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Entry

Lichen as Multipartner Symbiotic Relationships

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Definition: Lichens have long been considered as composite organisms composed of algae and/or cyanobacteria hosted by a fungus in a mutualistic relationship. Other organisms have been gradually discovered within the lichen thalli, such as multiple algal species, yeasts, or even viruses. Of pivotal relevance is the existence of the lichen microbiome, which is a community of microorganisms that can be found living together on the lichen surface. This community performs a growing number of functions. In this entry, we explore the journey of lichens being considered from a dual partnership to a multi-species symbiotic relationship.

Keywords: symbiosis; microbiome; partnership; mycobiont; photobiont; holobiont; bacterial layer

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1. General Context of Lichens

Lichens are just one of many symbiotic relationships that can be established between heterotrophic fungi and photoautotrophic partners, such as plants, mosses, cyanobacteria, and algae. Partnerships between fungi and vascular plants are highly diverse and ecologically relevant. Some of these partnerships, such as those of ectomycorrhizas [1], endomycorrhizas, or the unique orchid mycorrhizal associations [2], are well known. The relationships between fungi and cyanobacteria or algae are also well known and very diverse, including the relation between algicolous fungi and bacteria or algae [3] and lichens [4]. Algicolous fungi can parasitize algae or cyanobacteria [5,6] or alternatively can establish a mutualistic relationship, as in the case of mycophycobioses [7]. Lichen-forming fungi may establish symbiotic relationships with algae or cyanobacteria and form a unique identity, i.e., the lichen. However, although unique, lichens are just one example of the highly diverse partnerships between fungi and photosynthetic organisms.

More than 18,000 fungal species, comprising a highly diverse group and representing around 20% of those currently identified, participate in lichen partnerships. They occur in all terrestrial ecosystems, ranging from polar to tropical areas and from coastal to high mountain ecosystems. Lichens form vegetative structures called thalli and can grow on a large variety of substrates such as minerals, rocks, bare soil, and the wood or leaves of plants, even in streams and marine zones [8], as well as on synthetic material surfaces.

The symbiotic condition of lichens remained unknown for a long time and, until 1869 [9], they were thought to be individual organisms. The German mycologist Anton de Bary introduced the term "symbiosis" to describe the condition of dissimilar organisms living together [10] supporting Schwendener's theory that lichen is formed by two separate organisms, a fungus and an alga. From then on, lichens were considered to be an obligate partnership between a fungus (mycobiont) and either cyanobacteria and/or green algae,

that acted as a photoautotrophic partner (photobiont) [8,11]. Figure 1 shows the location of the mycobiont and the photobiont within heteromerous lichens, in which the algae and fungal components are arranged in definite layers. The stability of this symbiotic association depends on the mutualistic–antagonistic relationships of a multitude of interlinked organisms, also known as the "holobiont" [12,13]. This complex relationship is determined by the symbionts' interactive intimacy, stability of environmental conditions, and partner availability [14,15].

