ULTRASTRUCTURAL CHARACTERIZATION OF THE *IN VITRO* GAMETOPHYTE OF *BUCEGIA ROMANICA* RADIAN – A RARE LIVERWORT

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Ultrastructural characterization of the *in vitro* propagated gametophyte of *Bucegia romanica* Radian is presented in this paper. We have previously established an experimental protocol for *ex situ* conservation of this rare liverwort. Knop mineral nutritive medium without any source of carbon, temperature between 22 °C–25 °C and 16 hs light/8 hs dark photoperiod represent the experimental growth conditions. No anomaly of the thallus structure and ultrastructure has been observed. Much more, strong similarities at this level between Bucegia and other Hepatics were noticed, which allow us to consider the *in vitro* system established by us suitable for conservative purpose. The evolutive significance of the cell peculiarities, especially plasmodesmata biogenesis and features, was also underlined.

Key words: *Bucegia romanica* Radian, *in vitro* conservation, ultrastructure analysis.

INTRODUCTION

Bucegia romanica Radian is an interesting thallose liverwort from *Marchantiaceae* family, a species in Europe confined to the Carpathian chain. It was proposed by Romania to be included in the Directive for Habitats Species Conservation (Directive 43/92/EEC).

Because of its status of rare species, the establishment of a conservation strategy presents a special interest. In this frame, "*in vitro*" culture system has been used. A better understanding of the "*in vitro*" culture methods requires an efficient cytological study (photonic and electronomicroscopic).

In contrast with other hepatics (*Bazania, Lophozia, Marchantia*), structural and ultrastructural analyses of *Bucegia romanica* thallus are extremely scarce, almost inexistent.

MATERIAL AND METHODS

"*In vitro*" cultures were initiated from sporangia with spores. Biological material was collected from Bucegi National Reservation, at 2000 m altitude.

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After tap water washing for one hour, sporophytes were surface sterilized with 1% (w/v) solution of sodium dichloroisocyanurate (NaDCC) for 6 min, without detergent and followed by 3 sterile water washes (Duckett *et al.*, 2004; Rowntree, 2006). Spores were inoculated on Knop minimal nutritive medium (1865) solidified with 2g/l Gelrite, without sucrose or other carbohydrate source and adjusted to pH 5.8. Spores germinated 1-2 months after the inoculation. Cultures were maintained and monthly subcultivated on the same medium, in 6 diameter Petri-dishes, at 22.5°C (±3.5 °C), under a 16 h light/8 h dark photoperiod (Fig. 1).

