ULTRASTRUCTURAL PECULIARITIES OF THE *USNEA BARBATA* (L.) MOTT. MYCOBIONT AND PHYCOBIONT CELLS IN "IN VIVO" AND "IN VITRO" CULTURE

AURELIA BREZEANU[1], DIANA VOICU[1]

An electronomicroscopical study of native *Usnea barbata* (L.) Mott. species thallus in comparison with symbiotrophic appearance that developed in "in vitro" culture was achieved, in order to help elaborating an experimental system appropriate for artificial biosynthesis of lichen thalli. The inner structure of phycobiont in "*in vitro*" culture is from many points of view similar to the cells belonging to native thallus. Some changes such as the reduction of the cell dimensions, or of the number of chloroplasts, thyllacoids and pyrenoid globules (pyrenoglobules) randomly spread are recorded. As a characteristic is the presence of the "storage droplets", a possible reserve of lipids, proteins and other metabolic substrates which can be used by cells for future differentiation processes. These structures may also be involved in enabling the phycobiont to endure adverse environmental conditions and may represent adaptative modifications induced by "in vitro" culture conditions.

Key words: lichen, photobiont, mycobiont, *Usnea*, "in vitro" culture.

INTRODUCTION

Although all over the world as well as in some European countries there is interest for *in vitro* culture and artificial biosynthesis of lichens thalli, with the aim to produce high quantities of biomass for biotechnological applications (Armitage and Howe, 2006, 2007, Howe and Armitage, 2002, Ahmadjian V., 1990a) (Ahmadjian, V., L.A. Russell, and K.C. Hildreth, 1980), in Romania this kind of investigations are very scarce (Toma N. and Toma A., 1996, Toma *et al.*, 2001, Toma *et al.*, 2007).

In the past period of time, lichens have been extensively used in pharmaceutical investigations in various European countries. Many species have identified pharmaceutical effects such as: demulcent, febrifuge, antitumoral, astringent, tonic, purgative, etc. In some cases, for such identified characteristics, species such as *Usnea barbata*, *Xanthoria parietina*, *Parmelia saxatilis* were used (www.1911.encyclopedia.org/Lichens).

A considerable number of lichen species, European and exotic, seem to be endemic and that is why there is a great interest for their conservation and repopulation of areas of interest.

[1] Institute of Biology Bucharest, 296 Splaiul Independenței, 060031, Bucharest, Romania, E-mail: aurelia.brezeanu@ibiol.ro

Still, of great importance is the capacity of many species of energetic supplying the humans and animals because the lichens thalli contain starchy substances and, in some cases, small quantities of saccharine of mannite nature. *Cetraria islandica* represents one of the most useful nutritious species (www.1911.encyclopedia.org/Lichens). There are no thoroughly cytological studies, particularly at electronmicroscopical level, in order to understand the unique morpho-cytological and physiological peculiarities of these symbiotroph biological systems in "in vitro" culture. Without a deeper understanding of the cellular and molecular mechanisms involved in the establishment of the mutual relationships between the mycobiont and the phycobiont it will be extremely difficult to elaborate a proper experimental system for the *in vitro* culture of some lichen species.

The first developments of biotroph forms were reported by Y. Yamamoto, through cultivating "*in vitro*" explants of *Usnea* (Yamamoto, 1993). These are characterized by a poor morphoanatomical organization and an unstable functional potential. Subsequently, in 1980 Culberson also worked on more species belonging to the genera *Usnea*, *Cladonia* and *Parmelia*.

The aim of this paper is to realize an electronomicroscopical study of native *Usnea barbata* thallus, in comparison with symbiotrophic formations, which is developed in "in vitro culture" in order to elaborate an experimental system for artificial biosynthesis of lichen thalli. These preoccupations are useful for both classical and modern researches for the purpose of elucidating some recognition and compatibility phenomena between both partners and biotechnological applications in phytotherapeutics.

MATERIAL AND METHODS

Biological material was represented by *Usnea barbata* (L.) Mott. species (Fig. 1) fresh thalli collected from resinous tree barks (spruce fir, pine) and deciduous (beech, sycamore, maple tree) in mountainous regions. For native thallus studies on electron microscopy the conventional method was used (Mascorro and Bozzola, 2007). The fresh thalli were shaken to remove debris and washed in cold running tap water for 10 min. Small pieces of about 2 mm were fixed in a solution of 3 % glutaraldehyde in Na cacodylate buffer 0.2 M, at pH 7, over night, at 4 °C and postfixed in a solution of OsO_4 1 % over night at 4°C in the same buffer solution (0.1 M). After dehydration by using a gradual series of ethanol and propylene oxide, specimens were infiltrated in Epoxy resin; the sections were cut on a LKB ultramicrotome using a diamond knife and after being contrasted by the Reynolds method (1963) the sections were examined in an EM – 125 (Selmi-Ucraina) electron microscope.

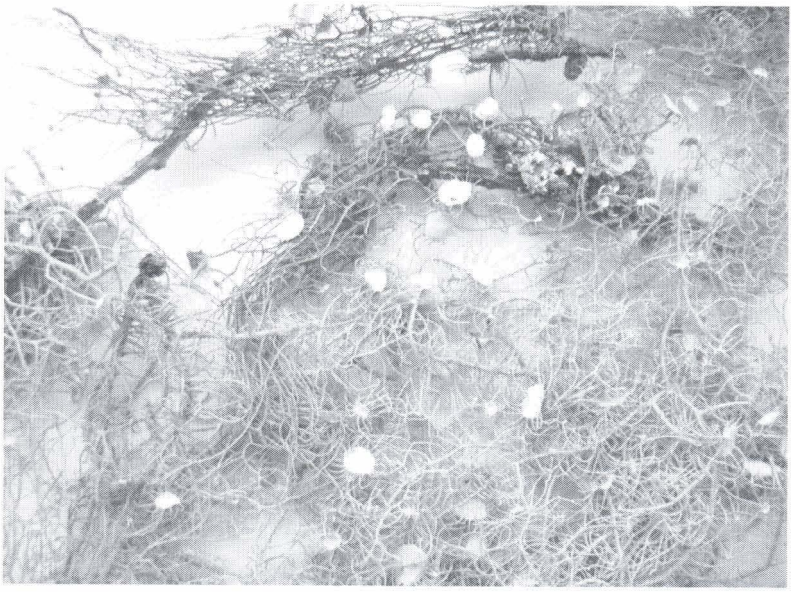

Fig. 1. *Usnea barbata* native thallus.

