Symbiosis, 7 (1989) 257–266
Balaban, Philadelphia/Rehovot

Characterization of Symbiotic and Cultured *Nostoc* of the Lichen *Nephroma laevigatum* Ach.

N. KARDISH[1], M. KESSEL[2] and M. GALUN[1]
[1] *Department of Botany, The George S. Wise Faculty of Life Sciences*
Tel-Aviv University, Tel-Aviv 69978, Israel
[2] *Department of Membrane and Ultrastructure Research*
The Hebrew University — Hadassah Medical School, Jerusalem 91010, Israel
Tel. 972-3-5459163, Telex 342171 VERSY IL

Received October 15, 1989; Accepted December 27, 1989

Abstract

The photobiont (algal symbiont) of the lichen *Nephroma laevigatum* is *Nostoc* sp. The symbiotic *Nostoc* appears in the intact thallus as enlarged, shapeless and usually separated cells. Upon isolation and culturing the *Nostoc* undergoes remarkable modifications until reverting and regaining its typical free-living filamentous growth form. Several stages of this process are demonstrated. Electron microscopical examinations and an indirect immunofluorescence assay revealed differences of cell wall structure and apparently molecular diversity between the symbiotic *Nostoc* and its cultured counterpart.

Keywords: *Nostoc*, *Nephroma laevigatum*

1. Introduction

Lichens are a symbiotic association between a fungus and an alga or/and a cyanobacterium. The interaction between these symbionts results in an organism which is morphologically and physiologically distinct from either of the components (Bubrick, 1988; Galun, 1988). Consequently, both components are subjected to remarkable modifications during integration and formation of the lichen thallus. Particularly cyanobacterial symbionts are often

0334-5114/89 /$03.00

altered to the extent that their origin becomes completely obscured (Marton and Galun, 1976). This led to taxonomic misidentifications and erroneous classification of many cyanolichens (Bubrick and Galun, 1984).

Resynthesis of a lichen from its separated components, under controlled conditions, is rarely successful. The few successful reconstruction attempts, report a protracted process with very limited yields (Ahmadjian, 1974, 1982; Bubrick and Galun, 1986). It is therefore impossible to sequence the events during a lichen thallus formation.

Upon isolation and culturing, the symbionts usually regain their free-living life style. This reverse process, from the symbiotic state to the free-living state, can more easily be documented.

We demonstrate here changes of *Nostoc* sp. of the lichen *Nephroma laevigatum* from its symbiotic form to its free-living form, in conjunction with our studies on the establishment of lichens and other symbiotic systems. (Part of the data have been published in *Proceedings of the IVth Colloquium on Endocytobiology and Symbiosis*, Lyon, Aug. 1989).

2. Materials and Methods

Nephroma laevigatum Ach. was collected from bark of oak trees, at Har Meron (Upper Galilee). Symbiotic *Nostoc* was isolated from the lichen thallus by delicately macerating thallus fragments, which were washed for 1 hr with running tap water. The slurry was then filtered through a nylon screen (20 mesh), centrifuged and the pelleted cells plated on BG-11 medium (Stanier et al., 1971), solidified with 1.2% agar, in 9 cm petri dishes. On top of this layer, additional approx. 3 ml of the same medium, warmed to approx. 50°C, were poured. Most contaminating organisms developed inside the medium (between the two layers), whereas the *Nostoc* filaments grew through the top layer. Pure cultures were achieved by transfer and repeated restreaking on the same medium. Cultures were grown at 20–22°C at a light intensity of 6.5 $J.m^{-2}\cdot sec^{-1}$ under a 16 hr light/8 hr dark daily cycle.

Thallus fragments prepared for Fig. 1 were fixed in 1% glutaraldehyde in Na cacodylate buffer 0.1 M, pH 7.4, for 1 hr and postfixed with 1% tetroxide in the same buffer. The material was then dehydrated in a graded series of ethanol solutions, 25–100% and embedded in Epon. Sections were cut with a Reichert OmU3 ultramicrotome using a glass knife. The sections were stained with lead citrate and uranyl acetate. Electron microscopy was performed with a Jeol 100B electron microscope at 80 kV.