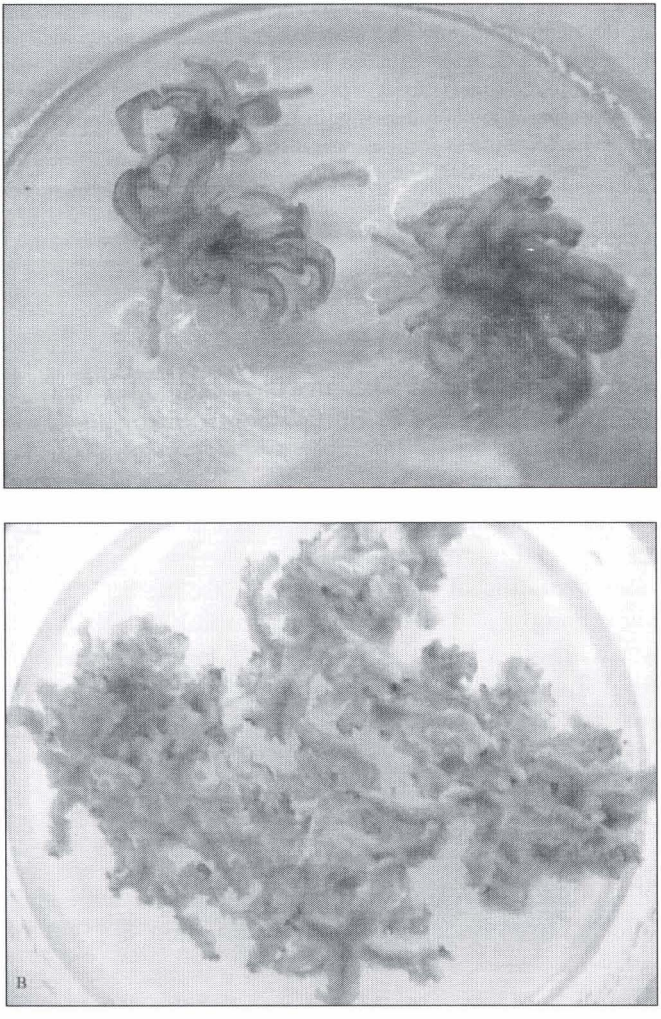


Fig. 1. *Bucegia romanica* species in *in vitro* culture.
A – detail of *Bucegia romanica* macroscopic aspect;
B – general view of *Bucegia romanica in vitro* culture.

For electronomicroscopy studies, small segments of young thalli were fixed in 6 % glutaraldehyde buffered at pH 7 with 0.05 N cacodylate buffer over night at refrigerator. After repeated rinses in buffer, the tissue was postfixed in 1% OsO_4 in the same buffer over night in the refrigerator.

After a short dehydration process in alcohol series, followed by propylene oxide, the specimens were gradually infiltrated and embedded in Epon 812. Thin sections were prepared using diamond knife on an LKB Ultratome microtome and double stained with uranylacetate and lead citrate (Reynolds, 1963) and examined at an EM-125 (Selmi-Ucraina). For light microscopy, 1 to 2 μm (semifine sections) were stained with 1% toluidine blue in 1% borax (Pickett-Heaps, 1966).

RESULTS AND DISCUSSION

The investigation of the structure of *Bucegia romanica* thallus by semifine cross sections permitted us to observe the vegetative differentiation into epidermis with pores in various stages of organization, air chambers, parenchymatous tissue, chlorophyllous filaments, rhizoids and scales (Fig. 2).

It was noticed that thallus regenerated in "*in vitro*" conditions presents a similar structure with those from the "ex vitro", so "*in vitro*" conditions do not seem to induce modifications at the structural level.

Electronmicroscopical observations revealed the main peculiarities of the cells and cellular organelles. After double fixation with glutaraldehyde and OsO_4 the appearance of the main features of different organelles like nucleus chloroplasts, mitochondria, ER and Golgi bodies is the same as those of higher plants described so far by different authors.

NUCLEUS. The nucleus is the dominating organelle in the mature cells (but not senescent), generally free from chromatic condensation. It presents round or ovoidal shape (Fig. 3), but in some cells it has irregular sinuous lobate outline (Fig. 4).

At the ultrastructural level, the interphase nuclei of the cells from the *Bucegia* thallus present a similar pattern of organization to the nuclei from another taxonomical group of *Hepatica*, green algae and even those from higher plants (Lafontaine and Luck, 1980; Jordan *et al.*, 1980). Their structure greatly resembles with that of the surrounding, homogeneous, finely granulated cytoplasm.

The nuclear envelope has relative uniform pores distributed with various dimensions. According to Lamprecht' hypothesis (1979) the dimensional variations may be interpreted as a result of the pulsatory activity of nuclear pore annuli involved in nucleo-cytoplasmic exchanges (Fig. 4).

NUCLEOLUS. A special interest presents the nucleolus. Generally, a unique nucleolus per nucleus has been observed attesting the haploid status of the cells. Its pattern organization manifested some peculiarities. Its structure could be described

as reticulate alveolar or "sponge-like" (Figs. 3-4). This model of nucleolus architecture was described for another taxonomic group of plants, as algae from genus Chara (Toma *et al.*, 1986) and higher plants (Chimenez-Martin *et al.*, 1977), arguing for its functional significance. It is possible that the products biosynthesized at the nucleolus level to be carried through this network of small channels towards the caryoplasm during interphase and early prophase (Figs. 3-4).

