Appendix C

METHODS OF ISOLATING AND CULTURING LICHEN SYMBIONTS AND THALLI*

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I. Fungal Symbiont

A. Isolation Techniques

1. Spores

The best way to isolate a lichen fungus is by its spores. The ascocarp is allowed to discharge its spores onto a sterile surface, the spores are collected, either before but preferably after germination, and transferred in groups or singly to a suitable culture medium. The ascocarps may be soaked in water before being used or they may be placed directly onto a moist filter paper in the discharge chamber. Excess water should be removed from the surface of the apothecium. Spores are discharged within a few hours, and discharge from one ascocarp may occur continuously for days. One apothecium of *Cladonia cristatella* discharged spores for 13 continuous days after which the experiment was stopped (V. Ahmadjian, unpublished results). Discharge was determined by placing the petri-dish cover with the apothecium each day over a new agar surface.

*For additional information on this subject see Ahmadjian, V. (1967). "The Lichen Symbiosis," Ginn (Blaisdell), Boston; and Richardson, D. H. S. (1971). Lichens. *In* "Methods in Microbiology," pp. 267–293. Academic Press, New York.

Spores may appear on the agar surface within minutes or several hours after the ascocarp is placed into position. Spores may be caught on the following surfaces: (a) *Agar*—an ascocarp is fixed by means of petroleum jelly to the inside cover of the top of a petri dish. The top cover is then placed over the bottom half which contains a thin layer of nonnutrient or mineral agar. Discharged spores fall onto the agar surface either singly or in groups. A variation of this method is to have the petri dish with its top cover down and allow the spores to discharge up onto the agar surface. This way prevents debris and foreign organisms from contaminating the agar. Care must be taken to ensure that the distance between the ascocarp and the agar is within the spore's normal discharge range (maximum recorded = 45 mm). The duration of discharge can be determined either by examining each day whether spores were discharged onto a fresh agar surface or by placing the ascocarp near the outer rim of the petri-dish cover and rotating the cover at periodic intervals. Each impact area below the fruit can be circled with a crayon. To observe the spores without opening the petri dish place the dish in an inverted position on a microscope stage and focus on the agar surface.

When the spores have germinated small blocks of agar that contain spores are excised and transferred to a culture medium. Since the spores of many lichen fungi remain together in groups of two–eight, and the discharge area soon becomes crowded, the isolation of single spores is difficult. To facilitate single spore isolation it is necessary to have the spores separated as far as possible from each other. This can be done by reducing the discharge time, thus allowing only a relatively few spores to be discharged onto the agar and to increase the distance between the ascocarp and the underlying agar surface. Drifting of the spores will occur over the greater distance and the impact area will be widened considerably. When a spore has been sighted that is well separated from others, the area above the dish is marked with either a fine crayon if the dish is glass or, if the dish is plastic, with a sharp implement. Under a dissecting microscope the petri-dish cover is removed, the mark on the dish is located, and the spore, along with a small piece of agar, is removed with a fine needle.

(b) *Glass*—spores can be discharged onto a glass slide that is either suspended over or is directly below an ascocarp. Discharge is effected in a damp chamber apparatus. More sophisticated techniques include passing a row of glass slides at a known rate over the ascocarps. Studies on spore discharge have provided information on the duration and periodicity of spore discharge and how factors such as light, temperature, humidity, and pH affect discharge (Kofler and Bouzon, 1961; Pyatt, 1968, 1969; Garrett, 1971). To determine the distance of spore discharge an ascocarp is fixed on the vertical surface of a block at the end of a marked glass slide. The spores discharge along the length of the slide (Bailey and Garrett, 1968) (See Chapter 4).

(c) *Parafilm*—quantities of spores may be collected by having them discharge onto a parafilm surface and then washing them off with distilled water. The parafilm is sterilized with ultraviolet light, laid across the opening of the bottom of the petri dish and then the cover of the dish is set in place. Apothecia are placed on the bottom of the dish on moist filter paper. The suspension of spores can be maintained as stock material for use in future investigations. Tests have shown that such stocks still germinated after 1 month's storage (Kofler, 1970).

