 4, right panel). This globular pattern is observed at all stages and in all squamules.

4. Discussion

Mycobiont fragmentation

Agitation of fungal cultures in test tubes with glass beads has several advantages over the use of homogenizers. It allows rapid fragmentation of a large number of samples without danger of contamination. Volumes can be as small as 100 μl or as large as 20 ml. For mutagenesis and plating the fragments can be treated as if they were spores, keeping however in mind that they are multinucleate. The method can be used in a variety of contexts since it allows the regulation of the amount of damage inflicted to the cells. Slight damage has allowed the introduction of foreign DNA into yeast (Costanzo and Fox, 1988) or the generation of small colony-forming units as shown here, whereas heavy damage is useful to extract the cell contents for biochemical studies (Cramer et al., 1983). With lichen mycobionts, caution must be exercised in defining the proper conditions to obtain optimal growth, maximal fragmentation, and minimal death.

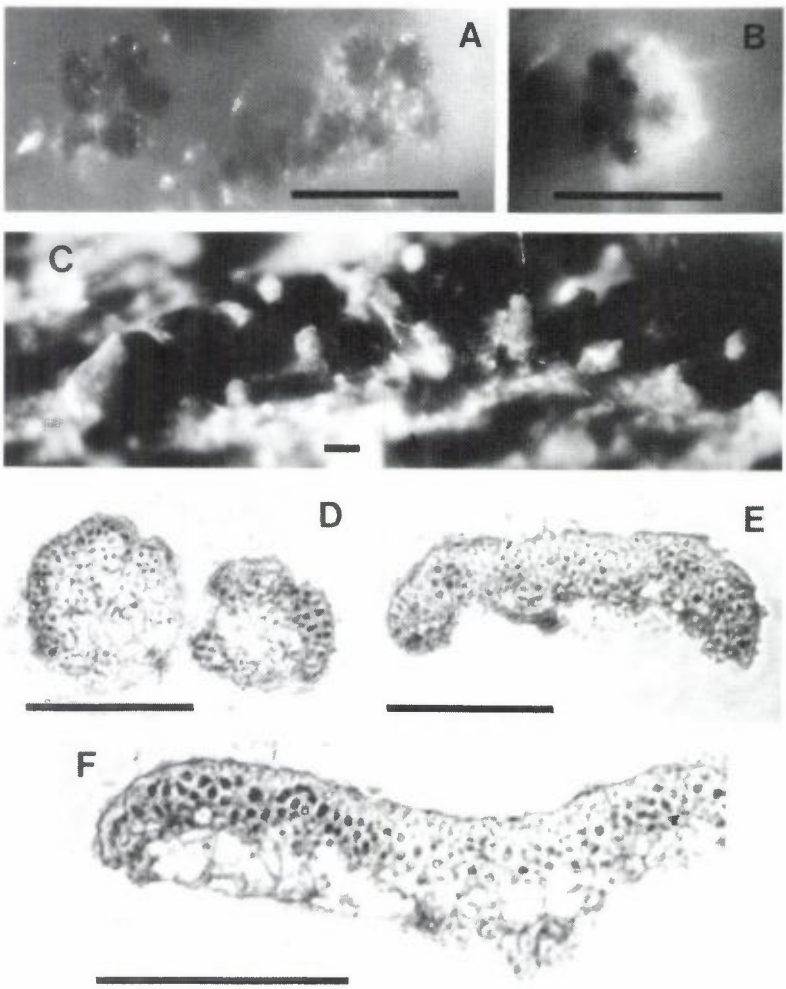

Figure 3. Stages of lichen development in the growth chamber. Bars represent 100 μ. (A) Polygonal spread of algal packets on the substrate. (B) Top view of the growing tip of a finger-like squamule; note the symmetrical arrangement of bulges, each enclosing an algal packet. (C) Overview of several finger-like squamules protruding from the substrate. (D) Cross-section of two neighboring finger-like squamules; note the peripheral disposition of algal packets, the beginning of a cortex on one side of the left squamule and of partial degeneration on the other, and the hyphae connecting the two squamules. (E) Cross-section of a young flattened squamule with recognizable cortex (top layer) and medulla (bottom layer) and a middle region in which algal packets still retain their individuality. (F) Mature squamule with well-defined layers; note the uniform distribution of photobionts in the algal layer.