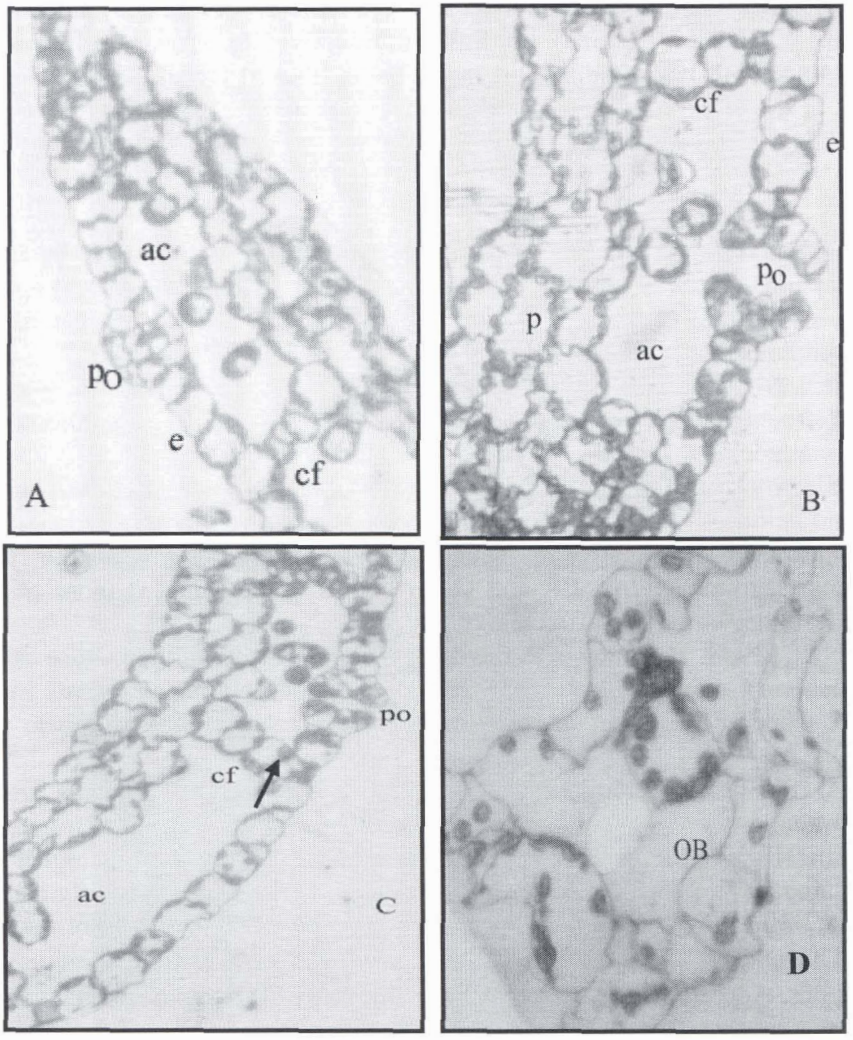


Fig. 2. The cross section of the thallus of *Bucegia romanica*. The vegetative differentiation into epidermis (upper and lower) (**e**), air chambers (**ac**), chlorophyllous filaments (**cf**), parenchymatous tissue (**p**), pores (**po**), oil bodies (**OB**) can be observed. **A** – oc.10×, obj. 20; **B** – oc. 10×, obj. 20; **C** – oc.10×, obj. 10; **D** – oc. 10×, obj. 40.