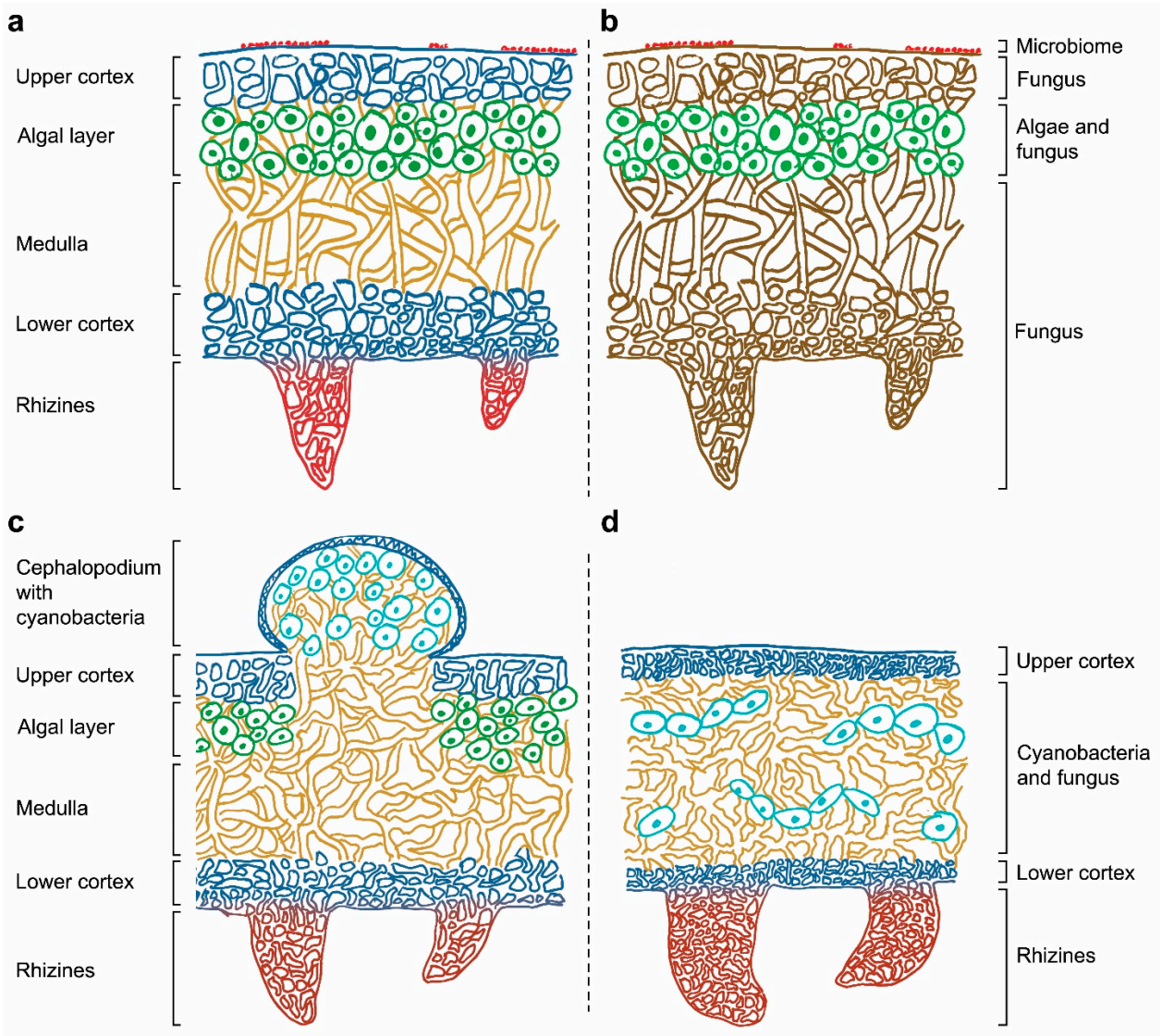

Figure 1. (**a**) Correspondence between the lichen structure and (**b**) their individual components in a heteromerous thallus. Depiction of tripartite lichen (**c**) and homoiomerous lichen (**d**).

Thalli are specialized structures unique to lichen-forming fungi and are not observed when the mycobiont grows in isolation. The thalli hold the photosynthetic partners and are one of the most complex structures in the entire fungal kingdom. Thallus structures are mainly determined by the fungal partner and can be grouped into three main growth forms: crustose, foliose, or fruticose. Crustose lichens, lacking the lower cortex, are completely attached to the substrate, while foliose and fruticose lichens are only partially attached through anchor like structures such as rhizines and hapters [16]. The internal space of the thallus can be made up of either an internal stratification with a fungal upper layer and underlying algal layer (heteromerous, Figure 1a), or an even distribution of the mycobiont and photobiont (homoiomerous, Figure 1d). Lichens do not have a waxy cuticle to isolate

the thalli from their surroundings and therefore everything in the lichen's environment is absorbed into its structure, including water and nutrients coming from air and rain. They also lack vascular tissues (such as xylem and phloem in plants) to distribute nutrients and water around their thalli.