2. Hyphal Fragments

Fragments of hyphae, either teased from the medulla, or those attached to algal cells, when placed onto a culture medium may grow into fungal colonies. The fragments should be small enough to minimize the chances of contamination. Sufficient numbers should be attempted to ensure that the fungal growth obtained is likely to be that of the mycobiont and not a foreign fungus growing on or in the thallus. The element of doubt with this technique is so high that it should be used only if spore isolations are unsuccessful.

B. Culture

When the spores have germinated, a process that takes several hours for some mycobionts and several days for others, they are transferred to a nutrient agar contained in a test tube, glass bottle, or Erlenmeyer flask. The medium that supports the strongest growth for many mycobionts is malt–yeast extract (malt extract, 20 gm; yeast extract, 2 gm; agar 20 mg; distilled water, 1000 ml). When grown on a solid medium the colonies that develop are compact and elevated above the agar surface. Mycobionts of some filamentous lichens may form long extensions and even lobelike structures. Not all lichen fungi do well on nutrient agar. Some, like *Endocarpon pusillum*, grow well only on media with limited nutrients, i.e., soil-extract agar (Bold's mineral solution, 960 ml; soil-water, 40 ml; agar, 15 gm). Malt–yeast extract inhibits the growth of this fungus. Incubation should be at about 20° C. After 6–8 weeks, small colonies of the fungus should be visible. If fungal growth occurs before this time the changes are high that the growth is that of contaminant molds.

Because lichen fungi grow slowly, cultures can be kept for months or even several years before they are transferred. Vegetative mycelia have been lyophilized successfully and stock cultures of representative lichen fungi are being maintained by the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852.

To obtain large quantities of mycelium for chemical analysis special laboratory-scale growth apparatus have been developed (Bloomer, 1968a, b).

II. Algal Symbiont

A. Isolation Techniques

1. Micropipette

The surest but most tedious way to isolate a lichen alga is with a micropipette. This method minimizes accidental isolation of a foreign alga that may be an epiphyte on the thallus and provides high assurance that the alga isolated is the true symbiont. All glassware and implements are sterilized before use, and if possible the slides should be soaked first in an acid bath and then washed well with distilled water. The reason for this is that the cells of many lichen algae adhere to a glass surface, particularly if the surface is dirty. Small thallus fragments are picked out from a clean part of the thallus and placed into a drop of water on a glass plate. These fragments are gently grinded with a glass slide until the water turns green. Fragments adhering to the slide are rinsed with a few drops of water over the stock suspension. The suspension is then placed into a small glass vial.

Micropipettes are made as follows: 9-inch segments of 3–4-mm glass tubing are plugged with cotton at both ends and drawn in the middle over a bunsen burner. The drawn tubing is separated into two gross pipettes either with a file or by melting the glass in the middle of the tubing. The latter method is preferred if the pipettes are to be stored since the burner can seal the openings and the pipettes can remain sterile indefinitely. A gross pipette is drawn into a micropipette over a flame that must be small enough not to heat the glass too rapidly. Such a small flame can be obtained by connecting to the gas supply tube a segment of glass that is tapered at one end. The tapered end of a gross pipette is grasped with a forceps, held over the flame until the glass softens and then pulled smoothly at the same time as the pipette is withdrawn from the flame. The softened glass will stretch into a thin tube. The opening of the micropipette should be about 50–75 μm in diameter. If the pipette is drawn too much the sealed end may be removed with a sharp pull from a fine forceps. Micropipettes that are not suitable, either because their openings are too large or their tips jagged, should be saved and repulled later. Because these pipettes are so fragile, about a dozen should be made before beginning the isolations.

A drop of the stock suspension is placed on one end of a glass slide and examined with a microscope at low power ($\times$ 100). About 15 algal cells, preferably those with bits of the fungus adhering to them, should be transferred from this drop through 3 or 4 new drops of water on the same slide. By the fourth drop the cell is usually removed from other impurities and can be then transferred to the surface of a nutritive agar slant. The pipette is drawn slowly along the agar surface and the liquid is blown out until air bubbles

appear. The tubes are incubated at 15°–20° C under dim illumination or in darkness for 6–8 weeks. Single cells or those in groups of two or three may be isolated.