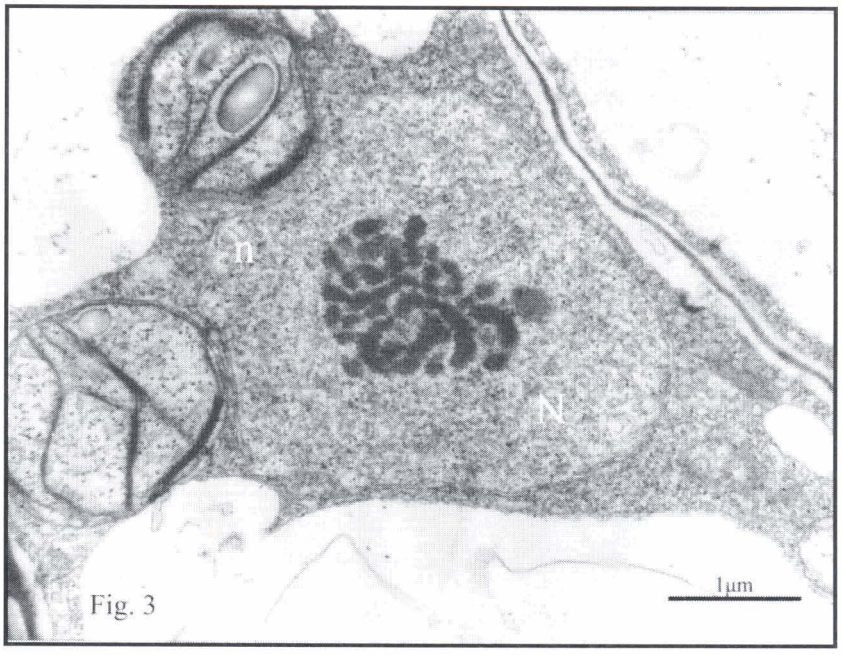


Fig. 3. The general pattern organization of the interphase nucleus (N). Nucleolus (n) with particular reticulate alveolar structure is obvious. A small nucleus spherule adjacent to the nucleolus (see arrow) is also present.

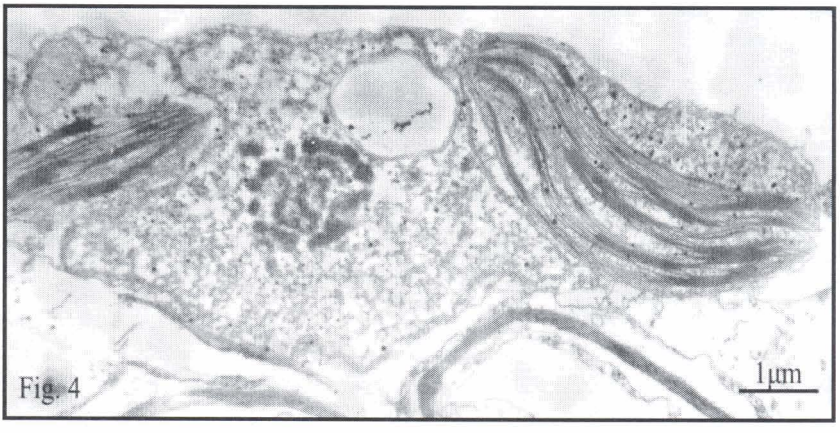


Fig. 4. Nucleus with irregular sinuous lobate outline in relation of contiguity with chloroplasts (Cl).

Electron microscope observations have also demonstrated the existence of a small spherical nuclear body, closely associated to the nucleolus or apparently free in the nucleoplasma (Fig. 3, see arrow).

These nuclear bodies were described during the time by different scientists in certain plant species too, and were variously called karyosomes (Sankaranarayanan and Hyde, 1965), micronucleoli or nucleolar organizers (Chally and Setterfield, 1975, Feldman and Torrey, 1977), micropuffs (Risueno *et al.*, 1978), nuclear bodies (Luck and Lafontaine, 1982), paranucleolar corpuscule, etc.

Their precise nature and role remain unclear so far, but surely they are involved in the stimulation of cell metabolic activity, especially of protein synthesis.

MITOCHONDRIA. Mitochondria with a typical structure were frequently present, singly or in groups (Fig. 9) and their location in the cytoplasm is quite often in the vicinity of the chloroplasts or/and of the nucleus. In the mitochondria, there are visible saculi, tubuli and osmiophilic dots near the outer membrane.

CHLOROPLASTS. The chloroplasts in *Bucegia* cells are polymorphic structures. Several have oval elongated shape and their ultrastructure appears similar to those of the mature leaf, except for the number of photosynthetic lamellae wich generally possess a reduced grana fret membrane system. Generally, they do not present starch grains (Fig. 5). This zone sometimes sends short arms into the cytoplasm (Figs. 4-5). These kinds of plastids are common in chlorophylous filaments which are composed by cells with a large vacuole and a small quantity of parietal cytoplasm.

Plastids from a second category are large, with numerous starch grains and a poor lamellae system and they were frequently observed in parenchymatous cells (Figs. 6-7). Generally, they are located in the cytoplasm, near the nucleus. Sometimes relations of contiguity with nuclear envelope or endoplasmic reticulum profile were established (Fig. 7).

Another category of plastids with atypical structure and oil bodies included have been observed especially in epidermal and subepidermal cells. These present a small number of large thylacoids grouped in the middle part of the organelles (Fig. 8).