The general structure of a heteromerous lichen is shown in Figure 1 and it is mainly composed of layers of fungus and alga. The upper cortex is the outer layer of the lichen thallus, and it is formed by the mycobiont. The cells in this layer are tightly packed to provide certain physical and chemical protection from the environment. The algal layer contains the photobiont, which frequently is a green alga. Cyanobacteria, if present, can be located in small vacuoles called cephalopodiums, which exist on top of the upper cortex or within the tissues of the lichen when there is a green algal layer already present (secondary photobiont, Figure 1c), or in a layer under the upper cortex (primary photobiont). Part of the lichen thallus is composed of filamentous fungal cells that form the medulla. This layer is loosely packed and has a threadlike structure. The lower cortex, with a similar structure to the upper cortex, protects the medulla and give support to the rhizines or other basal attachments that allow lichens to get linked to their substrate. Rhizines are fungal multicellular structures originated on the lower surface with no vascular purposes: no water or nutrients can be absorbed by them, and their unique function is to support the lichen attachment. Although this is the most frequent structural organization found in a thallus, some lichens present no distinguishable layers of mycobiont and photobiont. In these cases, the components are distributed in one big uniform layer, resulting in a gelatinous growth form (Figure 1d).

Most lichen-forming fungi belong to the phylum Ascomycota, while only 0.3% of lichenized fungi are known to be derived from Basidiomycota [8,17]. The majority of lichen-forming algae belong to the green algae (85%), and 10% have a cyanobacterium as primary photosynthetic partner, but also brown or yellow-green algae have been identified in these relationships [18,19]. Sexual reproduction of the fungal partner involves the growth of fruiting bodies from the thallus which produce ascospores to be dispersed. In addition to this sexual mechanism, lichens have also developed other processes of asexual reproduction to disperse both partners together in varied and specific joint propagules [16].

2. From a Dual Partnership to a Multi-Species Symbiotic Relationship

In this intimate and long-term partnership, the fungal partner provides water, mineral nutrients, and sheltering structures for the photobiont, which contributes photosynthetically fixed carbon as the energy source for the system. Although the fungus can be very specific when selecting its photobiont [20,21], generalist fungi have also been frequently described [22–25]. Yahr et al. [26] established three categories according to the range of photobionts with which they are able to lichenize: photobiont specialists, intermediates and generalists. Following this scheme, photobiont specialists partner with single algal lineages, while photobiont generalists accept a high variety of algal partners and can stablish associations with a number of strains according to environmental conditions. Intermediates form symbiotic relationships with a reduced number of algal partners. Photobiont generalists are frequently associated to lichens with wide both geographical range and ecological niches, which can associate with locally adapted photobionts in different climatic regions [25,27,28]. The exact factors that determine the photobiont selection are well known and appear to be related to phylogenetic specialization, fungus reproductive strategy, photobiont cell availability and ecological factors such as climate or substrate [29,30]. Surprisingly, multiple algal species have been observed in association with the same thallus in a large number of studies [24,31,32]. For example, Backor et al. [33] confirmed the presence of multiple algal genopypes in a single lichen thallus, Casano et al. [34] found that *Ramalina farinacea* thalli represent a specific and selective form of symbiotic association involving the same two *Trebouxia* phycobionts, and Del Campo et al. [35] concluded that ecological diversification and speciation of lichen symbionts in different habitats could include a transient phase consisting of associations with more than one photobiont in individual thalli. This

pattern of algal coexistence is probably promoted by their different and complementary ecophysiological responses which facilitate the proliferation of the lichen in a wide range of habitats and geographic areas [34].

In the last decade, it has been shown that lichens are far from being a simple association between two unrelated organismal groups, and instead involve a bacterial (and other fungi including yeast) component which is a key contributor to the biology of the holobiont. The inclusion of bacteria within the lichen partnership was first observed around the 1930's [36–38] (Figure 2), and it has been described as a discontinuous monolayer on the thallus surface (Figure 1). At that time, rudimentary methods only facilitated associating these bacteria with a possible and unspecific role in nitrogen fixation. At the beginning of the 21st century, the first molecular analyses started using bacterial isolates (e.g., Gonzalez et al. [39]). However, as culture-dependent methods can only account for 0.001–15% of the bacterial diversity [40], most microorganisms had remained unrevealed [41]. More recent studies allowed the observation that the structure of the nitrogen-fixing bacteria present in the cyanolichens is different from that of chlorolichens and that chlorolichens have a higher diversity of nitrogen-fixing bacteria than cyanolichens [42]. Also, in addition to the main cyanobiont, other cyanobacteria have been found within the microbiota of lichen thalli and substrates [43,44], which can also contribute with part of the nitrogen input to the symbiosis.

