

## A COMPREHENSIVE REVIEW ON LICHENS

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**Abstract.** This review analyses the interactions of lichens with in the *ex vitro* and *in vitro* environment, with an emphasis on structural and functional features, and factors that sustain their favourable evolution in the context of disturbance of certain parameters. An extensive bibliography highlights the lichens' tolerance to desiccation, heavy metals accumulation from polluted environment, cryoprotection mechanisms, by inducing repair mechanism as secondary metabolites production. The *in vitro* conservation of lichens is an efficient alternative reproduction strategy in the context of environmental changes and offers the possibility to understand this type of close symbiosis by promoting new studies of interactions between symbionts

**Keywords:** lichens, lichen symbiosis, conservation *in vitro*, heavy metal accumulation.

**Rezumat. Un review cuprinzător asupra lichenilor.** Acest review sintetizează cele mai relevante studii referitoare la biomonitorizarea cu ajutorul lichenilor, analizează interacțiunea lichenilor cu mediul *ex vitro* și *in vitro*, corelată cu o abordare aprofundată a trăsăturilor structurale și funcționale ale acestora precum și a factorilor care susțin evoluția lor favorabilă în contextul perturbării anumitor parametri. Bibliografia vastă consultată subliniază toleranța lichenilor la deshidratare, acumularea de metale grele din mediul poluat, mecanismele de crioprotecție prin inducerea mecanismelor de reparare precum și producerea de metaboliți secundari. Conservarea *in vitro* a lichenilor este o metodă alternativă eficientă de reproducere în contextul schimbărilor climatice și oferă posibilitatea de a înțelege acest tip de simbioză strânsă prin încurajarea efectuării de noi studii de interacțiune între simbioți.

**Cuvinte cheie:** licheni, simbioza lichenică, conservare *in vitro*, acumulare de metale grele.

### INTRODUCTION

**Lichens: An amazing model of symbiosis.** Lichens are enigmatic entities of the vegetal kingdom (HUANG et al. 2019) enclosing microecosystems of bionts (ZACHARIAH & VARGHES, 2018), with a prevalence of the fungal partner, the mycobiont and the algal partner, the photobiont, each of them building a new architecture, a long-living thallus. Lichens are distinguished by their unique chemistry and biotechnological potential (VOICU et al., 2019). The microhabitat and macrohabitat determine lichen abundance in lichens species (VICOL, 2016). Macroclimate and evolutionary events had a decisive role in establishing the symbiosis of lichens (SINGH et al., 2016).

Lichens are used to monitor climatic changes and pollution level (ABAS, 2021), because they are known to be colonizers of a wide range of habitats, from the Antarctica (Spielmann & Pereira 2012) and healthy environmental conditions with rich diversity in lichen species as the Baliem Valley region from Papua, Indonesia (SUHARNO et al 2020) to urban areas and high altitude (ARDELEAN et al 2015) due to the poikilohydric nature and correlation with climatic factors of an area (BAJPAI et al 2018), tolerance to desiccation (KRANNER et al., 2008), heavy metals accumulation (BOONPENG et al., 2020) and highly saline environments (HINOJOSA-VIDAL et al., 2018).

Lichen species include lichen extremophilic species with sensitivity/resistance to freezing; especially lichen algae have cryoprotection estimated as ice nucleation activity; the relatively thick cell wall composed of polysaccharides of the unicellular coccoid lichen phycobionts beside the mucilaginous sheet sustain this property (KVÍDEROVÁ et al., 2013; HÁJEK et al., 2016). The symbiotic lifestyle in lichens gives tolerance to freezing and changes in water relations to the symbionts (FERNÁNDEZ-MARÍN et al. 2020). To counteract with inconvenient factors including desiccation (GASSULA et al., 2021) or snag, lichens are sustained by a range of defence mechanisms, but respond to oxidative stress in different manner, depending on ecotype and species characteristics. Therefore, related to desiccation, lichenised fungi have mechanisms to transit between desiccation and rehydration state. Rehydration process to lichens *Ramalina lacera* determine the reinitiation of metabolic processes, the rapid increase of photosynthetic activity, the production of reactive oxygen species and nitric oxide (WEISSMAN et al., 2005). The capacity of inducing repair mechanisms also differs between lichen symbiosis partners. In this frame, against extreme saline conditions, the photobiont *Trebouxia* isolated from *Ramalina farinacea* developed alternative molecular pathways to avoid this.