ENDOPLASMIC RETICULUM (ER) consists of sparce short cisternae lying parallel to the cell wall sometimes surrounding the organelles or occasionally forming small vesicles. ER and plastids associations have been occasionally observed.

PLASMODESMATA. The cellular wall of the subepidermal cells, as well as the chlorophyllous filaments, presents a sinuous outline and is crossed by plasmodesma with a peculiar structure. They are very numerous and are involved in the intracellular transport regulation. The cell wall appears crossed by circular pores in transversal section and elliptical ones in oblique section. Longitudinal views reveled plasmodesmatal channels and did not clearly demonstrate ER crossing them. This could represent a cytological character which supports cellular

specialization in symplasmic transport in this species too (Fig. 9). The plasmodesmata of the liverworts which we examined possess similar features to those of the seed plants and charalean algae. According to Cook and collaborators' opinion (1997) the complex ultrastructure of modern seed plant plasmodesmata probably arose before the bryophytes diverged from the seed lineage.

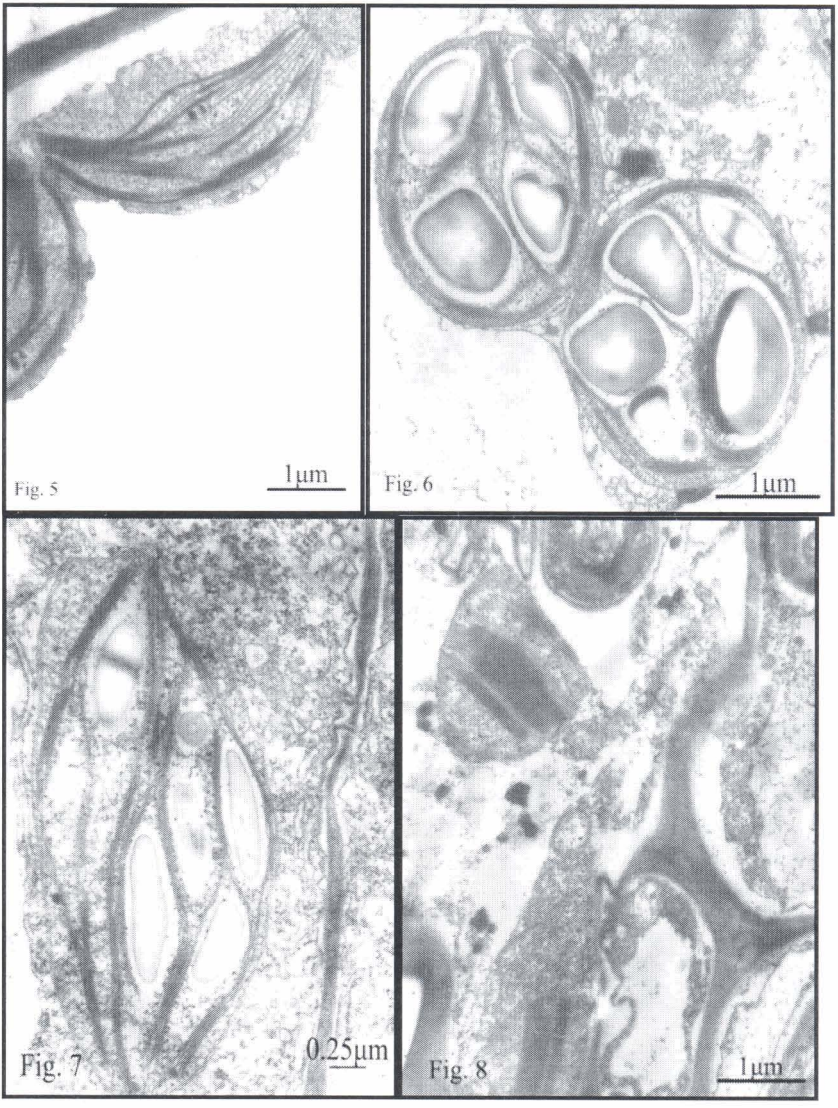


Fig. 5. Chloroplasts with a typical structure of the photosynthesizing cell of the thallus.
Fig. 6-7. Plastids with numerous and large starch grain and reduced grana fret membrane system.
Fig. 8. Plastids with atypical structure in an epidermal cell.