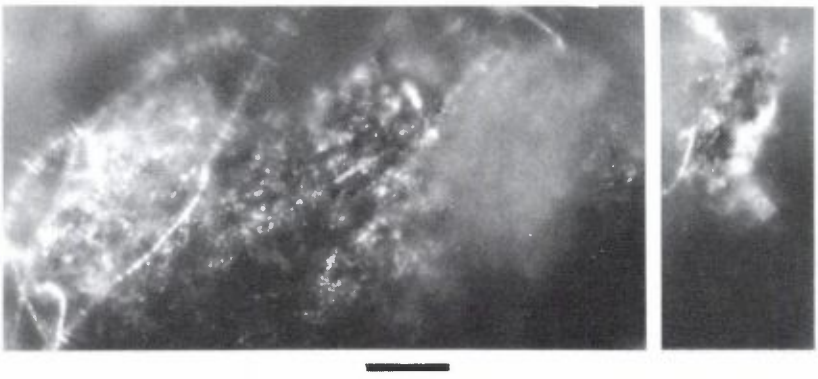

Figure 4. Top view of a flattened squamule. Left panel: This part of the squamule lies parallel to the substrate and the lumps on its dorsal (cortical) surface are the imprints of the underlying algal packets. The right portion of the squamule is out of focus as it bends toward the viewer. Right panel: Uppermost growing margin at the tip of the right portion of the squamule; note the spherical bulges. Bar represents 100 μ.

Growth chamber and morphogenesis

Although as yet tested with only one lichen and under a limited set of conditions, the chamber has produced encouraging results and is being used with a broader range of conditions and lichens and to perform resynthesis experiments.

Spherical algal/fungal bodies are an ubiquitous feature of early development in *Cladonia*, and their patterns suggest a model for lichen morphogenesis based on the biological superimposition of the individual modes of growth of the two symbionts. In isolation, fungal hyphae spread radially and centrifugally; trebouxoid algae (the photobionts of *Cladonia* and of many other lichens) in culture grow in size, divide into aplanospores (non-motile cells) contained in the mother-cell wall, and when eventually the latter is disrupted the individual aplanospores are released and begin a new cycle (Fig. 5). In the lichen, the convergence of these two growth patterns provides a key to interpret the observed morphologies (Fig. 6).

The growing hyphal shell starts breaking into and disrupting the algal core of the soredium (Figs. 6A,B). Hyphal disruption of aplanospore packets has been observed in both natural (Greenhalgh and Anglesea, 1979; Anglesea et al., 1982) and cultured lichens (Bubrick, as described by Galun, 1988). Individual hyphae and algae then make intimate contact with each other and, as the hyphae spread outwards from the original center of the soredium, they take the

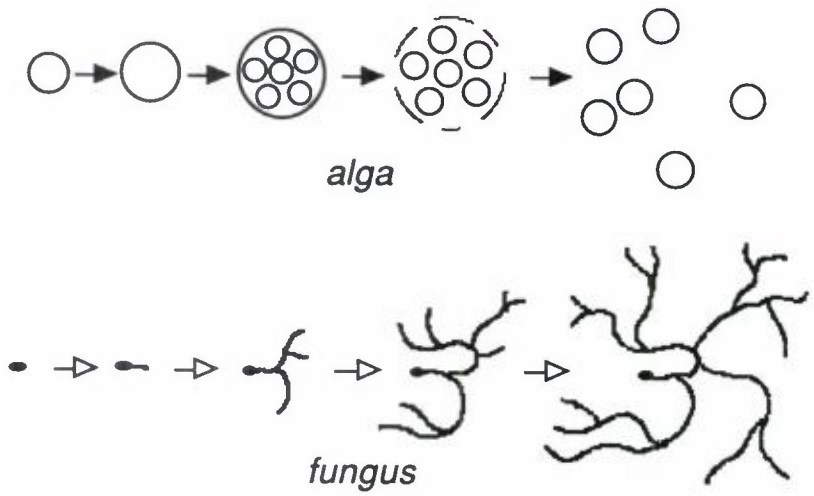

Figure 5. Growth patterns of the isolated symbionts. Details in text.

attached algae with them over a considerable distance. Each alga grows in size and becomes an aplanospore packet while being pushed outward (Figs. 6C,D). The mother wall surrounding the aplanospores provides temporary protection and delays the beginning of the next cycle of packet disruption, hypha/alga attachment, and expansion. As the cycle repeats from the central aplanospore packet translocated upwards, a new polygonal layer is formed over the preceding one. The finger-like squamule thus grows vertically by deposition of successive layers (Fig. 6G). Each cycle results in a morphological inversion: the algae are first packed inside, surrounded by hyphae, then end up near the surface with most hyphae inside. On the outermost circumference of every layer, hyphae progressively coalesce in the formation of a cortex and limit horizontal spreading (Figs. 6E,F). Lichenization thus appears as a multi-stage and cyclical process with different stages in different regions of the thallus.