Lichen metabolites are involved in the pollution tolerance of lichens and metal homeostasis (BHATTACHARYYA et al., 2016). Fungal hyphae are lined with secondary metabolites as crystals (GOGA et al., 2018). By the composition in metabolites of thallus extracts, as usnic, protocetraric and caperatic acid, many lichen species, including *Usnea florida* and *Flavoparmelia caperata*, have antibacterial activity (DIEU et al., 2020). Therefore, a wide range of lichens are used for synthesis of nanoparticles with antimicrobial potential (MIE et al., 2014; RATTAN et al., 2021) including *Cetraria islandica* (BALÁŽ et al., 2020) *Usnea longissima* (SIDDIQI et al., 2018) *Cladonia rangiferina* (RAI & GUPTA, 2019), *Parmelia perlata* (LEELA & DEVI, 2017).

Melanins, a type of lichen secondary metabolites with high - molecular - weight are photoprotective and antioxidants (RASSABINA et al., 2020). The production of secondary metabolites is influenced by temperature and precipitation at different altitude gradients (NEUPANE et al., 2017). Lichens function like biofilters (BAJPAI et al., 2019). Relevant from this point of view are the species *Parmotrema tinctorum* (Despr. Ex Nyl.) Hale, *Usnea longissima*

Ach. (with endangered status in our country) *Rocella montageni* Bel, *Heteroderma diademata*. The procedure to neutralise metal toxicity is laborious. The ability of lichens to function as natural filters is due to the high percentage of secondary chemical compounds rich in electron donor functional groups. This promotes lichens as useful bioresources for eliminating hazardous metal contamination, such as arsenic. As sources of novel bioactive molecules, lichens have a medicinal impact that has not been well studied yet (GAUTAM et al., 2021). The resistance of lichens to abiotic factors is sustained by the association of lichens with more than 800 types of bacteria representing the microbiome; the study of the microbiome on relatively fast growing lichens with ecological significance as *Lobaria pulmonaria* revealed the role on supplying lichens with nutrients, vitamins and trace elements (GRIMM et al., 2021). Traits such as resistance to extreme weather conditions make lichens useful for the genetic transfer of the genes responsible of these characteristics (BACKOR et al., 1998). Car traffic determines heavy metals accumulation in the central part of the thalli (VICOL, 2018). Rainfall can diminish air pollutants by washing and it can both add or remove pollutants in lichen thalli (GALLO et al., 2017). The *in vitro* culture of lichens is an alternative to cope with environmental factors because of the sterile conditions offered by the growth chambers, controlled conditions of temperature, light and humidity, the possibility to improve the input of nutritive factor by the medium culture composition, the possibility to multiply in a numerous number the *in vitro* regenerants and to acclimatise them, although lichens are some of the most recalcitrant vegetal organisms to these conditions, because of the different requirements of the symbionts. Also, the slow growth of the lichens in nature determined the scientists to adopt the method of *in vitro* culture.

**The relation of lichens with the *ex vitro* and *in vitro* environment.** The lichen body with a network architecture and physiological processes developed by symbionts is adjusted to the *ex vitro* and *in vitro* environment (VOICU & GAVRILOAIE, 2017). The reproduction strategy of lichenised fungi in nature is very elaborated (ANSTETT et al., 2014). The structures that contribute to these are assexuate lichen specialised propagules, sexuate spores and assexuate conidia (OTT, 1987; OTT & JAHNS 2002; TRIPP et al., 2018)

Also, the *in vitro* conservation of lichens is a reproductive strategy (VOICU et al 2017; STEINOVA et al., 2019). Fungal isolectins with arginase activity are involved in the mechanism of interaction between the mycobiont and photobiont (MOLINA & VICENTE, 2000). Also, arginase from lichens have a lectin function binding to the cell wall of algal cells, therefore being involved in the recognition and association of symbionts (DÍAZ et al., 2016).