GOLGI BODIES are not frequently seen in older cells and present typical peculiarities.

OIL BODIES. Many authors described in numerous marchantioids beside the cell wall a large number of highly electron dense osmiophilic lipid spherules named oil bodies (OB). They are both small and extensive dense bodies of varying shape. The enveloping membrane is not distinctly visible. Sometimes they are surrounded by ER membranes (Pihakaskik, 1968; Galates *et al.*, 1978) OB are considered by many authors to be fairly stable cell elements (Crandall-Stotler *et al.*, 2000).

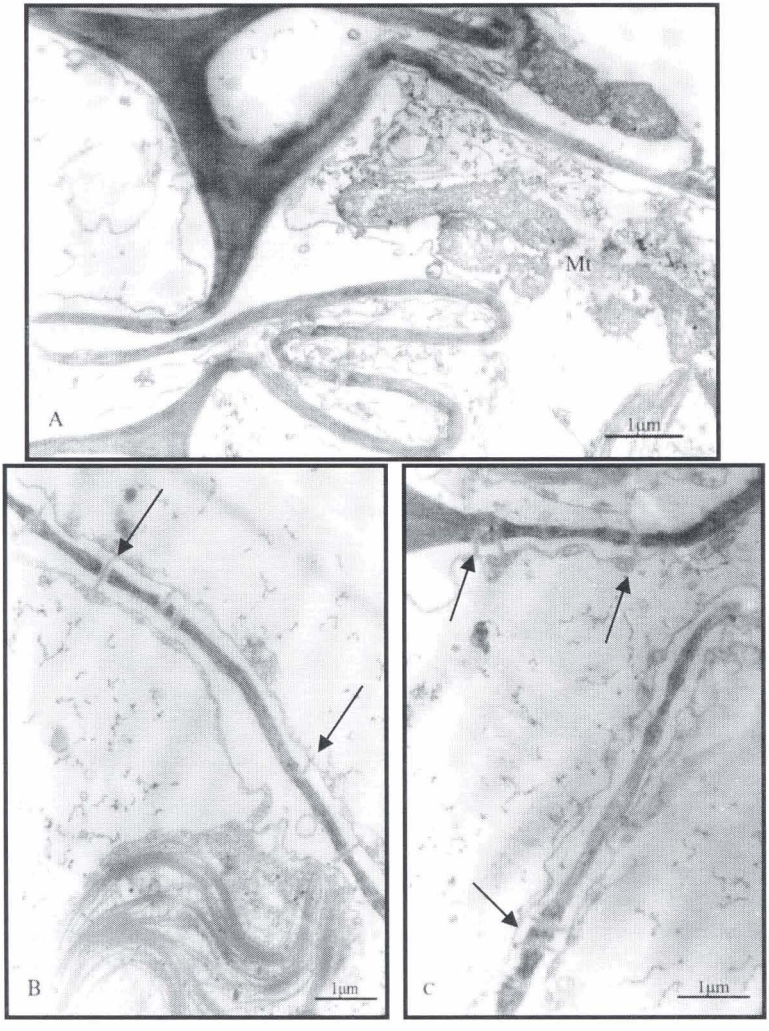


Fig. 9 (A, B, C). The plasmodesmata ultrastructural peculiarities of the *Bucegia romanica* thallus cells in the cross section. Frequent narrow tubules containing non discernable desmotubules, which cross the cell wall, are evident.