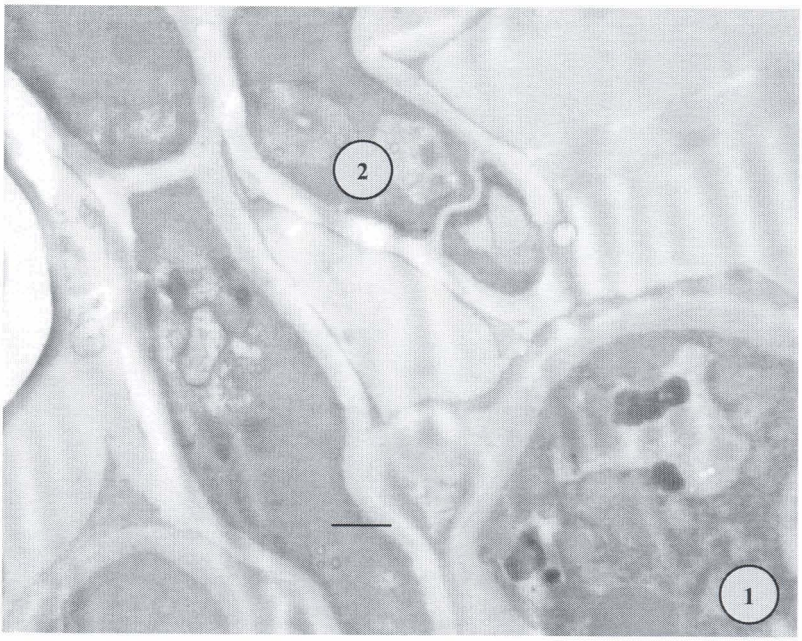

Fig. 2a.

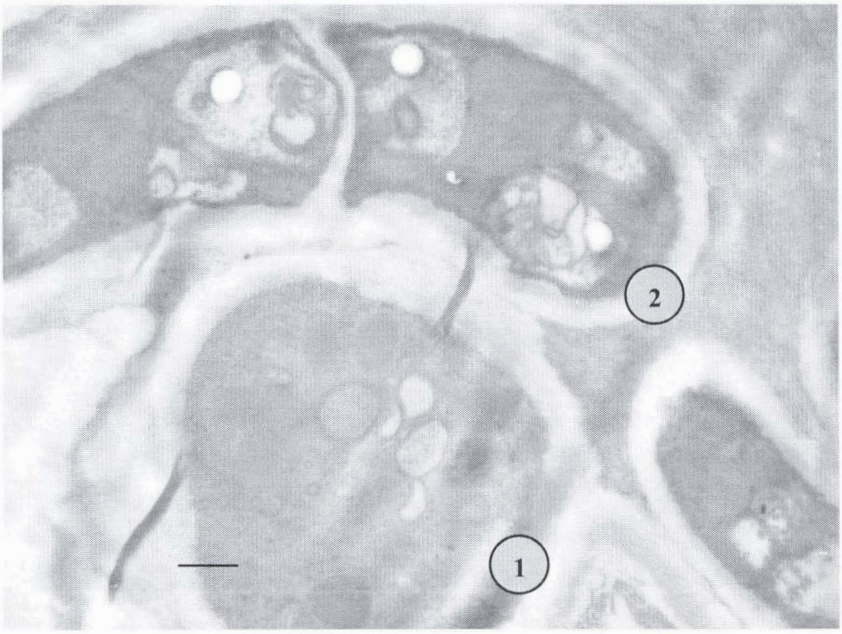

Fig. 2b.

Fig. 2a, b. Phycobiont cell (1) surrounded by few mycobiont cells (2) in a native thallus of *Usnea barbata* (L.) Mott. The scale bar is 1 μm.

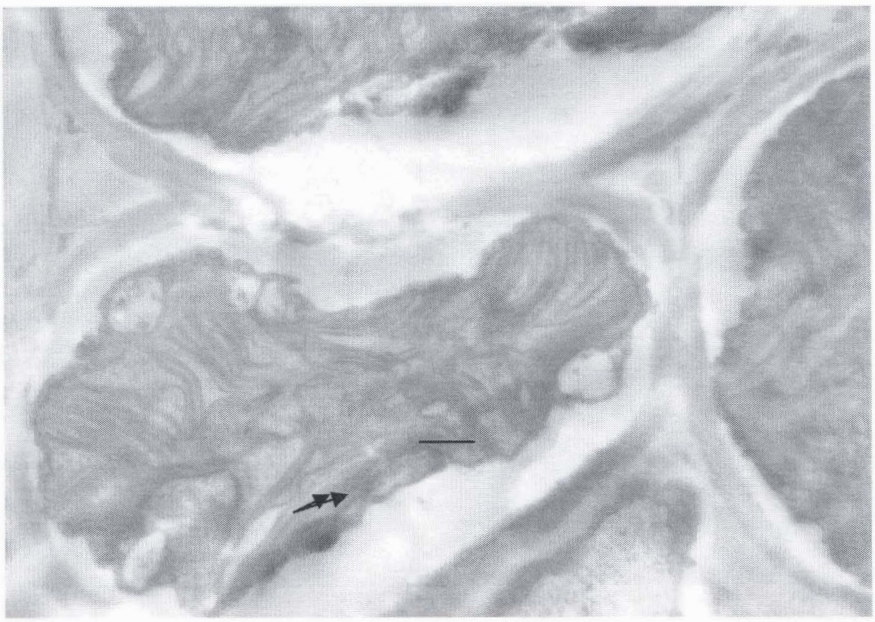

Fig. 3a.

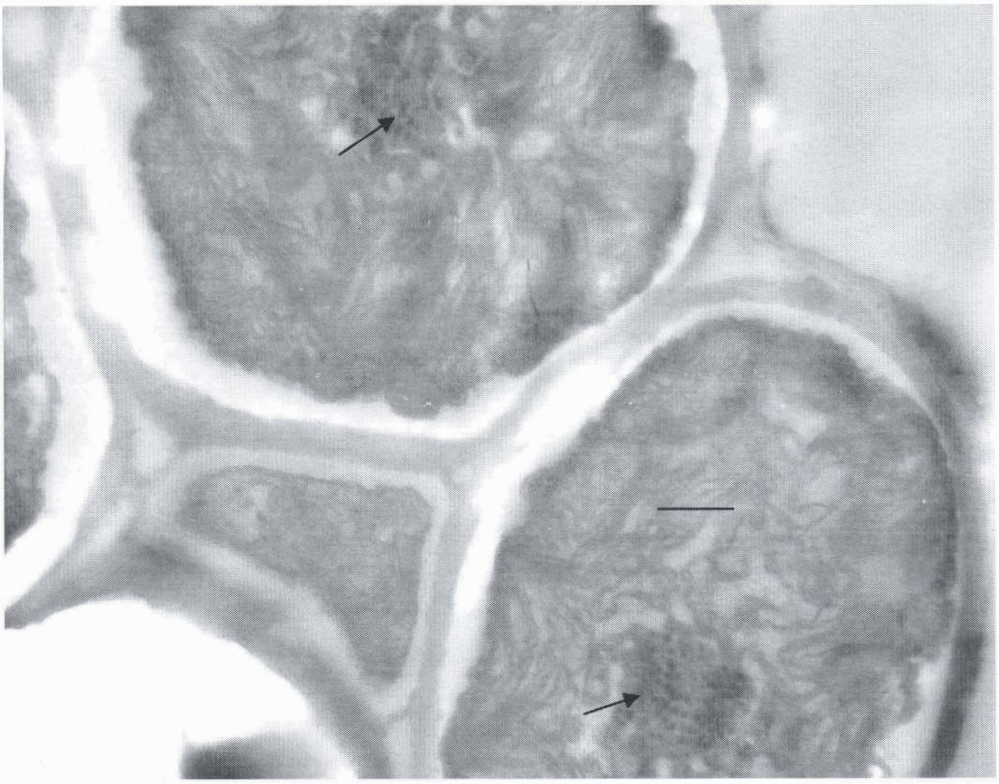

Fig. 3b.

