An overview to lichens

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Abstract. The lichen vegetative body, namely thallus, envelops two symbiotic partners, the green algae (the photobiont) or blue-green cyanobacteria and lichenised fungus (the mycobiont) with special characteristics like biopharmaceutical potential. Nature of the symbiotic association, finding factors that mentain the mycobiont-phycobiont stability by *in vitro* culture experiments, are some of the aspects discussed in this report. Recent advances for lichens *in vitro* culture on international research are mentioned. For Romania, introducing lichens in *in vitro* culture was a field unapproached until 2002 and a challenge developed for us until present. We listed in this review *in vitro* conservation strategies used for lichen species with common status or endangered status and biopharmaceutical potential, developed and improved during 2002-2017.
Key Words: symbiosis, biological indicator, threatened species, conservation, metabolites.

Background. To understand the nature of a symbiotic relationship is important to study the different stages of the symbionts, from free-living to symbiosis (Rafat et al 2015). The endangered status of a number of lichen species, listed in Habitats Directive, like *Cetraria islandica* Ach., *Cetraria laureri* Krempelh, *Cetraria oakesiana* Tuck., *Collema dichotomum* (With.) Coppins & Laundon, *Parmelia sinuosa* (Sm.) Ach., *Ramalina obtusata* (Ach.) Bitter, *Teloschistes chrysophthalmus* (L.) Th. Fr., *Usnea longissima* Ach. (Sârbu et al 2007), *Cetraria sepincola, Cladonia bellidiflora, C. cervicornis, C. crispata, Pychnothelia papillaria, Usnea florida, U. subfloridana* (Crisan & Ardelean 2010) beside the biopharmaceutical potential (Zambare & Christopher 2012; Stocker-Wörgötter et al 2014), are reasons for including them in medium and long term conservation strategies. In Romania, there are living 1700 lichen species, 4 of them being of European interest according to Habitat Directive Annex IIb (Banciu & Cristian 2015).

The first author's research in the lichen *in vitro* culture field started in 2002, with the PhD thesis. Moreover, the lichen species studied by us complete the variety of taxa introduced in *in vitro* cultures earlier (Brezeanu & Cogalniceanu 2016).

In situ conservation of threatened lichens sustain the *in vitro* approaches, by techniques like:

- transplantation of symbiotic propagules and thallus fragments in gardens from the nearby areas having similar climate conditions (Palmquist et al 2017);
- creating lichen gardens, these being effective methods to conserve them in their natural habitats that are considered like sanctuaries and gardens that would serve as educational and recreational sites (Upreti & Nayaka 2008).

Recent advances in lichenology regarding modern *in vitro* culture techniques (Guzow-Krzemińska & Stocker-Wörgötter 2013; Upreti et al 2015; Cornejo et al 2015; Muggia et al 2017) or antioxidant potential of lichen species and their secondary metabolites (Rankovic 2015; Fernandez-Moriano et al 2016) are the object of study for a number of reviews. Also, many protocols were useful for our beginning on this issue including Ahmadjian (1993), Kranner et al (2002), Nash III (2008), and Honegger (2008, 2012).

Elucidating the processes which contribute to the successive development of symbiotrophic formations, was an important objective and a challenge in Romania (Voicu & Gavriloaie 2007; Voicu & Brezeanu 2008a, b; Cristian & Brezeanu 2013; Cristian et al 2013; Voicu et al 2017) following the work of the famous lichenologists, mentioned in the previous paragraph.

Some considerations about lichens nature. Ahmadjian (1993) defines a lichen in agreement with the definition approved by the International Association of Lichenology (1982). More recently, lichens are considered a new biological entity resulted from the symbiotic association of two unrelated organisms with high degree of integration (Zarnea & Popescu 2011).