Lichen phenolics are related to the co-development and cooperation between symbionts. Therefore, algal cells release ribitol into the environment only when the mycobiont is present (MOLINA et al., 1997). There are many similarities between the profile of secondary compounds of the *in vitro* thallus cultures established on malt yeast extract (MY 10%) medium and natural lichen thallus SHANMUGAM et al. (2016). These were demonstrated by observations on the *in vitro* developmental stages of the mycobiont and secondary compound biosynthesis beside the whole thallus cultures of *Buellia subserotoides* along 120 days. Also, mycobiont cultures reach optimum biomass and biosynthesis of nine secondary compounds in MY 10% sucrose concentration.

As a result of symbiotic interrelations, not only does the mycobiont synthesize secondary metabolites, but the photobiont and lichen-associated bacteria also produce a range of potentially valuable molecules (CALCOTT et al., 2018). Also, the mycobiont can produce substances that are absent in symbiotic form in an aposymbiotic culture (FAZIO et al., 2018).

Polyketide (secondary metabolite) synthesis by mycobiont depends on the carbohydrate source from photobiont, as type and concentration. From this point of view, the fumarprotocetraric acid was detected by ELSHOBARY et al. (2016) only in cultures grown with 1 and 5 % ribitol concentration added to the agarised medium. The morphogenetic capacity of the mycobiont *in vitro* is enhanced by concentrations of 2 % malt extract with 0,2 % yeast extract, 1 % malt extract 0,4 % yeast extract and 10% sucrose (FAZIO et al., 2009). A long period of culture (over 5 and 10 months) on Lilly & Barnett medium (LB) stimulates the production of atranorin, correlated with a desiccation treatment. Developed on culture media as potato - carrot, malt extract - yeast extract (MY), Bold' s basal medium with nitrogen (NMBBM), oatmeal and yeast extract with supplements, a number of 25 mycobiont strains covering three classes and five orders of the *Ascomycota* were able to induce ascospore germination, fragmentation and distribution on liquid media for further culture (MCDONALD et al., 2013).

The liquid state of the culture media allows a complete contact of symbiotic components, ensuring the agitation of culture vessels and aeration are key points that stimulate with success symbiosis establishing (RAFAT et al., 2015). Resins added to the culture medium can absorb phenols that inhibit the germination spores of *Peltigera*. Elicitors delivered by the symbionts in *in vitro* culture triggers the three-stage gene activation responsive for recognition and the gene implied in protection (ATHUKORALA et al., 2014). Axenic cultures are useful to study the taxonomy, biochemical, molecular or physiological behaviour of symbionts outside the symbiosis (SPRIBILLE, 2019). In this context, the aposymbiotic culture of photobiont allows to compare the binding affinity of lectins. HONEGGER (1996) also studied the signalling process between the bionts. Symbionts reassociation from axenic cultures needed to modulate and tatonate the growth conditions provided *in vitro* by repeated experiments. The similarities between the vegetal tissues in the *in vitro* dedifferentiation process to higher plants in the initiation stage culture with the events conducted inside the lichen thallus inoculated *in vitro* are relevant. The similarities consist in the tendency of the lichen symbionts to dissociate when they are cultured *in vitro* like the initial stage of dedifferentiation to higher plants. A comparative study regarding the interactions between symbionts *in situ* and *ex situ* (*in vitro*) highlighted that these are tighter in natural conditions and weaker in *in vitro*.