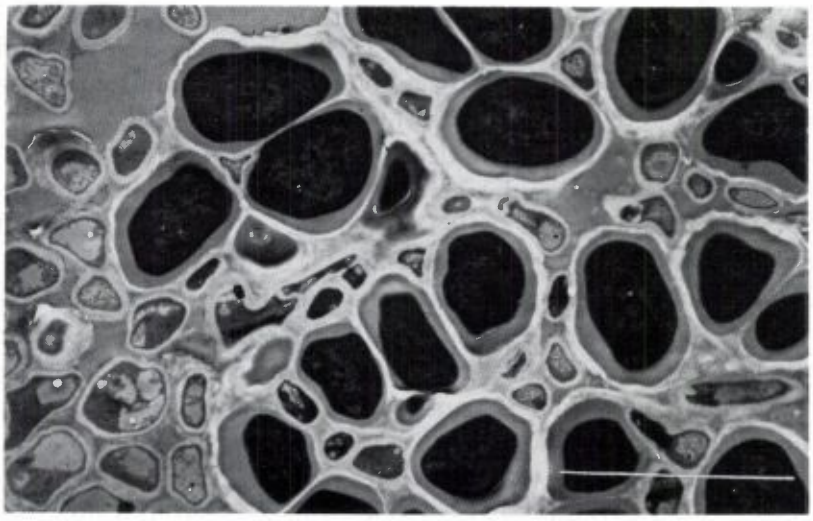

Figure 1. Ultrathin section of the algal layer of *Nephroma laevigatum*, bar = 10 μm.

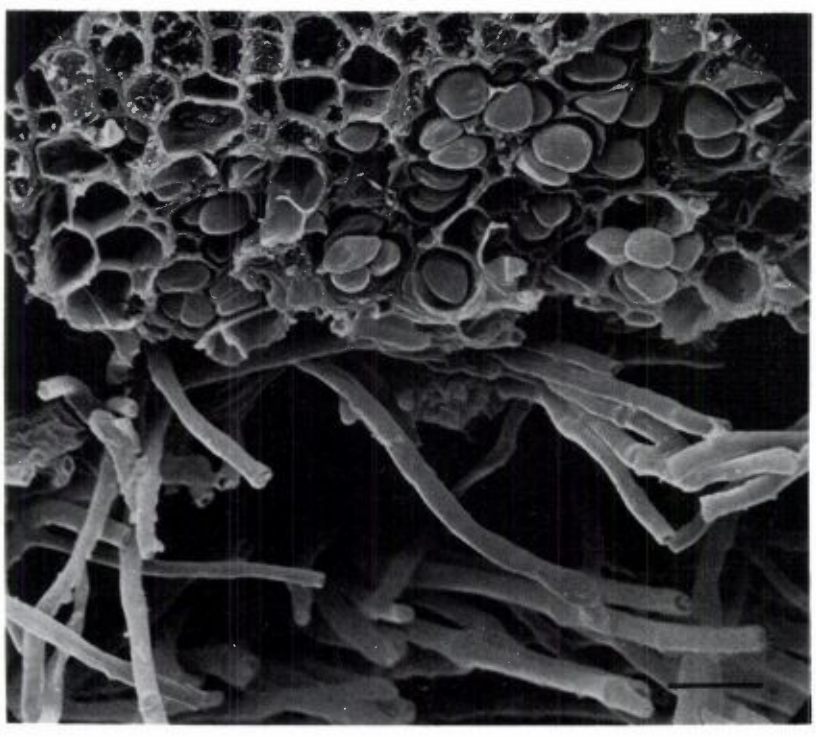

Figure 2. Scanning electron micrograph of the algal layer, cortex and part of the medulla of *Nephroma laevigatum* (Courtesy of Dr. J. Garty), bar = 10 μm.

For scanning electron microscopy, material was fixed overnight with 3% glutaraldehyde in Na cacodylate 0.1 M, pH 7.4, washed with the same buffer, dehydrated as above, dried with a critical point dryer and finally coated with gold. Examinations were by a Jeol 35 SEM operating at 25 kV.