Special interest for complex lichen study was amplified by unique characteristics of these, such as:

- latent life: their very slow growth - a few mm/year determined by catabolic and anabolic processes, their longevity – can achieve impressive ages – about 4500 years, the ability to resist in outer space confirmed by the viability of photobiont cells/clusters after 1.5 years of space exposure, photosynthetic and re-activation of *X. elegans* as indicator of post-flight viability (Brandt et al 2016), UV tolerance of symbiotic *Trebouxia* sp. in space (Sánchez et al 2014);
- adaptation to different ecological niches - encountered on the haughty mountain ridges (Muggia et al 2016), agents in soil development through species producing an oxalate at the thallus - substratum interface (Seaward 2015) making them pioneers of vegetation;
- symbiote with universal spreading in extreme environments (Zarnea & Popescu 2011; Palmquist et al 2017);
- the ability to synthesize metabolites (Çobanoğlu et al 2016) with antioxidant properties (Kosanić et al 2011; Yamamoto 2015).
- sensitivity to air pollutants, unselective absorbtion of toxic substances and heavy metal accumulation (Rola et al 2016), harmul for them in urban areas, making them indicators of pollution (Nimis et al 2002; Kuldeep & Prodyut 2015).

In another train of thoughts, lichens trigger the biodeterioration of mural paintings of churches (Gomoiu et al 2016). Also, considering the relation of lichens with substrate represented by plants, reports show that corticolous/epiphytic lichens on fir trees used with the term of lichen mycota (Çobanoğlu et al 2008) are detrimental to bark trees in different manner (Favero-Longo & Piervittori 2010) and we captured this aspect illustrated by two different fir trees, one at the beginning of the process and the other at an middle stage of branches deterioration (Figures 1 & 2).

Special attention is paid to *Cetraria islandica* L. Ach species (Figure 3) because of this sozologic status, properties and its great habitat from the top of the mountains (Cristian et al 2013; Vicol 2017).

The microhabitat plays an important role in conservation of the red listed lichen species living in subalpine and alpine areas (Vicol 2017).

Figure 1. *Parmelia furfuracea* on *Abies* branches (Cioplea peak, 1152 m, Piatra Mare mountain, original photo by Diana Voicu).

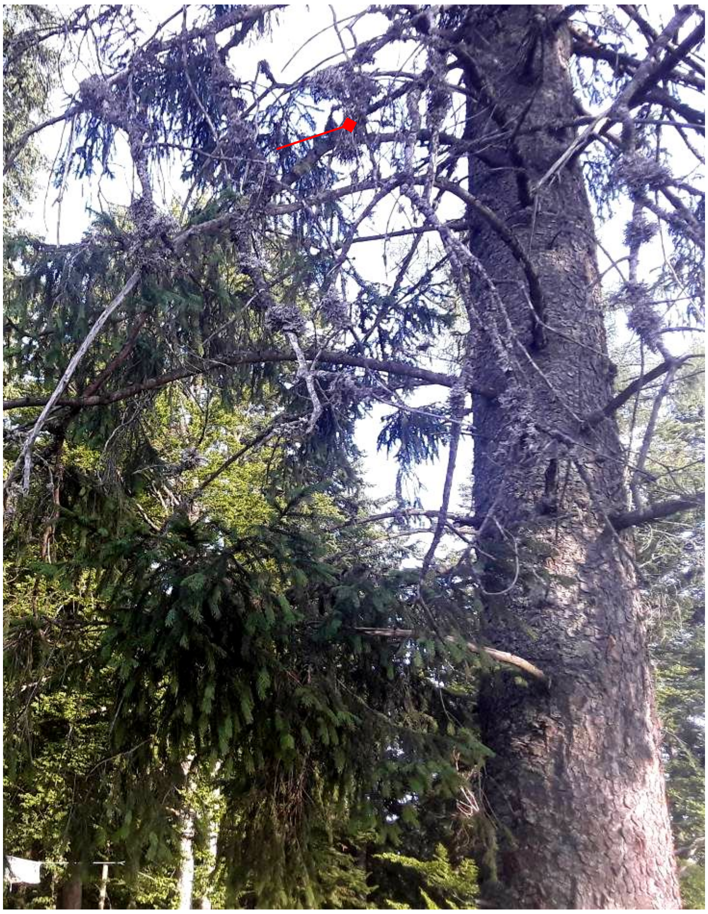

Figure 2. *Parmelia furfuracea* on fir trees branches (Cioplea peak, 1152 m, Piatra Mare mountain, original photo by Diana Voicu).