Phytohormones have a decisive role in stabilising lichen symbiosis, representing inter-kingdom signalling components (PICHLER et al., 2020). For example, experiments of PICHLER et al. (2020) demonstrated that *in vitro* isolated mycobionts release in the nutritive medium such as phytohormones as indole - 3 - acetic acid (IAA) that trigger physiological response to compatible corresponding photobionts of *Cladonia grayi*, *Xanthoria parietina* and *Tephromela atra*, lichens namely *Asterochloris glomerata*, *Trebouxia decolorans*, *Trebouxia sp* increasing water content with 4,4 %.

KONO et al. (2020) achieved a thalloid symbiotic structure with morphologically and biochemically similarities with the natural thallus by culturing together the isolates of the mycobiont of *U. hakonensis* and the photobiont *Trebouxia sp*.

## RESULTS

**Lichens as bioindicators of heavy metals accumulation.** The studies and methodologies on biomonitoring increased significantly in the recent years (ABAS, 2021). The process of bioaccumulation of heavy metals is closely correlated with the degree of pollution of the given biotope (SAWIDIS et al., 1995), although different species presents large variations. The excessive use of coal releases fuel in the air major contaminant in urban area as Ni, V and Cr (YAVUZ & ÇOBANOĞLU, 2019). Heavy metals resulting from industrial activities and vehicle traffic are absorbed by the aerial organs of plants or the entire body of lichens (SHAHID et al., 2016).



Species used like bioaccumulators encompass a broad spectrum of heavy metals, as Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Pb, and Zn (ȘTEFANUȚ et al., 2017). Also, biomonitoring methods used to plants, specially to trees (ȘTEFANUȚ et al., 2018) can be extrapolated to lichens. A common lichen species, frequently used in biomonitoring studies, is *Xanthoria parietina* (Fig. 1).

The lichen thallus is able to accumulate a maximum concentration of Cd, Cu, Mn, Ni, Zn in certain zones. A common lichen species, frequently used in biomonitoring studies, is *Xanthoria parietina*. Limiting the accumulation of heavy metals as Hg, Cd, Zn, Pb with toxic potential in plants with economic importance is an essential desideratum (BORA & BUNEA, 2019).

Lichens as accumulators of trace elements is a research area of great interest for air pollution monitoring (BRUNIALTI & FRATI, 2014). They represent ideal experimental models for physiological parameters assessment that are affected by this process (OZTETIK & CICEK, 2011). A wide range of foliose and corticolous lichen species are used as biomonitors, as *Flavoparmelia caperata*, *Leptogium gelatinosum*, *Collema subnigrescens*, *Xanthoria calcicola*, *Xanthoria parietina*, *Physcia adscendes*, *Physcia aipolia*, *Xanthoparmelia conspersa*, *Ramalina farinacea* and *Phaeophyscia orbicularis* (KINALIOĞLU et al., 2020).

Figure 1 *Robinia pseudacacia* L. trunk covered in abundance with *Xanthoria parietina* L. (original photo, Voicu Diana).

Heavy metal concentrations from the lichen thallus reflects their presence in the environment (CONTI & TUDINO, 2016). The main source of heavy metals uptake is the substrate and the accumulation mechanism implies three stages: trapping of solid particles, extracellular binding with exchange sites to the cell walls of the symbionts and intracellular uptake (ROLA et al 2016). The success of colonization of the substrate by lichens depends on the level of contamination with heavy metals; the accumulation pattern differs between different metals (ROLA et al., 2020).

The lichens have mechanisms of preventing heavy metals accumulation: the secondary metabolites of lichens form chelates with heavy metals; the substance from the lichen cortex, namely parietin, plays an important role in the protection of photobiont cells, photosynthetic pigments and chlorophyll-a fluorescence from the heavy metals deposited in excess, like cadmium (KALINOWSKA et al., 2015); binding cations at the extracellular sites (BAČKOR & LOPPI, 2009); formation of metal complexes by production of metal - binding proteins with suspensions of minerals and rocks, organic and inorganic precipitation, active transport, intracellular compartmentation, constituents of cell walls as chitin and melanin with significant metal-binding affinities; norstictic acid and other lichen substances determine tolerance to excess metal ions enabling colonisation of mineral substrates.