Thallus material for thin section electron microscopy, shown in Figs. 4a and b, was fixed in 1% glutaraldehyde in Na cacodylate buffer, pH 7.2, for 48 hr. The samples were washed three times in the above buffer, postfixed in 1% tetroxide in the same buffer for 2 hr and in 1% aqueous uranyl acetate for 1 hr, dehydrated in a graded series of acetone and embedded in Spurr's Low Viscosity Epon. Sections were cut on a LKB Ultratom III using a diamond knife. Sections were stained with lead citrate and uranyl acetate. Micrographs were recorded on a Philips EM 300 operating at 80 kV.

Antiserum was raised in female, approx. 3 months old "gelbsilber" rabbits, against whole cells of cultured *Nostoc* isolates (αCN), grown in liquid BG-11 medium, shaken on a gyratory shaker (New Brunswick) at 100 rpm, under the conditions described above. Cells were washed with phosphate buffered saline (PBS, containing NaH_2PO_4, 20 mM; Na_2HPO_4, 80 mM; NaCl 2.25 M, pH 7.4) and injected according to the schedule described by Frensdorff et al. (1979). Thirty-five microliters of antiserum, at a final dilution of 1:1000 in PBS, was incubated in Eppendorf tubes with approx. 35 μl of 2×10^6 cells, cultured or fresh isolates, for 30 min at room temperature with continuous rotating (VortexGenie 2) and then centrifuged for 3 min in a Hettich Microliter centrifuge at 12, 000 rpm. The supernatant was discarded. The cells were then washed in PBS. Thirty-five microliters of fluorescein isothiocyanate-labeled goat anti-rabbit IgG antiserum (FITC-GARIG, Bio-Marker) diluted 1:30, were added. After 30 min incubation, the cells were washed as above. Finally, the cells were suspended in borate-buffered glycerol (glycerol — 0.2 M borate buffer, pH 9.5, 9:1). The preparations were examined with a Zeiss fluorescence microscope (BG-12 exciter filter, no. 50 barrier filter) using epi-illumination optics.

Controls used were: (1) Cells incubated with primary antibodies only; (2) Cells incubated with secondary antibodies only; (3) Cells incubated in preimmune serum.

3. Results and Discussion

Inside the intact thallus of *N. laevigatum* the *Nostoc* cells are separated, their shape is irregularly distorted and they reside usually as single cells, in honeycomb-like chambers, a tissue formed by the fungal partner (Figs. 1 and 2).

In culture, these cells revert to the filamentous growth form characteristic of free-living *Nostoc* species. Figs. 3a–d demonstrate stages of this reversion process. The separated and enlarged symbiotic cells plated, at first develop into densely coiled, ensheathed clusters (Fig. 3a). These clusters expand, apparently by continuous cell divisions, revealing dense colonies of thick-celled chains (Fig. 3b), which gradually (Fig. 3c) recover into long and slender filaments (Fig. 3d). Not all colonies change simultaneously. Several stages, originating from the same inoculum, can be seen in one and the same plate. Similar stages have been observed in *Nostoc* isolates of *Peltigera praetextata* and *P. horizontalis* (Boissière et al., 1987).

Lichenization seems to have a much more dramatic effect on the *N. laevigatum* — *Nostoc*, than on the *Nostoc* of *P. canina* in which the filamentous structure is sustained and filaments of more than 100 cells have been observed in the intact thallus, according to Bergman and Hällbom (1982). Koriem and Ahmadjian (1986) report on aggregates of single cells in the thallus of *P. canina*.

Striking differences between the symbiotic *Nostoc* cells of *N. laevigatum* and their cultured counterparts were observed in high magnification electron micrographs of thin section preparations. The cultured cells show the typical cyanobacterial (gram negative) cell wall, composed of a cytoplasmic membrane, peptidoglycan layer and an outer membrane (Jost, 1965) (Fig. 4a), whereas the cell wall of the symbiotic *Nostoc*, although built of the same components, has an unusual undulated appearance. Depending on the direction of sectioning, the outer membrane appears as finger-like projections (Fig. 4b), which in grazing sections (Fig. 4 – insert) appear as a fibrillar network on the cell surface. The periplasm appears unusually electron dense, obscuring the peptidoglycan layer. A somewhat similar undulation of the cell wall can be discerned in the micrograph of the lichenized *Nostoc* of *Collema crispum* (Boissière, 1976). These finger-like projections have some resemblance to the blebs described by Honegger (1984), which are released from the outer membrane into the gelatinous sheath surrounding symbiotic *Nostoc* cells.