Figure 3. Morphological aspect of *Cetraria islandica* (L) Ach. (original photo by Diana Voicu).

In vitro cultivation methods are supplementary and recruiting ways of stimulating thallus growth in order to get significant amounts of biomass rich in bioactive compounds, although unlike plants, lichens are recalcitrant to *in vitro* conditions. To achieve this objective, we used first in our experiments a common encountered and studied species, *Xanthoria parietina* Beltr. (Figures 4, 5, 6) (Cristian & Brezeanu 2013) and then *Usnea barbata* L. Mott. (Figure 7) (Voicu & Brezeanu 2008a, b).

Also, studies are useful theoretically, for implementation of resynthesis experiments and the study of signaling and interaction between the two partners.

Figure 4. *Xanthoria parietina* Beltr. on forest wood (original photo by Diana Voicu).

Figure 5. *Xanthoria parietina* Beltr. thallus redifferentiated by in vitro culture of thallus explants on Knop with bark extract agarised, after three months of incubation (original photos by Diana Voicu).

Figure 6. *Xanthoria parietina* Beltr. *in vitro* mycobiont in liquid malt yeast extract medium (original photos by Diana Voicu).

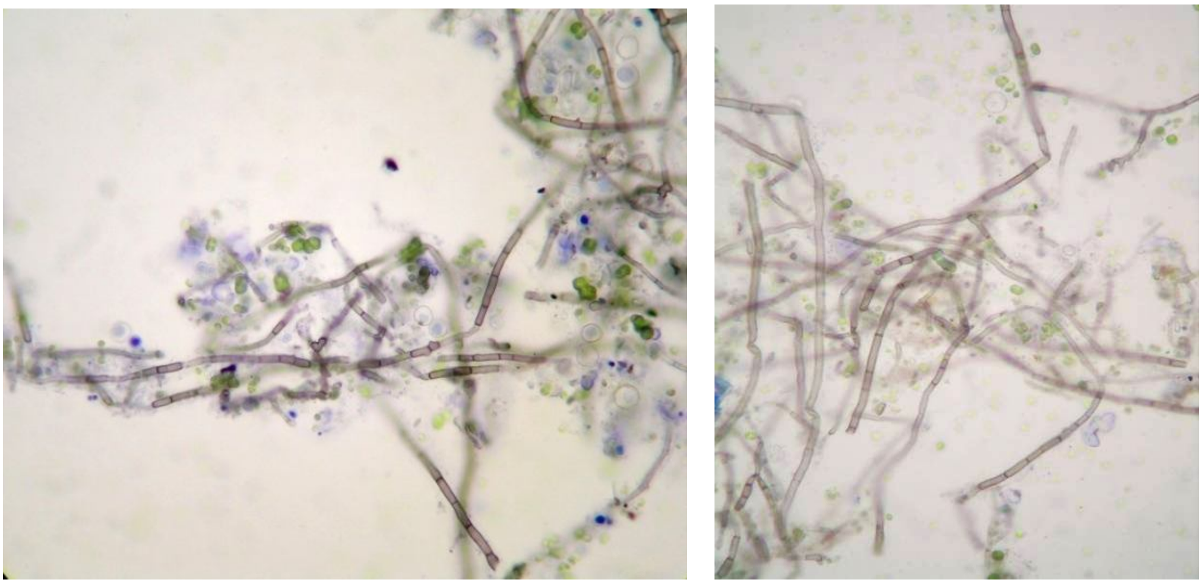

Figure 7. Incipient stages of morpho-physiological interaction between mycobionts with photobionts isolated from *Usnea barbata* L. Mott. preceded and induced by signaling and recognition events (three months cultures) (x 20) (original photos by Diana Voicu).