Lichens also have adaptations to tolerate metal toxicity. A physiological adaptation of lichens in metalliferous habitats is that they grow very slow. The positive effect of lichen association with metal is that copper lichen complexes and iron compounds function as sun-screening pigments (PURVIS, 2014). Out of the lichen symbionts, the most sensitive to abiotic stress are the photobionts. The photobiont decreases the impact of heavy metals through the sulfhydrylate groups. In photobionts subjected to the action of heavy metals the concentrations of cysteine and proline are

increased, forming stable complexes with free radicals (BACKOR & FAHSELT, 2008). Photobionts produce phytochelatins when are exposed to different amounts of heavy metals as Cd, Pb and Zn (PAWLIK-SKOWRONSKA et al., 2002); Cd and Cu are strong activators of phytochelatins biosynthesis in the lichen photobiont *Trebouxia erici* (BAČKOR et al., 2007).

Their uptake is positively influenced by a long exposure time (24 h) in 5 mM HEPES in 24 h (BAČKOR et al., 2007). Phytochelatins have not been observed in aposymbiotically grown lichen mycobionts. The cultured *Cladonia cristatella* mycobiont also did not produce phytochelatins (BAČKOR et al., 2006). To some extent, the metabolites produced by the mycobiont are involved in the tolerance of heavy metals.

Plants have detoxification mechanisms as a result of heavy metals accumulation like calcium inhibiting Cr ascension from root to shoot in rice seedlings (BRUNIALTI & FRATI, 2014; MUKTA et al., 2019). The high density of the heavy metals from the atmosphere influences the development of lichens, especially the early stages (SCHUSTER et al., 1985; OTT, 1987). In lichens, heavy metals get inside atoms of the thalli and accumulate by complexes with lichen secondary metabolites (LONGTON, 1997). Heavy metals influence the lichens' physiology. The phenotypic traits of the thallus influence bioaccumulation (OSYCZKA et al., 2018).

Heavy metals like Cu are phytotoxic and inhibitory on photobiont growth, viability and chlorophyll-a fluorescence (BIĚOVÁ et al., 2019). The establishment of lichen symbiosis is influenced by the accumulation of heavy metals. Therefore, the *in vitro* culture experiments proved that the molecules implied in signalling and recognition between symbionts are inhibited by sulphur-based pollutants  $ZnSO_4 \times 7H_2O$  (VOICU & GAVRILOAIE, 2007). Sulphur compounds also affect the physiological processes like plasmalemma permeability and enzymatic activity of the symbionts (SUNDSTROM & HALLGREN, 1973). The process of accumulation depends on a range of factors, like pH (decreasing the PH increases the solubility of some metals, from the atmosphere or substrate), temperature, location of lichens (correlated with weather conditions, precipitation allowing solubility), location (lichens from the isolated trees contain a higher quantity of heavy metals than those from a forest), soil stability (WEGRZYN et al., 2016).

