Particularities of the methods used for *in vitro* lichen photobiont culture

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Abstract. Lichens include both primarily and secondary producers, thus being miniature ecosystems. The aim of the study was to elaborate a method of aposymbiotically culturing the photobiont from a lichen with a wide distribution, namely *Xanthoria parietina* (L.) Th. Fr. by improving the recipes of the culture medium with coconut milk, a mixture of vitamins and organic substances. Furthermore, in the separation protocol of the symbionts, we also included a gradient of concentration of NaCl in order to remove the mycobiont hyphae, and a colouring substance specific for mycobiot cells namely cotton blue. The selected media for *in vitro* culture promoted the growth of the photobionts.
Key Words: coloration, culture, mycobiont, symbiosis.

Introduction. As lichens include both primary and secondary producers, and have their own carbon cycles, they actually represent miniature ecosystems rather than individuals or populations; their symbiotic nature is not limited to the thallus level biology of individual lichen species (Rikkinen 2015). To understand lichens as symbiotic models, symbionts obtaining in culture is useful to study their characteristics as structural and functional aspects of the lichen symbiosis interface (Honegger 1991), the exchange of nutrients and enzymes between photobiont and mycobiont (Richardson et al 1968; Legaz et Vicente 1983; Legaz et al 2001), the cell surface molecules involved in the recognition process, like lectins (Galun 1988; Molina et al 1993).

There have been numerous studies concerning lichens component isolation, namely mycobionts (Crittenden et al 1995; Cristian & Brezeanu 2013), bacteriobionts (Jiang et al 2017), and photobionts (Wiedeman et al 1964; Fontaniella et al 2000; Rafat et al 2015). Methods of algae isolation and culture have been performed and subsequently improved (Ahmadjian 1993; Perez et al 1985; Fontaniella et al 2000; Calatayud & Etayo 2001; Yoshimura et al 2002; Voicu 2009; Rafat 2015; Muggia et al 2018; De Carolis et al 2022). Gasulla et al (2010) developed micro- and macro-methods for photobionts isolation of nine lichen species, namely *Acarospora hilaris* (Dufour) Hue, *Aspicilia desertorum* (Kremp.) Mereschk., *Lobaria pulmonaria* (L.) Hoffm., *Parmotrema perlatum* (Huds.) M. Choisy, *Ramalina farinacea* (L.) Ach., *Ramalina siliquosa* (Huds.) A. L. Sin., *Seirophora villosa* (Ach.) Frödén, *Teloschistes chrysophthalmus* (L.) Th. Fr., and *Xanthoparmelia tinctina* (Matheu & A. Gillet) Hale.

Lichen green algae can be isolated quantitatively directly from lichen thalli after the method of Richardson (1971) undergoing different centrifugation regimes and stages of the thallus macerate, depending on species type. Liquid or agarized mineral media are amenable for maintaining cultures (Voicu et al 2017).

The aim of this the study was to elaborate a method of culturing aposymbiotically the photobiont from a lichen with a wide distribution, namely *Xanthoria parietina* (L.) Th. Fr., amenable to axenic cultivation, by improving the recipes of the culture medium with coconut milk, a mixture of vitamins and organic substances.

Material and Method. As a source of lichen material for photobiont isolation we used *Xanthoria parietina* collected from Sinaia forests, 1400 m altitude. For pure culture of the photobiont we used basal Bold (Deason & Bold 1960; Bischoff & Bold 1963) semisolid culture medium or Knop semisolid culture medium. As supplements for photobiont *in vitro* basal culture medium we used cortex extract, after the method of Ahmadjian (1993) or soil or coconut milk extract medium.

After reviewing more methods of algae isolation from literature, we elaborated a simple method, centred on thallus homogenisation, filtration, and inoculation *in vitro* on culture medium. We added a gradient of concentration of NaCl (Gassula et al 2019), and a colouring substance, cotton blue, as in a previous research (Voicu et al 2017).

Lichen inoculum preparation for isolation and photobiont aposymbiotic culture had the following steps:

- washing thallus in cold running tap water, for one hour;
- tapping the optimum treatment for surface sterilization with sterile distilled water and 70% ethanol (30 seconds), dichloroisocyanuric acid sodium salt - 0.5 g/100 mL (in two rounds of 3 minutes);
- changing the water 3 times;
- samples mixing in a sterile mortar and pestle containing distilled water;
- ultrasonnication, for photobiont contaminants colonies (bacteria, mycobionts) attached to the cell walls;
- mixture centrifugation (1000 rpm, 5-10 minutes);
- succesive filtration using a sets of ultrathin and dense silk clothes embebbed with cotton blue, a recognised stain agent for mycobiont hyphae;
- separation of the blue layer of mycobiont hyphae from the green layer of algae;
- removing the supernanant and adding 1 mL of sterile water and a few drops of Tween 20 to the photobiont cells that remain in centrifugation tube;
- prelevating about 1 mL of algae solution for spreading with a sterile pasteur micropipette on the culture medium variants: Knop semisolid culture medium with bark extract, BBM solid culture medium with bark extract, BBM semisolid culture medium with coconut milk;
- the cultures maintaining at 18°C with a photoperiod of 16 hours and a irradiation of 30 µmol m^{-2} s^{-1}.