The requirements of the symbionts for triggering the lichen. What requirements meet the partners of the symbiosis one to each other is the questions to answer for experiments understanding and triggering the resynthesis experiments. Trying to determine what maintains this union in a stable cohabitation/companionship and what are the involved factors in this perfect union we've documented about their free-state life style and we found that their ecology explain the success.

First of all, symbionts association needed an efficient signaling between them (Rikkinen 2002). Genetic water ''memory'' of each, besides the experience gained during evolution submitted in the mycobiont and phycobiont, improved this and determined them to ''call'' and find each other based on selectivity and specificity (Insarova & Blagoveshchenskaya 2016).

In this regard, the algae evolutionary transition from aquatic to terrestrial environment (Delwiche & Cooper 2015; Lipnicki 2015) was intercepted by a a special hydrated body named the fungus. The internal hyphae medium shields the sunlight for it, maintaining the same refraction index. The distribution of the algae inside the thallus use like a model the distribution of chloroplasts in leaf assimilation tissues. The sponge form of the mycobiont, inspired from the animal connective tissue, beside the disc-shaped algae is designed to counter the periods of drought.

Physiological processes like water balance and thermoregulation are controled by the hyphae central distribution in the medullary layer and the secondary metabolites - a product of the symbiosis which is on the hyphae surface that function like a hydrophobic coat (Honegger 2006). Water is absorbed quickly through the entire surface of the thallus and stored mainly in the medullary hyphae - relaxing network in the middle. It sees it like a groundwater. Lichens have no vascular system for conducting water or nutrients.

In nature, lichens have little biological control over gas exchange, and air pollutant gases are assumed to readily diffuse down to the photobiont layer.

The mycobiont ability to lichenize different photobionts, genome characteristics (Wang et al 2014) allows them to colonize a wide range of ecological habitats, the substrates being stones, walls buildings, soil, cortex in wet conditions. Algal partner plays a determing role in the ''phenotypic expression of the genotype of the fungal partner'' and they are closely connected.

The most relevant informations regarding this aspect is given by their *in vitro* axenic culture. Detailed results about mycobiont *in vitro* (Figure 6) requirements are presented in Cristian et al (2013). Photobiont *in vitro* development is less studied. We mention here most of the techniques used by the lichenologist to obtain axenic photobiont cultures: the micropipette (Ahmadjian 1993) or the whole suspension and centrifugation methods (Calatayud et al 2001, Yoshimura et al 2002, Yamamoto et al 2002), successfully used in our protocol in a combined manner (Voicu et al 2017). Also, we tried to copy the recipes of the natural substratum. Inoculum preparation was most laborious step of the experiment consisting in (Voicu et al 2017):

- hydration of the thallus by washing in cold running tap water, for one hour;
- tapping the optimum treatment for surface sterilization by washing the samples with sterile distilled water and 70% ethanol (a few seconds), then with dichloroisocyanuric acid sodium salt - 0.5 g/100 mL (in two rounds of 3 minutes);
- homogenizing the samples in a sterile mortar and pestle containing distilled water and the specific liquid culture medium for lichen algae (BBM solid with bark extract; Knop semisolid culture medium with soil or bark extract);
- succesive filtration of by two nylon sieves of 500, respectively 150 µm mesh, of the grinding paste thallus, this double filtration removing small fragments resulted from crushed cells and bigger thallus parts;
- selecting thallus fragments between 150 and 500 µm;
- spreaading about 1 mL of resulted solution with a sterile pasteur micropipette on the culture medium.