Chemical compounds are closely involved in the bioaccumulation of heavy metals. The secondary metabolites of lichens secondary have an increased reactivity to increased concentrations of metal ions. The experimental results revealed that the level of secondary metabolites decreases as thallus accumulates heavy metal ions. Therefore, increased concentrations of copper induce a reduction of the secondary metabolites level in lichen *Stereocaulon japonicum* (NAKAJIMA et al., 2019). Comparative studies regarding air pollution in urban areas and control sites in nearby forests using different species of lichens, as *Parmotrema arnoldii* and *Tillandsia usneoides* (BENITEZ et al., 2019), are required to evaluate heavy metal depositions. For a comparative study related to the different degree of pollution, lichen samples are collected from urban, peri-urban and rural areas. Lichen samples from urban areas are enriched with metals originating from vehicle emissions and road dust (DAIMARI et al., 2020). The absence of sensitive lichen species in the parks of Bucharest beside the morphological changes in the moderately sensitive species reflect the high degree of pollution by intensive traffic (GOMOIU & ȘTEFĂNUȚ, 2008). Bioaccumulation with lichens presents two methodological approaches: bioaccumulation with lichens collected *in situ* and bioaccumulation with lichen transplants (CANSARAN & DUMAS, 2015). Different transplanted sites, as the vicinity of industries, evidence a particular type of metal accumulation (LOGESH et al., 2014). Transplanted lichens are also used to investigate indoor air quality and outdoor air quality in urban and rural areas, mostly in schools; studies indicate that in indoor conditions lichens accumulate Cd, Cu and Pb and maintain their viability (PAOLI et al., 2019). The bioindication parameters of lichens can be improved by calculating various ecological variables and estimation of environment quality.

Lichen abundance as an indicator of metal pollution is a better indicator than the index of atmospheric purity (AGNAN et al., 2017). Soil microbiota sustain plants to neutralize the noxious effect of heavy metals; the use of genetically engineered plants equipped with useful remediation traits according to the requirements of contaminated sites can be a highly effective tool to support this technology for on-site application (KHALID et al., 2016). For the safety of consumers regarding the plants' content of heavy metals, the cultivation of the plants must be realised in the areas without contamination with heavy metals (LAJAYER et al., 2017). Terricolous fruticose lichens absorb heavy metals from the corresponding substrata. They are used to monitor Cu, Zn and As pollution in surface soils (SUEOKA et al., 2016). In *Acarospora smaragdula* and *Lecidea lactea*, norstictic acid form complexes with copper from the cupriferous substrates (PURVIS et al., 1987). Interpretative scales are useful instruments to detect the amplitude of the pollution process in biomonitoring (CECCONI et al., 2019). Chlorophyll fluorescence emission is a parameter measured to highlight health lichens sample status. The lichens' viability influences the bioaccumulation capacity. From this point of view, CECCONI et al. (2019) determined 24 of chemical elements (Al, As, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Mg, Mn, Mo, Na, Ni, P, Pb, S, Sb, Sr, Ti, Zn) to samples of *Pseudevernia furfuracea* devitalised by spectrophotometric method. The desiccation/rehydration state of the lichens influences the effect of some heavy metals on lichens ROS induced especially by the photobiont photosynthetic pigments; therefore, the concentration of ROS decreases (ÁLVAREZ et al., 2015). Differences are seen between species in terms of bioaccumulation level. In this context, FORTUNA & TRETIACH (2018) demonstrate that *P. furfuracea* accumulates three times more Hg than both *X. parietina* and *R. pseudoacacia*.

Their results are discussed in the context of the actual European Union directives concerning air pollution monitoring and assessment, revealing that both active and passive biomonitors are efficient tools to provide a reliable estimation of the spatial changes of Hg concentrations in the environment.

One of the longest biomonitoring studies of Hg air pollution ever carried out at a local scale concern five biomonitors performed in the proximity of a waste incinerator over a 16-year period according to both active (biomonitor transplants) and passive (sampling native biomonitor populations) techniques. Our study demonstrates the high capacity of lichens to biomonitor pollution level with heavy metals.

Lichen nutrients and their implications in health benefit, beside secondary metabolites, promote lichens in future studies (ZHAO et al 2021).

## CONCLUSIONS

The partners of the symbiosis ensure the morphological, physiological, biochemical unity of lichens system. The study lichen symbiosis implies deciphering numerous important aspects of development and reproduction strategies of this system in nature and *in vitro*. The *in vitro* conservation of lichens is an efficient alternative reproduction strategy in the context of environmental changes and offer the possibility to understand this type of close symbiosis by promoting new studies of interactions between symbionts.

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