A length of rubber tubing added to the micropipette facilitates its use. The tubing is placed in the investigator's mouth during the isolation process and it helps to increase the natural capillary action of the micropipette. The worker can apply suction or blow out the contents of the pipette.

2. Thallus Outgrowths

Small pieces of a thallus are placed into a mineral solution or onto an agar surface and incubated under light. After several weeks, or even longer with blue-green phycobionts, the algal cells will grow out of the confines of the fungal hyphae. The thallus fragments may have to be transferred to fresh media several times because of the dense growth of foreign algae. Identification of the phycobiont can be made from observations of the fragments at a stage where the algal cells still are loosely associated with fungal hyphae or, with filamentous algae, where the filaments can be traced back to fungal tissue.

3. Centrifugation

This technique is used to obtain quantities of algal cells that will be more or less free of fungal filaments. Lichen thalli are ground up with a mortar and pestle in distilled water and the resulting suspension then centrifuged at the following speeds: (a) *Blue-green algae*—375 *g* for 3 minutes; the green algal zone of the precipitate is resuspended and centrifuged at 125 *g* for 30 seconds. This procedure is repeated several times with greater speeds up to 90 seconds. (b) *Green algae*—60 *g* for 10 seconds; the supernatant is recentrifuged at 375 *g* for 5 minutes. The precipitate is resuspended and centrifuged for another 5 minutes. These speeds are guidelines and will vary slightly according to the different types of lichens (Richardson, 1971). Lichens that have algal symbionts of small size, i.e., species of *Peltigera* that have *Coccomyxa* as phycobiont, are especially suited to this technique because of the wide differences in size between the algal cells and fungal hyphae.

B. Culture

Lichen algae can be separated into two groups on the basis of their cultural requirements, i.e., *Trebouxia* phycobionts and other phycobionts. Most strains of *Trebouxia* grow well only on a medium supplemented with organic

compounds. On such a medium they grow well even in complete darkness. A basic mineral medium with additives (per liter) of glucose (20 gm), proteose peptone (10 gm), and even coconut milk (140 ml) will support strong growth. *Trebouxia* is sensitive to high light intensities and should not be grown under light intensities of more than 150 ft-c. Other lichen phycobionts grow well on organic medium but they will develop also on inorganic media.

III. Lichen Thallus

Culture of the whole thallus is difficult and complicated by the sensitivity of lichens to air pollution, their need for varying environmental growth conditions, and the contaminant organisms that grow on the thallus. In recent years, because of increased studies in whole thallus physiology, greater attention has been focused on cultivating thalli for long periods of time. A special controlled environmental lichen growth chamber was developed by Kershaw and Millbank (1969) that kept thalli of *Peltigera aphthosa* in a healthy condition for 6 months and Dibben (1971) achieved new growth of 5 terricolous lichens under Phytotron conditions. Other studies on whole lichen culture are those of Tobler (1939), Pearson (1970), and Galun *et al.* (1972). Kershaw and Millbank (1970) studied the development of isidia excised from *Peltigera*.

Alternate drying and wetting are essential for successful lichen growth as they are also for the recombination of the separated symbionts (see Chapter 18). Sustained moisture conditions bring about contamination and dissociation of the symbionts. The substrates on which lichens have been cultured include soil, sand, silica gel, and moist filter paper on a sandy surface. The essential property of these substrates has been their ability to dry slowly.

Axenic cultures of lichen thalli are not possible because of the bacterial flora on the thalli. Bacterial cells are found lodged in chinks and cracks of the lichen thalli and embedded within the extracellular polysaccharide material found throughout the thalli (Jacobs and Ahmadjian, 1971). Attempts to sterilize thalli with plasmolyzing agents or radiation have not been successful. The only possible way to achieve lichen thalli that are free from contaminants is to begin with the separate symbionts and recombine them under sterile conditions. Such a possibility now exists with *Endocarpon pusillum* (Ahmadjian and Heikkilä, 1970).

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