As the squamule increases in size, the combined effects of a microenvironment that is not uniform in all directions will interfere with the symmetrical expansion of an ever-growing cylindrical body. The changing topology (compare C and F in Fig. 6); the exact number and orientation of aplanospores in every new algal packet; the polarities of substrate vs air, of light, water and nutrient availability; selective algal death; cortex formation; and fusions with neighboring squamules (Fig. 3D) will determine the transitions from spherical (soredium) to cylindrical (finger-like squamule) to dorsiventral (flattened squamule) symmetry or to completely irregular shapes (Fig. 6H). Despite the progressive loss of obvious symmetry, the basic generative pattern of successive

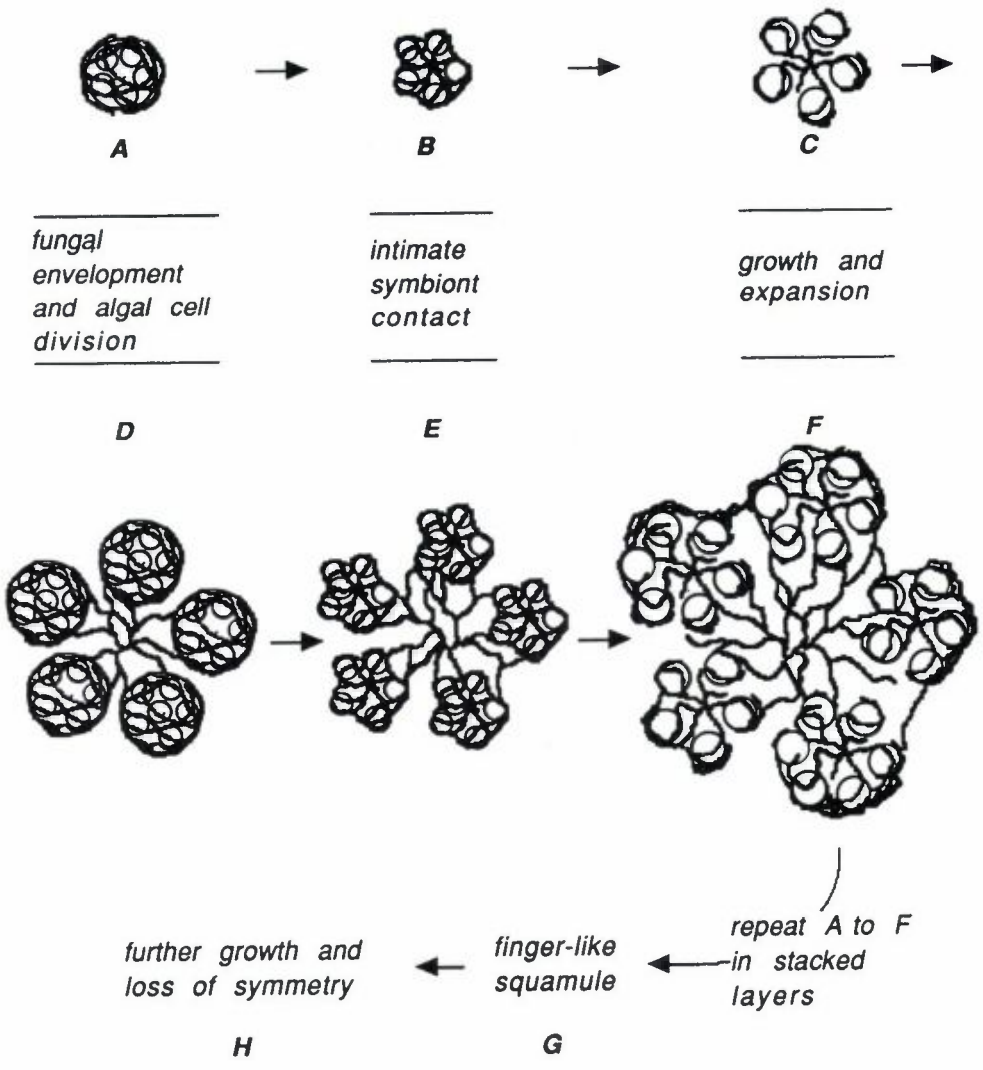

Figure 6. Scheme of presumed major route of early squamule development, top view. The lined captions emphasize the main characteristics of stages D, E, F, considered essentially amplified repetitions of stages A, B, C, respectively. The central aplanospore packet translocated upwards (toward the viewer) has been omitted for design clarity. See text for discussion.