Symbiotic status. Lichen symbiosis represent a long-term partnerships (Palmqvist et al 2017) with the highest degree of integration in nature. The integration creates new structures, diversify metabolic network by which it is synthesized the metabolites that are not produced by individual components ever produced by partners separately and amplified resistance to certain environmental factors.

The symbiotic products named secondary metabolites with unique properties, are involved in the maintaining of the symbionts relations and in the regulation of important physiological processes (Voicu 2007):

- maintaining water balance - by microcrystalline hydrophobic nature, poor solubility and their arrangement on the surface of the hyphae, prevent medulla water saturation; beside them, hydrophobins, seal the both partners with a hydrophobic coat which prevents free water from accumulating and forces the passive fluxes of solutes from the thalline surface to the algal layer and vice versa to flow underneath this hydrophobic lining (Nash III 2008). Wall surface properties of lichen - forming fungi play key roles in thalline water relation and thus in the functioning of the symbiotic relationship;
- photosynthesis- by focusing light, because of the cristalls, with optical active properties.

In mycobiont-phycobiont interaction, preceeded by recognition, cellular shells plays an important role, because they contain receptors. It was hypothesized that the receptors belong to the mycobiont cell wall and the ligands, to the photobiont cells. It appears that the elicitation is made by photobiont. Moreover, proteins expressed by the lichens (fungal proteins ensuring vesicle transport, algal proteins functioning in photosynthesis) (Eymann et al 2017) extend our knowledge about mutlifaceted interfacebetween symbionts.

The factors that sustain resynthesis. Tasks distribution argues symbiosis success, besides common points that strengths - bind the symbionts. The fungus bring it out the alga, and the alga is that who shed it light. Algae meet to the mycobiont shelter for high light intensities, water storage from the hyphae. Territory preferences brings them together. Fungi prefer shaded areas.

There is a mutual care and benefit of the both partners. Therefore, the algae produce tryptophan which is transformed into auxin by the fungus. The auxin increase the growth of the alga *Trebouxia*, control glycolysis in the algae, or increase the permeability of the algal cell membrane. Also, low concentration of the hormone stimulate growth while higher concentration inhibite growth (Remmer et al 1986).

So, the mycobiont meets the phycobiont needs, with the more so as they absorb minerals from the soil, providing thus ingredients for the photosinthetic partner preparing food. Task sharing between partners is one of the well factorlife style for the symbionts.

The *in vitro* conditions modulated the parameters. Resynthesis experiments allow us to observe a number of biological processes which can provide important details regarding this unique type of symbiosis, such as the signaling mechanisms between symbionts, the behavior of symbionts after isolation; the characteristics of aposymbiotic cultures.

The high affinity binding between symbionts result from greater intermolecular forces ligand - receptor (Singh et al 2017).

Also, our investigations allowed us to decipher ontogenetic stages of lichens thalli rediferentiation and thus to develop alternative methodologies to obtain lichen biomass of biotechnological interest. This desideratum brings the degree of novelty for the biotechnology of lichens from Romania. All the experiments demonstrated the fastest proliferation of the mycobiont in relation to the photobiont. Our experimental data regarding lichen structure differetiation revealed that for symbiotrophic formations obtaining, the lichen thallus grinding paste is most suitable. Also, the presence of the cytokinines (kinetin respectively) in nutritive media and liquid state of the culture media achieved good results (Voicu et al 2017).

Lichens are an model of coexistence and a mutually beneficial cooperation from which we can learn (Nash III & Gries 2002). Given the biotechnological value, namely secondary metabolites synthesized by the lichen species, the methodology used by us presents real socio-economic resonance (Voicu et al 2017).

Conclusions. This self-supporting association has a functional strategy that sustains the harsh ecological environments. Morphologically and physiological adaptative changes are interrelated. Nutrient status condition allows a highly coordination of survival and evolution processes. Lichens are an model of coexistence and a mutually beneficial cooperation. Due to the secondary methabolites synthesized by lichens, they are very valuable species for biotechniology applications.

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