The cytoplasm of the cultured cells show typical cyanobacterial cytological features: photosynthetic lamellae, ribosomes and fibrillar DNA. The cytoplasm of the symbiont appears at first not to have been fixed as well. Close examination, however, will reveal the double track membrane of the photosynthetic lamellae. The other cytoplasmic features may therefore be obscured by an unusually dense groundplasm indicative of the special physiological state of the symbiotic cell.

Antiserum raised against whole cells of the cultured *Nostoc* isolates (α CN) binds to the surface of these (homologous) cells (Fig. 5a). The correspond-

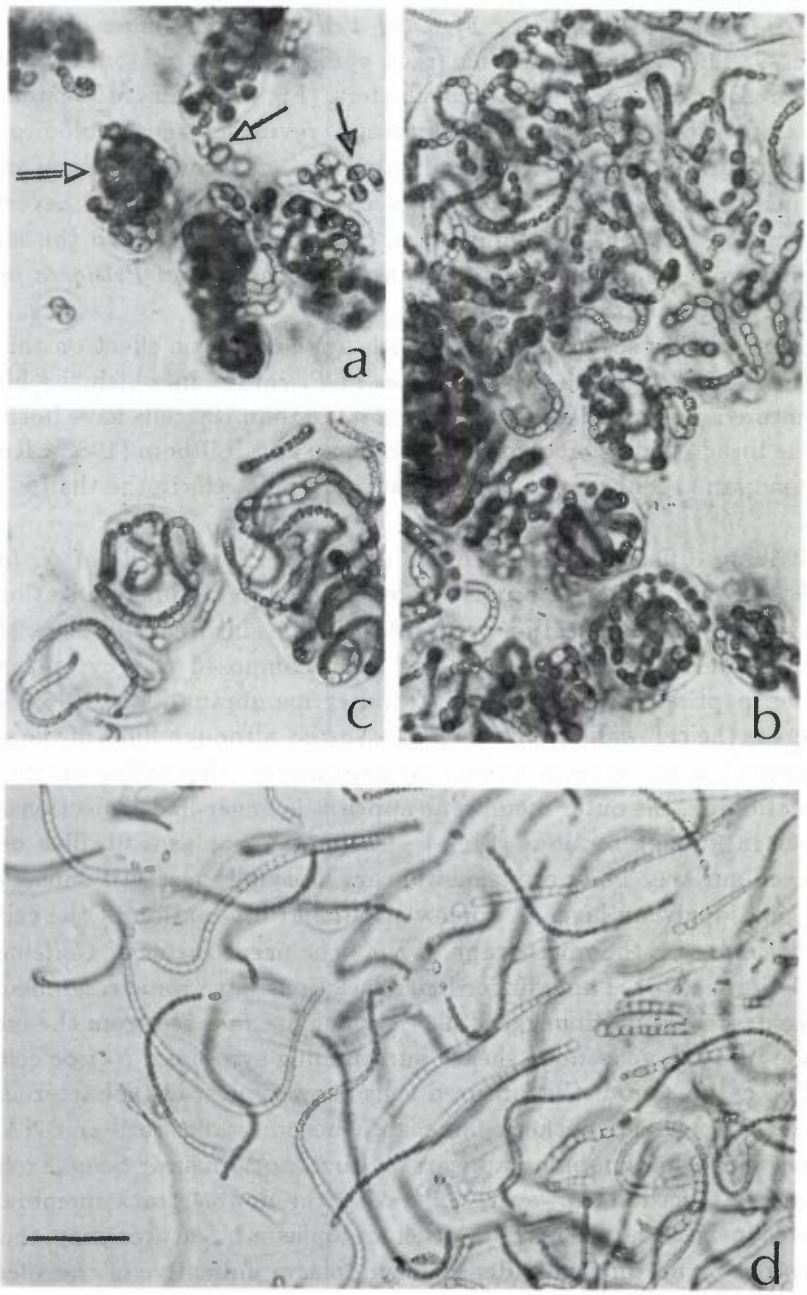

Figure 3. *Nostoc* isolates in culture: (a) enlarged symbiotic cells as inoculum (⟶), densely coiled, ensheathed clusters (⟹); (b) dense colonies of thick-celled chains; (c) stage between (b) and (d); (d) slender filaments, bar = 25 μm.