Fig. 3a, b. Cross sections through a phycobiont of *U. barbata* in native thallus. Note the parallel arrangement of the thylakoid and the presence of pyrenoglobuli (see arrows). The scale bar is 1 μm.

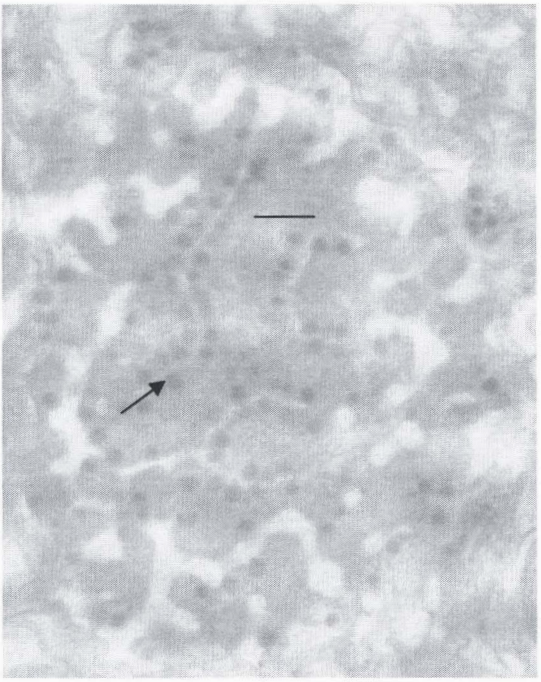

Fig. 4. A pyrenoid penetrated by a single thylakoid and pyrenoglobuli arranged along the chloroplast thylakoid (see arrows).

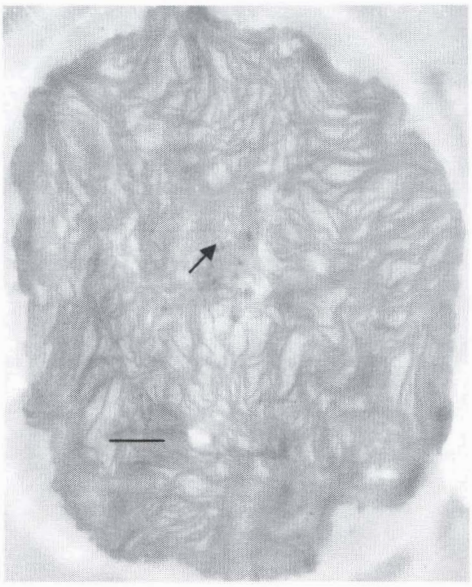

Fig. 5. Cross sections through phycobionts. Some structural details can be observed. Grouping of pyrenoglobules (see arrows).

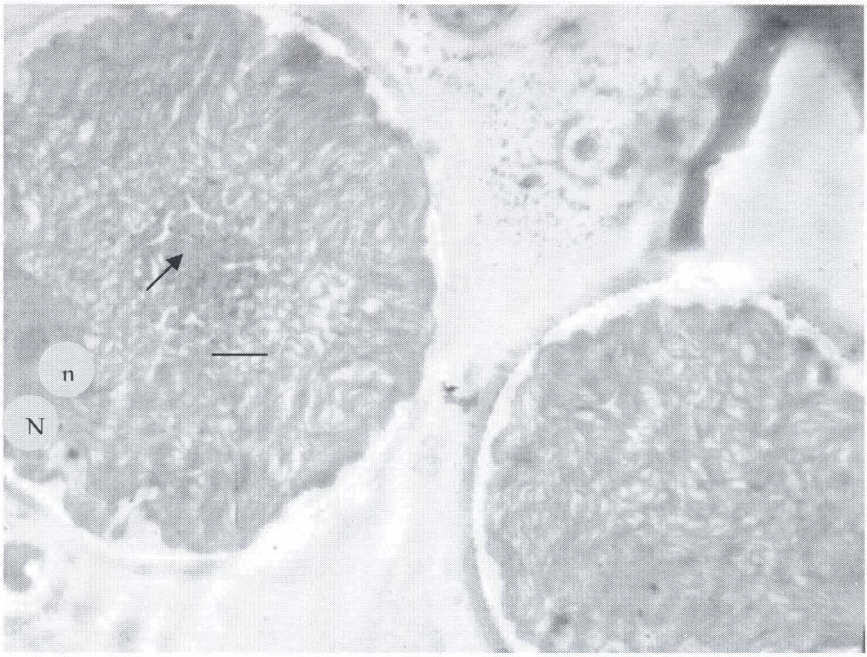

Fig. 6. Cross sections through phycobionts. Some structural details can be observed. Grouping of pyrenoglobules (see arrow), nucleus with nucleolus (N, n). The scale bar is 1 μm.

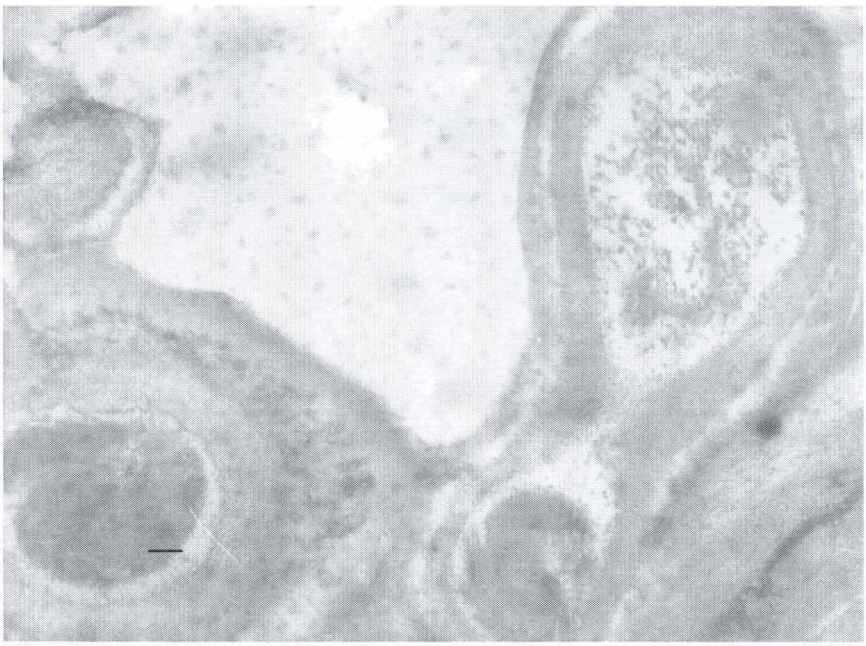

Fig. 7. Electronomicroscopical image of the mycobiont cells in native thallus of *Usnea barbata*. The three stratification structures of the cell wall can be observed. The scale bar is 1 μm.