"micro-explosions" remains clear and active near the growing margins of the small thalli (Figs. 3B and 4) and may be involved in maintaining the morphological plasticity observed in lichens (Jahns et al., 1982). A similar pattern has been noted at the growing margins of *Parmelia* and *Usnea* (Greenhalgh and Anglesea, 1979; Anglesea et al., 1983).

Symbiont contact, intercalary growth, and differentiation

The model described rests on a fundamental assumption: During early development, individual algal cells are pushed a considerable distance outwards as they remain locked in intimate contact with hyphae that continue to grow centrifugally beyond the algae. Under these conditions, pushing requires intercalary hyphal growth on the proximal side of the algal cell and not just at the hyphal tip. Each alga could be moved directly by the hyphae attached to it or by the progressive intercalary crowding of the internal space of the lichen primordium. While hyphal elongation is generally apical in fungi, intercalary growth also occurs occasionally (Bergman et al., 1969; Gooday, 1975). The chamber will permit the monitoring of new hyphal growth with labeled cell wall precursors (Galun et al., 1976) provided during early lichen development. Intercalary growth is the simplest explanation for the observations made and has the following schematic and speculative implications for the differentiation of cortex, algal layer, and medulla in lichens (Fig. 7).

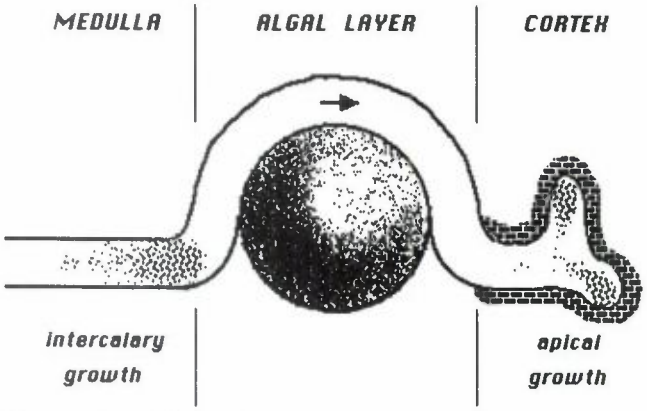

Figure 7. Scheme of the lichen differentiation hypothesis. The arrow emphasizes the directionality of hyphal metabolic flow. The dots inside the hypha represent regions of hyphal elongation. The brick pattern symbolizes wall thickening. See text for discussion.

Hyphal growth depends on the polar transport of nutrients toward the growing tip where synthesis of new cell membrane and wall material normally occurs. We shall assume that, in lichen fungi, the alga has a stimulatory effect at close range on hyphal growth (Lallement and Bernard, 1977; Greenhalgh and Anglesea, 1979). The same signals that may induce the hyphal tips to grow into and break up the aplanospore packet may then be thought to persist in the region of intimate contact between alga and fungus and induce intercalary growth in the hyphal segment proximal to the alga (Fig. 7, left panel). The diversion of hyphal nutrient flow towards intercalary growth may partially starve the region of the hyphae distal to the algae and slow down apical growth. In several non-lichen fungi, the artificial inhibition of metabolism (mostly with antibiotics) reduces growth and almost universally induces hyphal branching (Baratova et al., 1968; 1975) and in some cases thickening of the cell wall (Sternlicht et al., 1973; Dominguez et al., 1978). In lichens then, the short branched hyphae with thickened walls characteristic of the cortex (Anglesea et al., 1982; 1983) could be the consequence of a natural downturn in the metabolism of the apical regions of the hyphae (Fig. 7, right panel). The tripartite differentiation of lichens may therefore originate from a perturbation of metabolic traffic at the alga/fungus interface (Fig. 7, center panel). Thus, the future algal layer would be embedded between the proximal region of intercalary growth that generates the medulla and the distal starved region forming the cortex.

The morphogenetic model described suggests that both symbionts play major roles in lichen differentiation (as exemplified by photosymbiodemes and reviewed by Hawksworth, 1988, and Jahns, 1988), and that several (but certainly not all) specific features of the lichen thallus arise as novel combinations of pre-existing properties characteristic of some free-living fungi and algae. This scheme is of course not intended to discount the genetic and biochemical interactions unique to lichen development and differentiation. It is rather meant to highlight an ontogenetic principle that might have its roots in lichen phylogenesis: When two organisms evolutionarily as distant as an alga (or a cyanobacterium) and a fungus negotiate a common road to symbiosis, each will try to follow its own biology as far as possible while building the subtle bridges needed to tighten and stabilize the interaction over time.

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