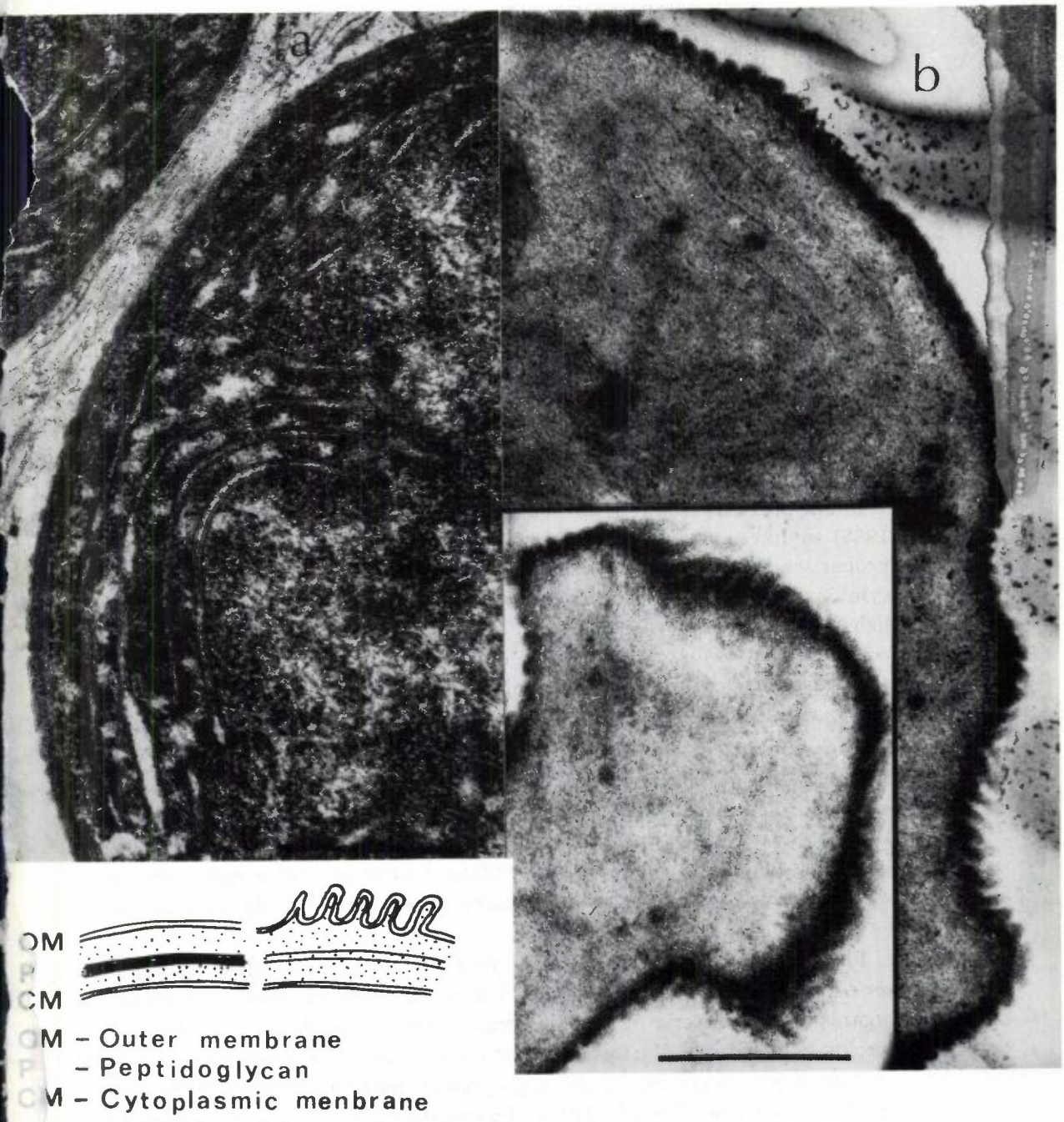

Figure 4. Electron micrographs of thin sections of cultured *Nostoc* isolates (a) and *Nostoc* symbiotic in the intact thallus (b); insert: grazing section from symbiotic *Nostoc*; bar = 0.5 μm.