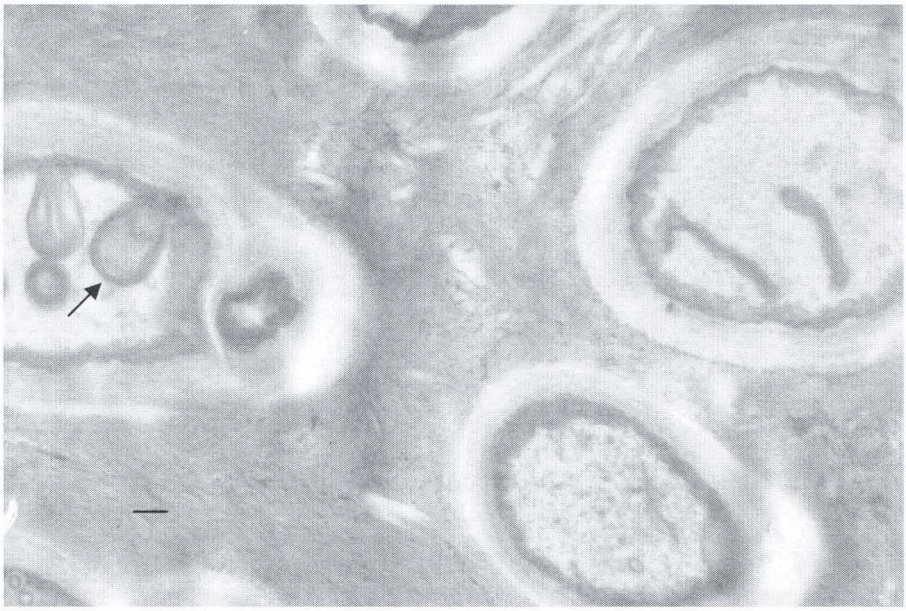

Fig. 8. The highly folded plasmalemma sometimes connected with vacuolar and myelin figures are present (see arrow). The scale bar is 1 μm.

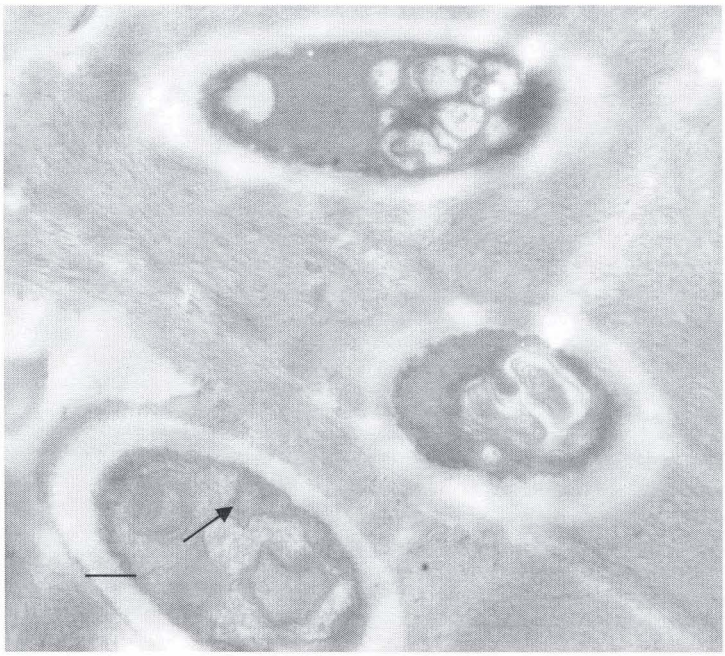

Fig. 9.

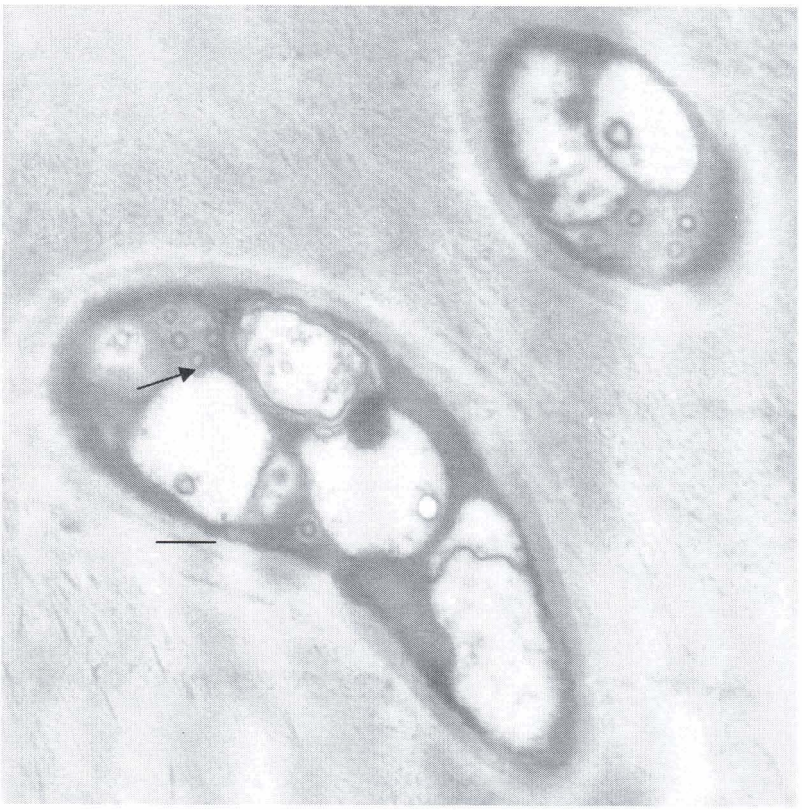

Fig. 10.

Figs. 9-10. Oil bodies at the periphery of the mycobiont cytoplasm (see arrow). The scale bar is 1 μm.

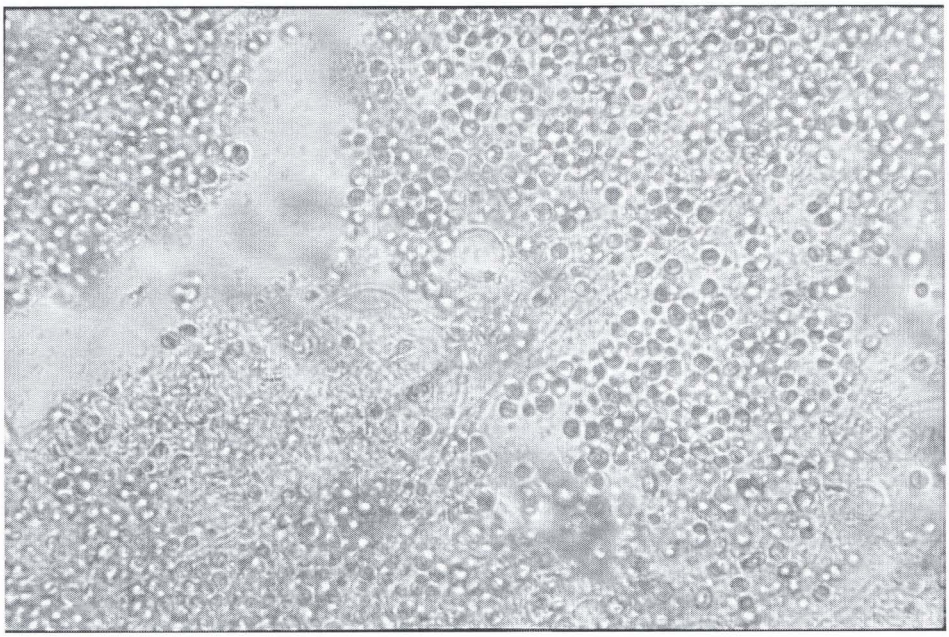

Fig. 11.