Cultures maintaining. In weak light conditions, photobionts have been maintained in culture for a long period of time (about one year). The subcultures have been performed at an interval of three months, by cutting from the agarised culture medium of some cubes of 5 mm and their transfer, in total asepsis conditions, on fresh medium poured into Petri dishes of 6 cm diameter. *Trebouxia* sp. (the photobiont) has an optimum of temperatura between 15 and 20^{o}C, an optimum pH of 4.0-7.0, and light between 16 and 27 µmol m^{-2} s^{-1} (PPFD).

Results and Discussion. Brushing thallus mainly on the portion attached by the substratum is a pre-cleaning required method. Double filtration of the grinding paste thallus removes small fragments resulted from crushed cells and bigger thallus parts. After ultrasonication and centrifugation of the double filtered solution, a clean and free of debris cells suspension resulted. A subsequently filtering of this solution on a filter embebed with cotton blue allowed to stain in blue the mycobiont hyphae layer in order to differentiate them from the green layer of the algal cells; the sodium salt gradient concentrations allowed to separate the layer of mycobiont from the photobiont after subsequently centrifugations. We prelevated the algae with a micropipette after removing the mycobiont cells layer (Figure 1, Figure 2). The micropipette method resulted in clonal and axenic cultures of photobionts (Figure 3, Figure 4).

According to the results of Ahmadjian (1993) *Trebouxia* sp. grow much faster and in larger quantities after the addition of glucose and proteose peptone to the culture medium, sustaining the facultative heterotroph nature of photobionts. According to the findings of Ahmadjian (1993), coconut milk, also a mixture of nutriments and enzimes, improved the photobiont culture. Furthermore, supplementing BBM and Knop medium

with bark extract or soil extract prelevated from the natural substratum, stimulated algal cells proliferation. Green algal photobiont cultures required low intensities and moderate temperatures (15^{o}C) as in experiments of Yamamoto et al (2002). Specific *in vitro* culture media selected for photobiont promoted the growth of the photobionts.

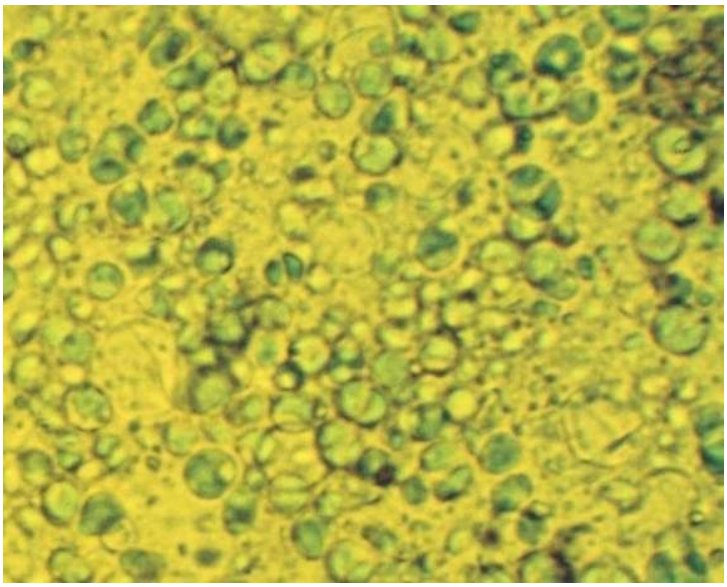

Figure 1. *Xanthoria parietina* gonidial cells serving like inocullum for photobiont culture (oc 10, ob 40).

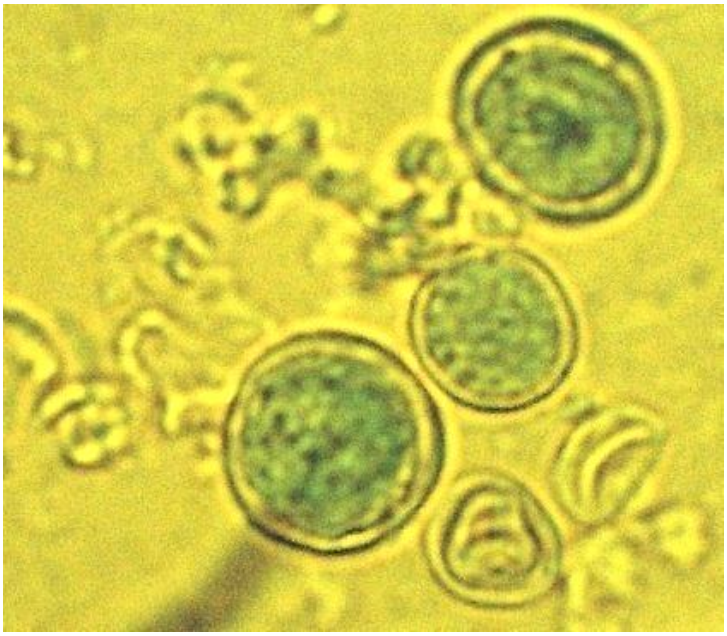

Figure 2. *Trebouxia* photobiont (oc. 10, ob. 20).