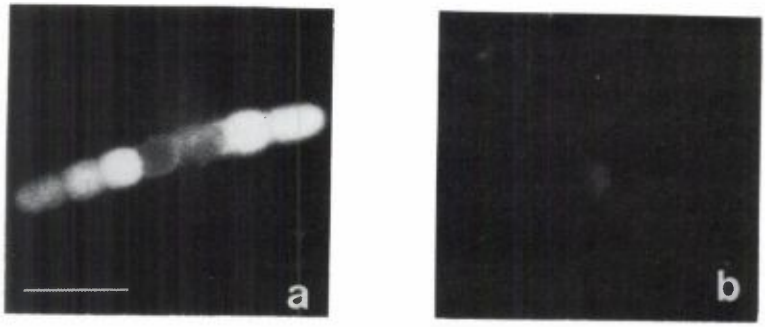

Figure 5. Indirect immunofluorescent staining of cultured *Nostoc* isolate (a) (homologous antiserum) and fresh isolate (b). (a) positive reaction, (b) negative reaction; all controls were negative, bar = 10 μm.

ing, freshly isolated (FN), i.e. the symbiotic *Nostoc* cells (immediately after separation from the intact thallus) did not react with the antiserum raised against CN (Fig. 5b), indicating molecular diversity of the cell wall surface.

In our previous studies (Bubrick and Galun, 1980a, b; Bubrick et al., 1982, 1985) we have shown that there are a number of differences in the cell wall properties between freshly isolated and cultured photobionts (green algal symbionts) from the lichen *Xanthoria parietina*, as well as from several other lichen species. Moreover, antiserum to the cultured *X. parietina* photobiont could be used to detect this alga in nature (Bubrick et al., 1984) suggesting similarity (or identity) of cultured photobionts and their free-living counterparts, at least in respect to surface molecules.

The modifications of cell surface properties correlate with their functions. At the initial stages of symbionts' interaction, cell surfaces play an essential role in selection and recognition (Galun and Bubrick, 1984; Galun 1988), whereas in the established lichen the photobionts act as transfer cells (Gunning and Pate, 1969) with an enlarged surface/volume ratio. The cell wall protrusions of the symbiotic *Nostoc* shown here (Fig. 4) obviously enlarge the surface area of these cells.

The microbionts of all well established symbiotic associations undergo a series of modifications as a result of their intimate relation with another organism, e.g. bacteria of the *Rhizobium* – legume symbiosis turn into bacteroids; fungi form a Hartig-net in ecto- and vesicular-arbuscular structures in endotrophic mycorrhiza; and algae, which participate in many types of symbiotic systems (Trench, 1979). The modifications of the *N. laevigatum* cyanobiont described here are, therefore, not surprising.

Acknowledgements

This work was partly financially supported by a grant from the National Council for Research and Development, Israel and the European Economic Community (to M.G.). The authors are greatly indebted to Dr. P. Keller, Ms. H. Lehr, Ms. I. Leizerovich and Ms. E. Sadovnic for their skillful technical help.

REFERENCES

Ahmadjian, V. 1974. Resynthesis of lichens. In: *The Lichens*. V. Ahmadjian and M.E. Hale, eds. Academic Press, N.Y., pp. 565–579.

Ahmadjian, V. 1982. Algal/fungal symbiosis. In: *Progress in Phycological Research 1*. F.A. Round and D.J. Chapman, eds. Elsevier Biomedical Press, Amsterdam, pp. 179–233.

Bergman, B. and Hällbom, L. 1982. *Nostoc* of *Peltigera canina* when lichenized and isolated. *Can. J. Bot.* **60:** 2092–2098.

Boissiére, M.-C. 1976. Cytologie du *Peltigera canina* (L.) Wild. en microscopie électronique. II. Le *Nostoc* libre et lichénisé; étude comparative. *Rev. Cytol. Biol. Veg.* **39:** 139–199.

Boissiére, J.-C, Boissiére, M.-C., Champion-Arnaud, P., Lallemant, R., and Wagner, J. 1987. Le cycle des *Nostoc* des genres *Peltigera* et *Collema* en cultures *in vitro* et dans le thalle lichénique. *Can. J. Bot.* **65:** 1468–1477.