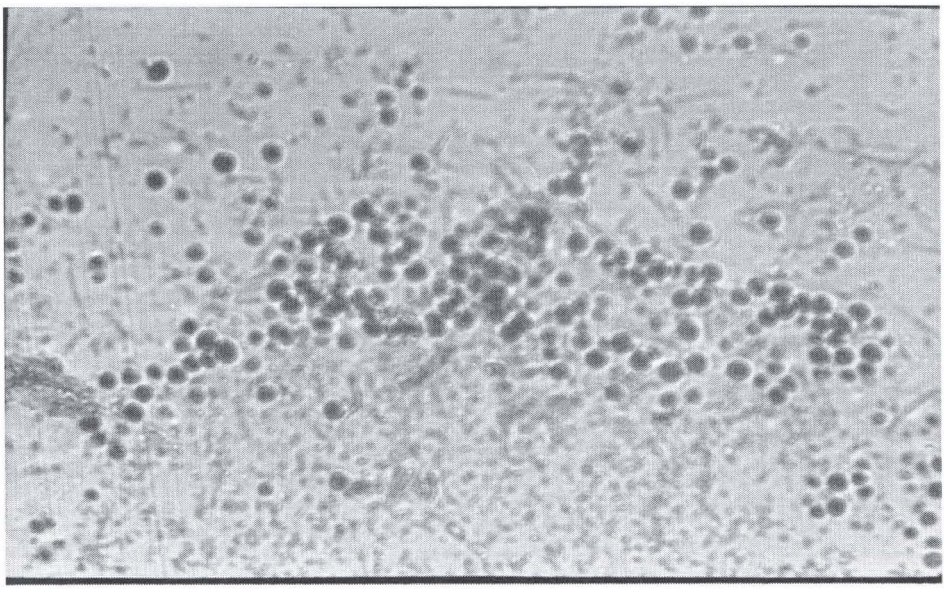

Fig. 12.

Figs. 11-12. Thick sections through the symbiotrophic formations of soredial type regenerated by "*in vitro*" culture. Numerous algae cells surrounded by fungal hyphae (see arrow) can be observed (oc. 10, obj 40). The scale bar is 1 μm.

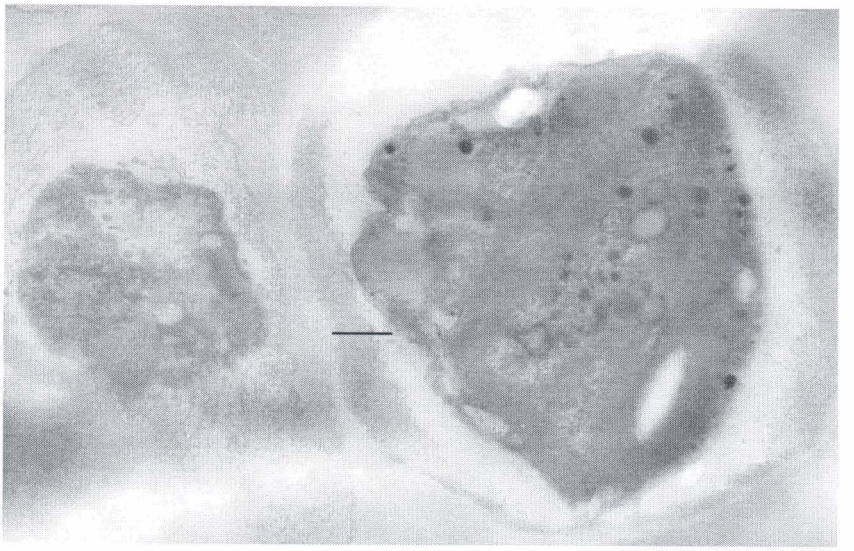

Fig. 13a.

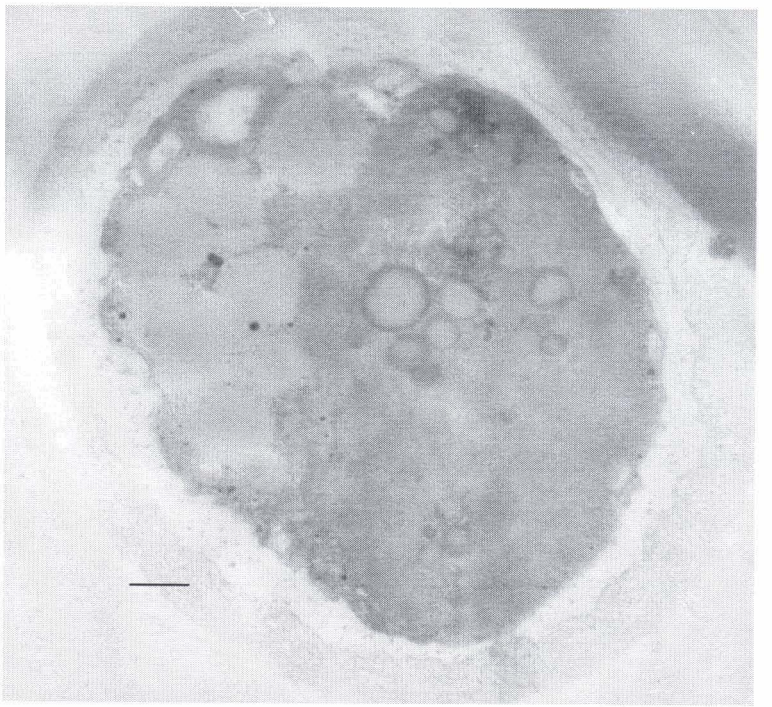

Fig. 13b.

Fig. 13 a, b. The ultrastructural features of the phycobiont cells in symbiotrophic formations regenerated in "in vitro" culture. The small number of thylakoids and pyrenoglobules randomly spread are evident. The scale bar is 1 μm.