Once new methods were developed to complement the previously applied techniques, the limitations of the bacterial isolation were overcome, which allowed researchers to holistically explore the bacterial community. Fingerprinting techniques [45] and molecular cloning methods (e.g., Hodkinson and Lutzoni, [46]) allowed the production of microbial community profiles of lichen-associated microbiota. Bates et al. [47] revealed the microbial community associated with lichens based on next generation pyrosequencing for the first time. Thus, multi-omics approaches, that allow the integration of multiple omics research and datasets explaining the mechanisms underlying biological processes and molecular functions [48], along with bioinformatic tools have put the spotlight on the host-specific bacterial microbiome [49–52]. Recent findings about bacterial associations with lichens support their relationship as a multi-species symbiosis in which different roles are played by an increasingly recognized diversity of organisms associated with the thalli (see [49] and references therein; Figure 2).

3. Potential Roles of Recently Discovered Partners

Bacterial and secondary fungal communities inhabiting lichens have been found through next-generation sequencing techniques (e.g., Grube et al. [45], Tuovinen et al. [53]). Key roles as functional components in structuring the thalli and modulating the response to environmental factors could be performed by these overlooked organisms [49,50,54]. Since the first half of the 20th century, individual strains of bacteria have been isolated from lichens, with Alpha proteobacteria making up the dominant group, first observed by Cardinale et al. [55]. Studies of the diversity of lichen-associated bacteria suggest that different parts of the thallus, providing different chemical and physiological micro-niches, can influence microbial colonization ([45,46] and references therein). Another main factor driving the bacterial composition is the fungal partner. Accordingly, Aschenbrenner et al. [56] demonstrated that the lichen *Lobaria pulmonaria* presented a core and shared fraction of its bacterial biome, as well as a transient fraction. They also demonstrated that bacteria were present in the lichen's vegetative propagules which allowed them to vertically transmit through asexual reproduction. Traditionally, it has been accepted that the mycobiont builds up the thallus as a result of the specific interaction with a suitable algal partner, and then numerous associated and potentially interacting bacterial and other partners colonize the lichen more or less specifically. Recently, it has been hypothesized that the microbiome contributes to the lichenization process [57].

Similar questions to those posed regarding the role and specificity of bacteria within lichens have also been raised in relation to secondary fungal communities inhabiting lichens.

Spribille et al. [58] found that a specific group of basidiomycetous yeasts played a part in the microbiome of two epiphytic lichens (Figure 2), and that the abundance of the yeast within the lichen was correlated with concentrations of vulpinic acid, which is a secondary metabolite associated with lichen defenses. More recently, Cernajova and Skaloud [59] discovered previously unknown cystobasidiomycete symbionts in a number of *Cladonia* species in the northern hemisphere.

The consistent presence of basidiomycetous yeast within lichens in these studies suggested these were the third partner within the lichen complex. However, Millanes et al. [60] considered *Cyphobasidium* spp. to be a lichen-related fungi that can form galls on the thalli instead of as a previously unseen third mutualistic partner. The relevance of these lichen-related yeasts was also discussed by Oberwinkler [61], who pointed out that "it is obvious that basidiomycetous yeasts in lichen thalli are not a third component of symbiosis, but rather the vegetative propagules of mycoparasites". Supporting this view, Lendemer et al. [51] failed to detect basidiomycete yeasts in over 97% out of 339 lichen species from the Appalachian Mountains in North America in a metagenomic study. Although their metagenomic approach is likely less sensitive than PCR assays with specific primers, these findings raise questions about the ubiquity and specificity of yeasts in lichens.