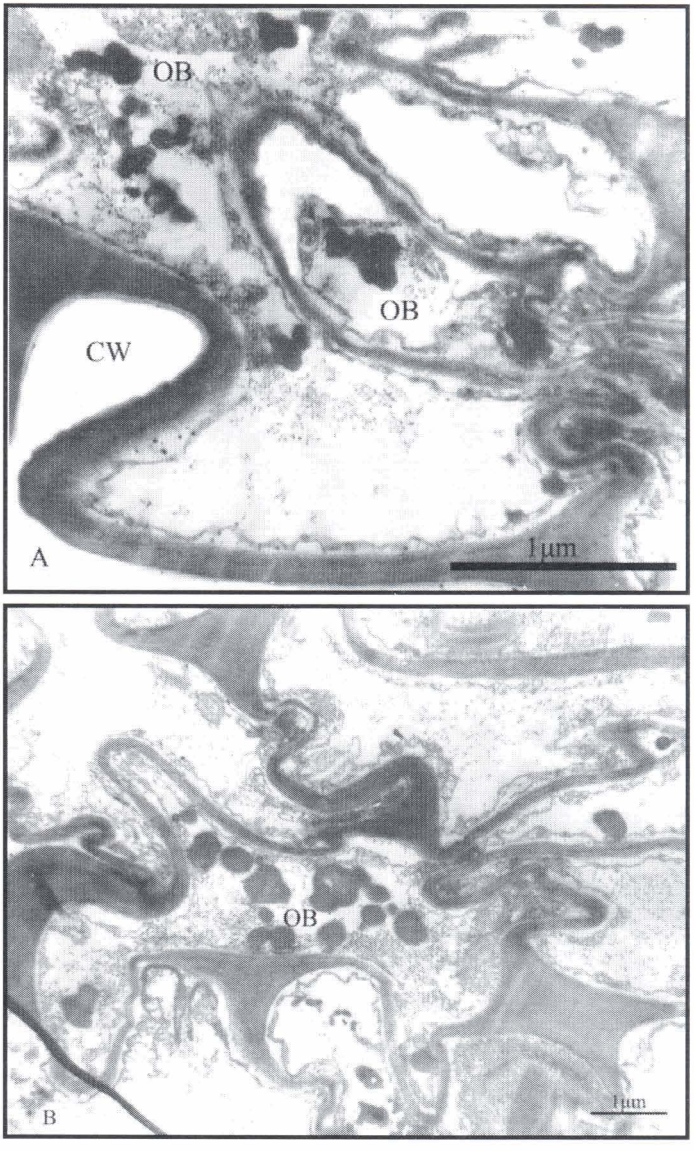


Fig. 10 (A, B). Sectors of the epidermal cells with oil bodies (OB) in different stages of development.

Their structure is not affected when the thalli develop under different conditions of humidity and temperature, light or darkness or in different culture media (Galates *et al.*, 1978).

Regarding OB function, various hypotheses have been formulated including suggestions that they deter herbivores and provide protection from cold and UV

radiations (Crandall Stotler *et al.*, 2000). The speculation that this organelle arose early in liverwort phylogenesis strongly supports the monophyly of *Marchantiophyta* (Crandall Stotler *et al.*, 2000).

In our experimental conditions OB were not frequently observed (Figs. 10-11). We can presume that their degradation process induced by a prolonged glutaraldehyde prefixation during specimen processing for electronomicroscopy could be involved. Galates and collaborators (1978) and Crandall Stotler and collaborators (2000) revealed that OB are well preserved with OsO_4 fixation or with double one with glutaraldehyde performed at 0-4 °C or at room temperature for a short time. On the contrary, a prolonged glutaraldehyde prefixation causes OB disruption at *Marchantia poliacea*.