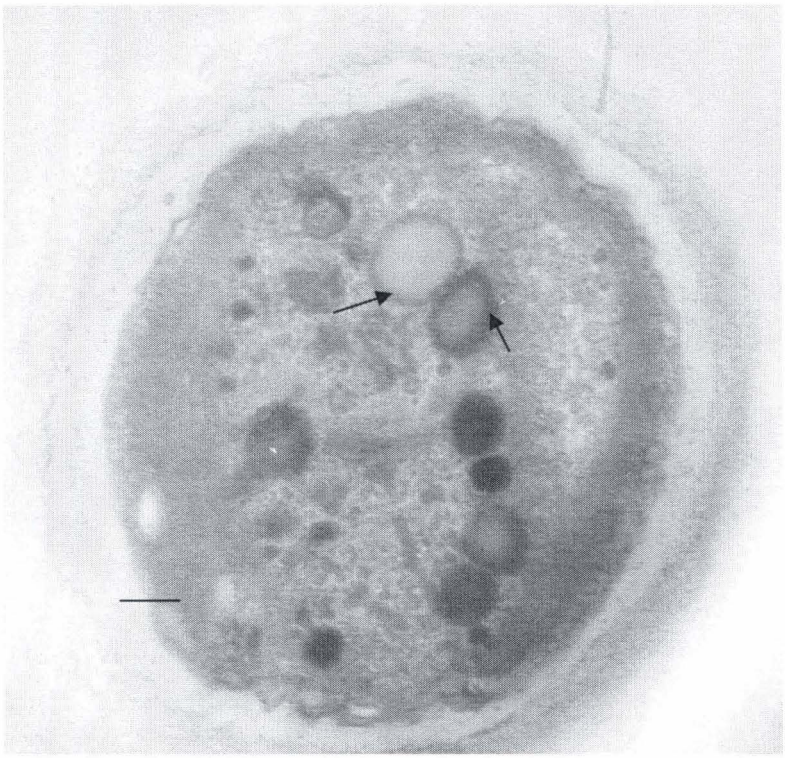

Fig. 14.

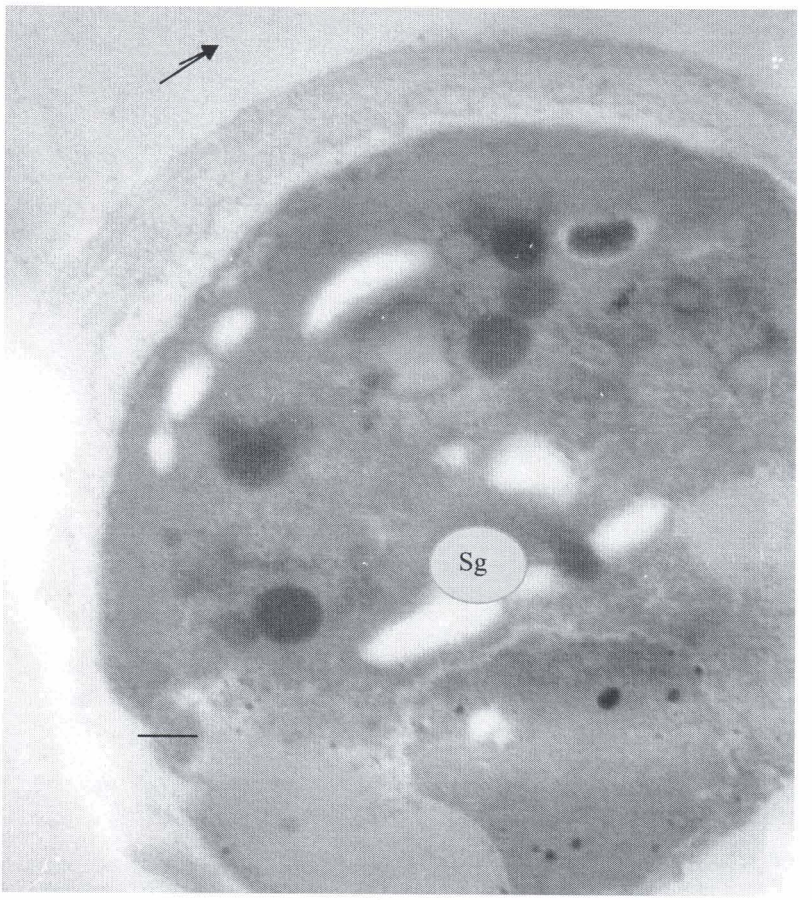

Fig. 15.

Figs. 14-15. The presence in the cytoplasm of "storage droplets (see arrow)" or "storage bodies" containing reserve material as well as starch granules (Sg) is revealed. The scale bar is 1 μm.

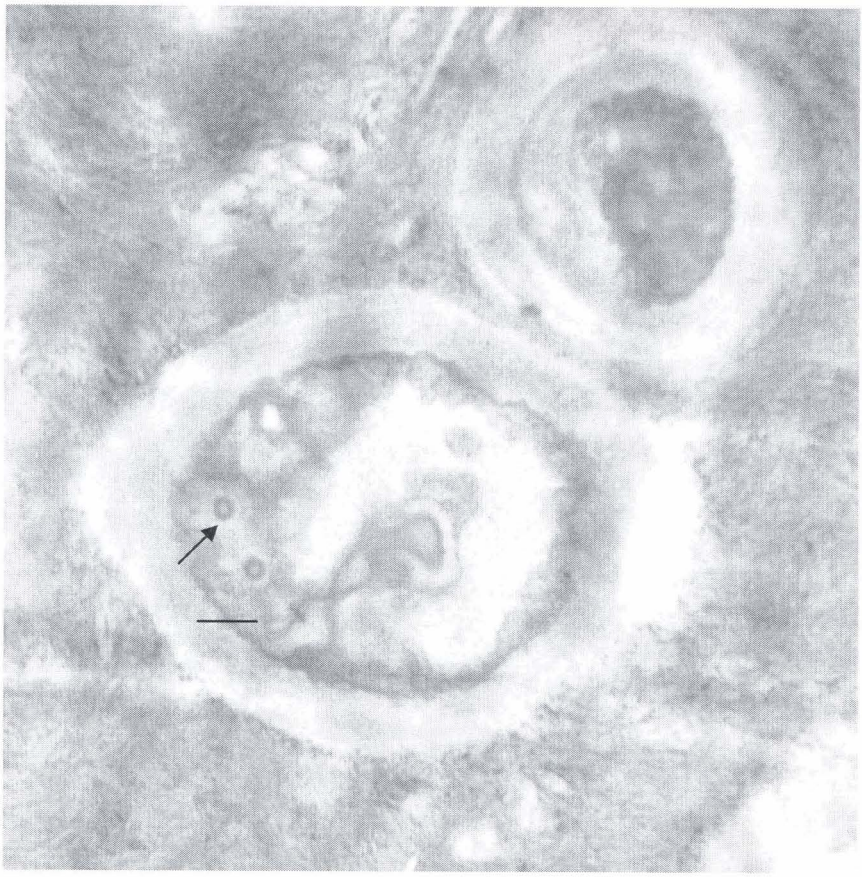

Fig. 16. Cross section through the mycobiont cell in symbiotrophic formations regenerated in *in vitro* culture. Concentric bodies or ellipsoidal bodies are visible (see arrow). The scale bar is 1 μm.