Bubrick, P. 1988. Effect of symbiosis on the photobiont. In: *Handbook of Lichenology*. Vol. II. M. Galun, ed. CRC Press Inc., Boca Raton, FL, pp. 133–144.

Bubrick, P. and Galun, M. 1980a. Symbiosis in lichens: Differences in cell wall properties of freshly isolated and cultured phycobionts. *FEMS Microbiol. Lett.* **7:** 311–313.

Bubrick, P. and Galun, M. 1980b. Proteins from the lichen *Xanthoria parietina* which bind to phycobiont cell walls. Correlation between binding patterns and cell wall cytochemistry. *Protoplasma* **104:** 167–173.

Bubrick, P. and Galun, M. 1984. Cyanobiont diversity in the Lichinaceae and Heppiaceae. *Lichenologist* **16:** 279–287.

Bubrick, P. and Galun, M. 1986. Spore to spore resynthesis of *Xanthoria parietina*. *Lichenologist* **18:** 47–49.

Bubrick, P., Galun, M., Ben-Yaacov, M., and Frensdorff, A. 1982. Antigenic similarities and differences between symbiotic and cultured phycobionts of the lichen *Xanthoria parietina*. *FEMS Microbiol. Let.* **13:** 435–443.

Bubrick, P., Galun, M., and Frensdorff, A. 1984. Observations on free-living *Trebouxia* De Puymaly and *Pseudotrebouxia* Archibald, and evidence that both symbionts from *Xanthoria parietina* (L.) Th.Fr. can be found free-living in nature. *New Phytol.* **97:** 455–462.

Bubrick, P., Frensdorff, A., and Galun, M. 1985. Proteins from the lichen *Xanthoria parietina* (L.) Th.Fr. which bind to phycobiont cell walls. Isolation and partial purification of an algal binding protein. *Symbiosis* **1:** 85–95.

Frensdorff, A., Wald, A., and Galun, M. 1979. Relationship between unicellular cyanobacteria established by indirect immunofluorescence of intact cells. *Curr. Microbiol.* **3:** 105–110.

Galun, M. 1988. Effects of symbiosis on the mycobiont. In: *Handbook of Lichenology.* Vol. II. M. Galun, ed. CRC Press Inc., Boca Raton, FL, pp. 145–151.

Galun, M. 1988. Lichenization. In: *Handbook of Lichenology.* Vol. II. M. Galun, ed. CRC Press, Inc., Boca Raton, FL, pp. 153–169.

Galun, M. and Bubrick, P. 1984. Physiological interactions between partners of the lichen symbiosis. In: *Cellular Interactions.* H.F. Linskens and J. Heslop-Harrison, eds. Enc. Pl. Physiol. New Series. Vol. 17, Springer-Verlag, Berlin, pp. 362–401.

Gunning, B.E.S. and Pate, Y.S. 1969. Transfer plant cells with wall ingrowths specialized in relation to short distance transport of solutes: their occurrence, structure and development. *Protoplasma* **68:** 107–133

Honegger, R. 1984. Fine structure of different types of symbiotic relationships in lichens. In: *Lichen Physiology and Cell Biology.* D.H. Brown, ed. Plenum Press, New York, pp. 287–302.

Jost, M. 1965. Die Ultrastruktur von *Oscillatoria rubescens. Arch. Mikrobiol.* **50:** 211–245.

Koriem, A.M. and Ahmadjian, V. 1986. An ultrastructural study of lichenized and cultured *Nostoc* photobionts of *Peltigera canina Peltigera refescens* and *Peltigera spuria. Endocyt. C. Res.* **3:** 65–78.

Marton, K. and Galun, M. 1976. *In vitro* dissociation and reassociation of the symbionts of the lichen *Heppia echinulata. Protoplasma* **87:** 135–143.

Stanier, R.Y., Kunisawa, R., Mandel, M., and Cohen-Bazire, G. 1971. Purification and properties of unicellular blue-green algae (order Chroococales). *Bact. Rev.* **35:** 171–205.

Trench, R.K. 1979. The cell biology of plant animal symbiosis. *Ann. Rev. Plant Physiol.* **30:** 485–531.