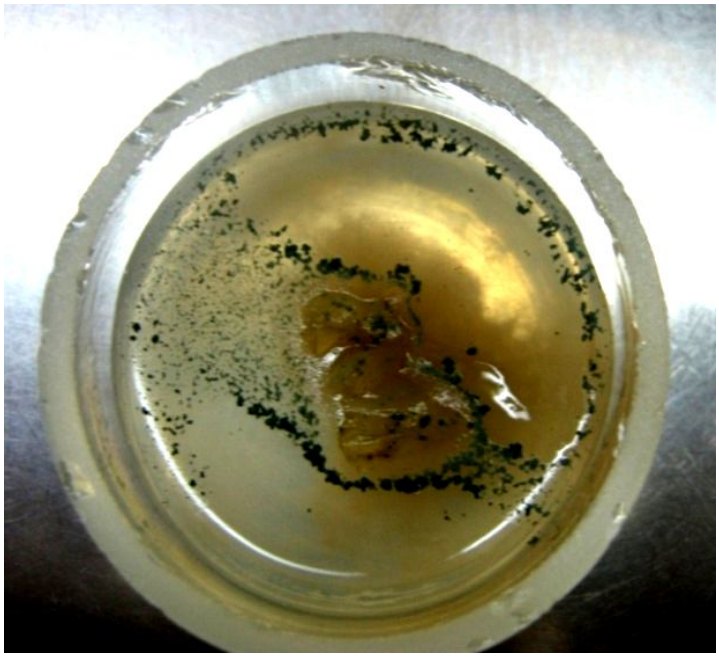

Figure 3. Aposymbiotic culture (*Trebouxia* sp.) of *Xanthoria parietina* on BBM semisolid with bark extract.

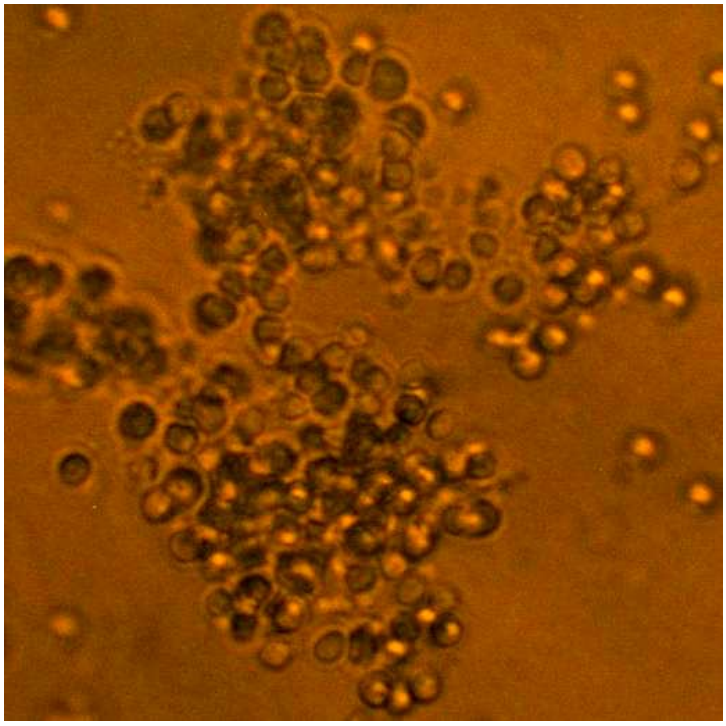

Figure 4. Photobiont cells from aposymbiotic culture (oc 10, ob. 40).

Conclusions. The centrifugation of the double filtered grinding paste and distribution with a micropipette of the resulting suspension is a suitable way to obtain clonal and axenic cultures of photobiont. Double filtration through the silk cloth embebbed with bleu cotton is a reliable way to highlight the hyphae beside the algal cells. The recipes of the natural substratum as bark extract or soil extract are suitable for stimulating algal cells proliferation; coconut soil extract added to the culture medium as a source of vitamins and organic substances sustain in a favourable manner the algae proliferation. Low intensities of light and moderate temperatures are required for green algal photobiont cultures. The previous methods of isolation process were more laborious than our method that is less time consuming and assure the reduced contamination to very low levels.

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Conflict of interests. Author declares that there is no conflict of interest.

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