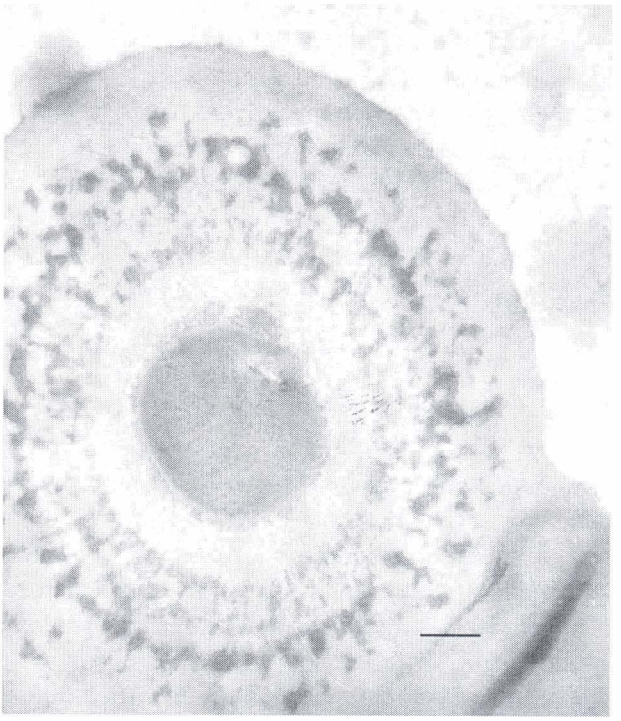

Fig. 17.

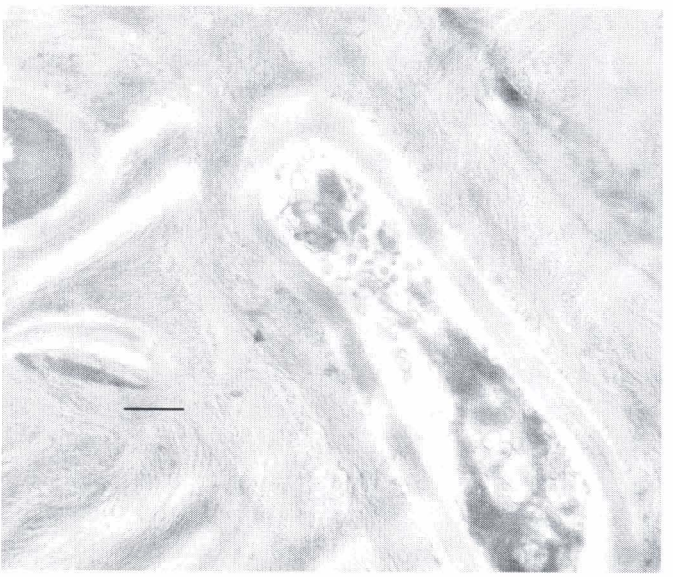

Fig. 18.

Figs. 17-18. Details of mycobiont cell wall structure in "in vitro culture". The scale bar is 1 μm.

For electronomicroscopical analyses of the symbiotrophic thalli that were redifferentiated by "*in vitro*" culture using cellular aggregates of the thallus, the same method was used but for a higher stability of these samples after glutaraldehyde fixation it was necessary to include them in agar (2 %).

To initiate lichen tissue culture from vegetative thalli Yamamoto *et al.* (1993) method was used (Voicu and Brezeanu 2008), lichen tissue culture has been grown in a culture chamber at a temperature of ± 2 ° C, a light intensity of 1000 lux and a photoperiod of 16 h light.

RESULTS AND DISCUSSIONS

a) *Ultrastructural characteristics of the mycobiont and phycobiont cells from native lichen thallus*

The transmission electronomicroscopic observations on native *Usnea barbata* thallus revealed general peculiarities of the thallus cells of other lichens described by different authors (Armitage and Howe, 2007, Toma *et al.*, 2007).

As is very well known that lichens are a life form composed by a fungus called the "mycobiont" growing in a close relationship with some algae known as the "phycobiont" (Howe and Armitage, 2002). Lichen algae and fungi are woven together forming "tissues-like structures" that resemble the tissues of unrelated "higher plants" in a general fashion (Armitage and Howe, 2006). Generally, many fungal cells are surrounding one phycobiont cell (Fig. 2). The chloroplast occupies most of the cell alga volume and presents numerous lamellae randomly distributed that extend throughout the stroma. Some of the thylakoids at the periphery of the chloroplast are parallelly disposed (Fig. 3). Within the chloroplast structure resides a large central pyrenoid that can be penetrated by a single thylakoid (Fig. 4). Pyrenoglobuli are generally not numerous, often located in the peripheral position, or arranged along the chloroplast thylakoid (Fig. 5) which traverse the pyrenoid and sometimes they are distributed throughout the pyrenoid. The functions of the pyrenoglobuli are not clear so far but probably serve as storage centres for lipids and carotenoid pigments. Several other functions have been proposed for pyrenoglobuli including, involved in the development of chloroplast lamellae, septum (Brown and Wilson – 1968) and in the protection of algae from radiation (Jacobs and Ahmadjian 1969). It has been also proposed that they serve as respiratory substrates under conditions of water stress. The cytoplasm occurred as a thin layer between the chloroplast and the cell wall and contained a nucleus with a larger nucleolus (Fig. 6). Starch granules in our specimens were in the shape of small disks outside the pyrenoid sometimes in addition to pyrenoglobules.

Cross sections of the mycobiont hyphae in these specimens revealed a thick cell wall that consists of an outer layer, multilaminated layer in the middle and amorphous inner layer adjacent to the plasmalemma (Fig. 7). Its thickness also varied with hyphal age. The plasmalemma is highly invaginated and folded and sometimes connected with a myelin-like structure (Fig. 8). The invaginations of the fungal plasmalemma provide an increased protoplast surface allowing a high metabolic activity that can be an "adaptation" which stimulates an exchange between symbionts (Peveling 1973).

The fungal cytoplasm includes oil droplets and storage granules, concentric bodies, oil bodies occurred along the periphery as an electronolight spherical area and it seems to be a peculiarity for old cells. They appear to be intimately connected in the internal membrane system of the cell (Figs. 9-10). Concentric bodies are located in the proximity of the plasmalemma and probably they are involved in membrane synthesis and in the movement of protein molecules although Ahmadjian (1990a) suggested that they could be even viruses (Armitage and Howe 2007).

b) *Ultrastructural features of the symbiotrophic formations regenerated in "in vitro" culture*

The interest for "*in vitro*" lichen culture experiments was stimulated by the encouraging results of some scientists. Yamamoto *et al.* (1993), for example, developed an original method to initiate lichen tissue culture using vegetative thalli and established "*in vitro*" system tissues isolated from three fruticose genera such as: *Alectoria*, *Ramalina* and *Usnea*.

Our previous studies (Voicu and Brezeanu, 2008) revealed that by "*in vitro*" culture of cell aggregates of *Usnea barbata* thallus symbiotrophic formations of "sorediale" type with an intense growth were regenerated. This suggested us that the methods used by us could be a possibility of lichen multiplication, in "in vitro" system.

In thick sections (1 μm) through these symbiotrophic formations numerous algae cells surrounded by fungal hyphae were observed (Fig. 11). The BBM liquid nutritive medium supplemented with 0.5 mg/l kinetin offers satisfactory conditions for "in vitro" culture.