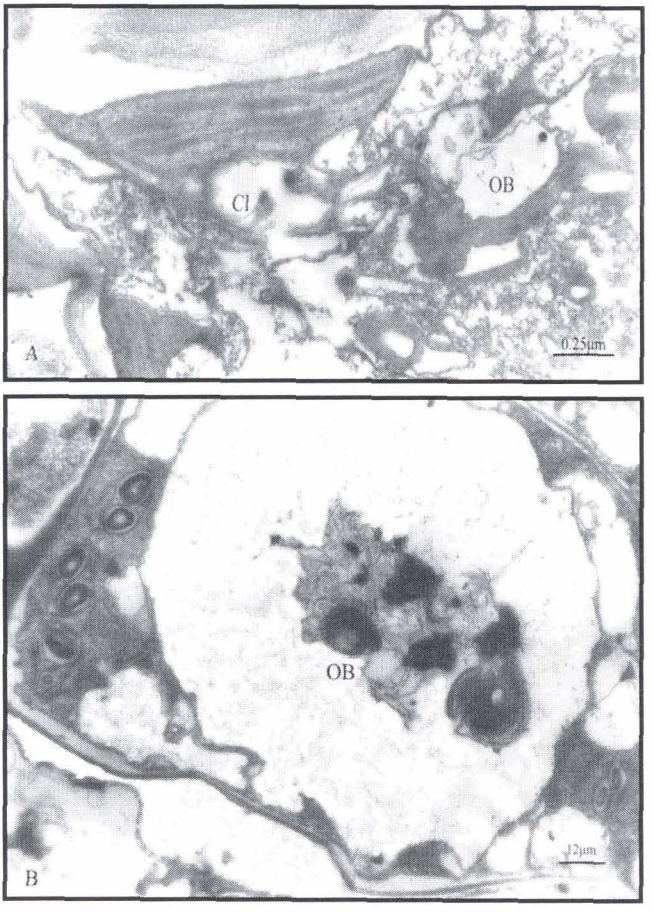
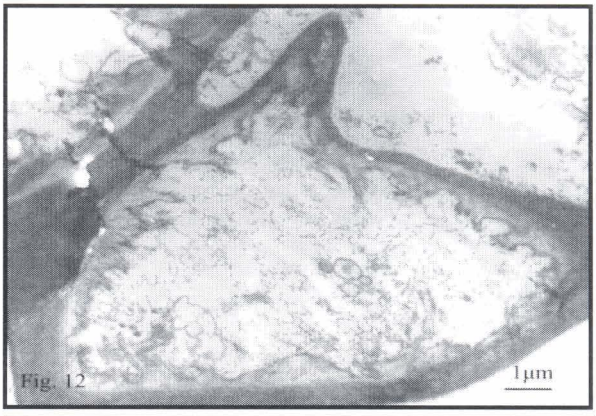
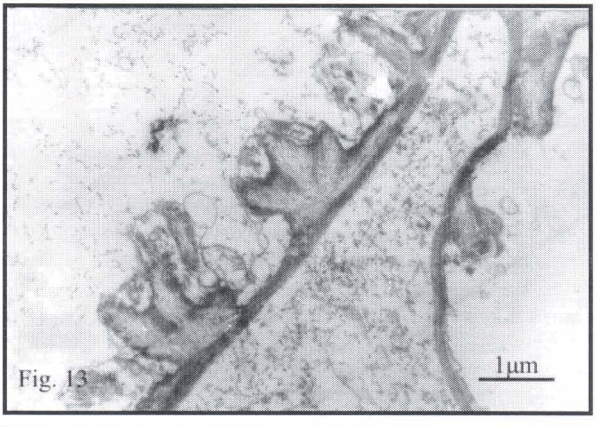
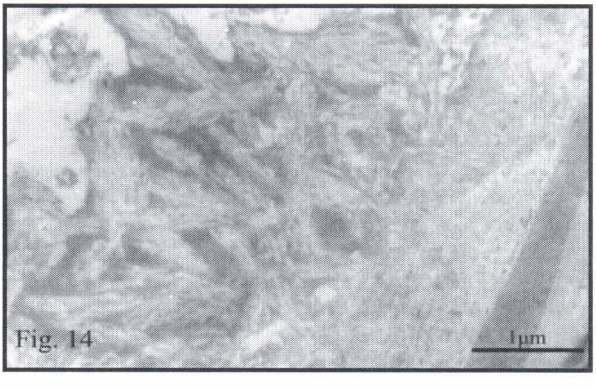


Fig. 11 (A, B). Mature epidermal oil body (OB) containing one globule. The density of matrix material and the size of the globules have been obviously increased.







Figs. 12-14. Scales in different stages of development.

The scale cells present particular characteristics. A deposit of stratified amorphous material on the inner site of plasmalemma and numerous filaments which developed from this area were observed. This structure occupies gradually all cellular content (Figs. 12-13, Fig. 14). In these cells, other cell organelles were not observed.

CONCLUSIONS

The cell structure of the *Bucegia romanica* thallus presents similarities with other *Hepatics*, as reported by different authors.

The "*in vitro*" system used by us did not affect the thallus structure which permitted us to consider it a suitable technique for "ex situ" conservation.

Electronomicroscopical analyses which were performed in this study could be used in plant cell biology research for tracing the evolution of some complex features of seed plants, especially plasmodesmata formation and its peculiarities.

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