On ultrathin sections it is often revealed that grouping of round algae cells is very frequently associated or not with mycobiont. As a characteristic their dimensions reduced significantly. A reduction in their height up to 50 % was observed (Fig. 12). The inner structure of phycobiont is from many points of view similar to cells belonging to the native thallus. However, some modifications appeared and the most affected is the chloroplast with a small number of thylakoids and a small number of pyrenoglobules which are randomly spread (Fig. 13). Another characteristic is the presence of so-called "storage droplets" (Armitage and Howe 2007) (Figs. 14, 15).

These kinds of storage bodies are associated with the cytoplasm and may represent reserves of lipids, proteins and other metabolic substrates. Two categories of storage bodies were identified. One of them presents electronodense content and the other one electron light content, probably in relation with their composition. Their presence may be related with an inhibitory effect of the culture conditions especially on the thylakoid system development. Possibly, they are involved in enabling of phycobiont to endure adverse environmental conditions, and may represent adaptative modification.

The fine structure of the mycobiont is from many points of views similar with that of the old mycobiont from the native thallus. Concentric bodies or ellipsoidal bodies are visible and are present in the cytoplasm like small formations, singly or in clusters with a core body surrounded by electron transparent halos (Fig. 16).

The cell wall also consists of the cell layers and the thickness of the outer layer, varying according to the age (Figs. 17-18). As a peculiarity is that the outer layer consists of an amorphous electronodense material and a fibrillar matrix and the plasmalemma is highly folded.

In these experimental conditions, in this symbiotrophic stage, the algal cells do not present haustoria.

These experimental results enabled us to appreciate that this procedure, for "in vitro" culture of *Usnea barbata* thallus, could be improved and represents a promising starting point for artificial biosynthesis of lichen thalli.

It is known that the evolution of the symbiotrophic structures depends on the culture medium and that the soil and bark extract present a positive effect.

For the future we are intending to test more complex nutritive media particularly by enrichment with soil extract and optimal light regime.

CONCLUSIONS

1. TEM studies on native *Usnea barbata* (L.) Mott. thallus revealed general structural peculiarities of the thallus cells of other lichens described in different papers;
2. *Usnea barbata* (L.) Mott. is a species which responds to the *in vitro* conditions, the cells aggregates grinding paste thallus being an efficient source of inoculum;
3. The BBM liquid nutritive medium supplemented with 0.5 mg/l kinetin offers satisfactory conditions for *in vitro* culture;
4. The ultrathin inner structure of phycobiont in *in vitro* culture is; from many points of view, similar to the cells from the native thallus. Some changes were identified such as the significant decrease of the cell size; also most affected were the chloroplasts which showed a small number of thylakoids

and pyrenoglobules randomly spread and the presence of "storage droplets". This could represent adaptative modifications to *in vitro* culture conditions (the nutritive medium composition and the intensity of light, particularly);

5. The fine structure of the mycobiont is similar from many points of view with that of the old mycobiont from the native thallus;
6. The procedure used by us for *Usnea barbata in vitro* culture represents a promising starting point for artificial biosynthesis of lichen thalli.

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REFERENCES

1. Ahmadjian V., L.A. Russell, and K.C. Hildreth, 1980, *Artificial reestablishment of lichens. I. Morphological interactions between the phycobionts of different lichens and the mycobionts Cladonia cristatella and Lecanora chrysoleuca.* Mycologia, **72:** 73-89.
2. Ahmadjian V., 1990a, *What have synthetic lichens told us about real lichens? Contributions to Lichenology*. In honour of A. Henssen. Bibliotheca.
3. Armitage H. M.and F. G. Howe**,** 2006, *Lichens in cross section: Evidence for design and against macroevolution*, CRSQ, **42:** 252-264.
4. Armitage H. M. and F. G. Howe, 2007, *The ultrastructure of Lichen Cells*, *Support Creation not Macroevolution*, CRSQ, **44**, 1, 40-53.
5. Jr. Brown R. M. and R. Wilson, 1968, *Electron microscopy of the lichen Physic aipolia* (Ehrh) J. of Phytology, **4:** 230-240.
6. Howe G. F. and M. Armitage, 2002, *Lichens: A partnership for life*, CRSQ, **39**, 81-88.
7. Culberson C. F. and V. Ahmadjian, 1980, Artificial reestablishment of lichens.II. Secondary products of resynthesized *Cladonia cristatella* and *Lacanora chrysoleuca.* Mycologia, **72:** 90-109.
8. Jacobs B. J. and V. Ahmadjian, 1969, *The ultrastructure of lichens* I., *A general survey*, J. of Phycology, **5**, 3, 227-290.
9. Jacobs B. J. and V. Ahmadjian, 1971, *The ultrastructure of lichens II. Cladonia cristatella: The lichen and its isolated symbionts*. J. of Phycology, **7**, 1, 71-82.
10. Mascorro A. J. and J. J. Bozzola, 2007, *Processing Biological Tissues for Ultrastructural Study, Electronmicroscopy, Methods and Protocols*, Second Edition, Ed. by J. Kuo, Humana Press, 19-35.
11. Peveling E., 1973, *Fine structure.* In: V. Ahmadjian and M. E. Hale (eds.), *The Lichens*, Academic Press, New York, 147-182.
12. Reynolds E. S., 1963, *The use of lead citrate at high pH as an electron – opaque stain in electron microscopy*. J. Cell Biol, **17**, 208.
13. Toma N. and A. Toma, 1996, *Cercetări citologice asupra unor formațiuni simbiotrofe obținute prin cultura "in vitro" a fragmentelor taline de Usnea barbata Mott.*, Acta Bot. Horti Bucurestiensis, 85-90.
14. Toma N., L. Ghetea, R. Nitu and D. I. Corol., 2001, *Progress and Perspectives in the Biotechnology of Lichens*, Roum. Biotechnol. Letters, **6**, 1, 1-15.

15. Toma N., D. Voicu and F. A Toma, 2007, *Aspecte ale organizării ultrastructurale a talurilor de Pseudoevernia furfuracea (L.) Zopf, Xanthoria parietina (L.) Th. Fr.*, An. Grad. Bot. Univ. Macea, **1**, 78-93.
16. www.1911encyclopedia.org/Lichens.
17. Voicu D. and A. Brezeanu, 2008, *Comportamentul în cultură "in vitro" a speciilor lichenice Xanthoria parietina Beltr. şi Cetraria islandica Ach.*, Biotehnologie vegetală pentru secolul XXI, Ed. Risoprint, Cluj-Napoca, 101-112.
18. Voicu D. and A. Brezeanu, 2008, *In vitro reactivity of Usnea barbata (L.) Mott.* Rom. J. of Biol.-Plant Biol., **53**, 2.
19. Yamamoto Y. *et al.*, 1987, *Effects of culture conditions on the growth of Usneaceae lichen tissue culture*, Plante Cell Physiology, **28**, 1421-1426.
20. Yamamoto Y., Mizuguchi and Yamada, 1993, *Using lichen tissue cultures in modern biology*, The Bryologist, **96** (3), pp. 384-393.