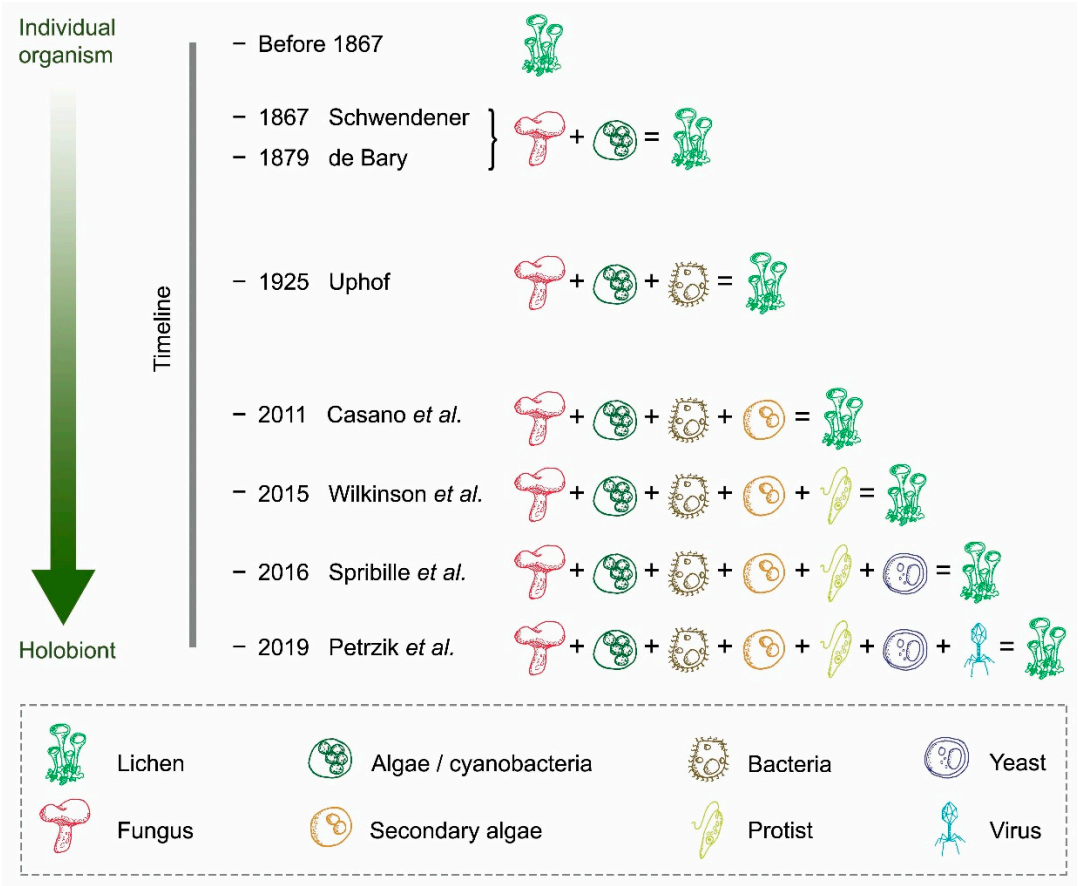

Figure 2. Temporal line indicating the timing at which the different components of the lichen partnership were discovered. This figure reflects the journey from considering lichens as a single organism to the complex multipartner symbiotic relationship known nowadays [9,10,34,36,58,62,63].

4. The Increasing Complexity Surrounding the Concept of Lichen

The discovery of potential new partners increased the complexity of species interactions within these supraorganisms. However, the lack of experimental evidence regarding the lichen microbiome hindered our ability to reveal the network of interactions within this holobiont. As opposed to what was traditionally believed, the photobiont should not be

limited to a single strain of algae [34] and protists and even viruses can form symbiotic associations with lichens [62,63] (Figure 2).

Conceptualizing a lichen means accounting for a vast array of related microorganisms, providing the ideal example of a holobiont, composed of a dominant mycobiont and diverse microbiome [64]. This evolved network of biotic connections whose morphology is shaped by the mycobiont, is at the service of the fitness of the entire superorganism. Hawksworth and Grube [65] re-defined the lichen symbiosis as: 'a self-sustaining ecosystem formed by the interaction of an exhabitant fungus, an extracellular arrangement of one or more photosynthetic partners and an indeterminate number of other microscopic organisms' [66].

While a detailed analysis of the scientific scrutiny focused on lichens is beyond the purpose of this text, a rough indication of the increasing interest in the lichen microbiome can be obtained by looking at the publications of the last 20 years (Figure 3). Although a linear relationship is missing, since 2014, the concept of a lichen symbiotic association, including more than just the mycobiont and the photobiont was well established. The development of "omic" technologies is a key element that has allowed the proliferation of studies in this research field [67]. The number of citations of a specific paper can increase with the time from its publication, which can explain the low number of citations for the most recent works; however, since 2015, the annual number of citations has exceeded 50, testifying to an increasing interest in this specialized topic.

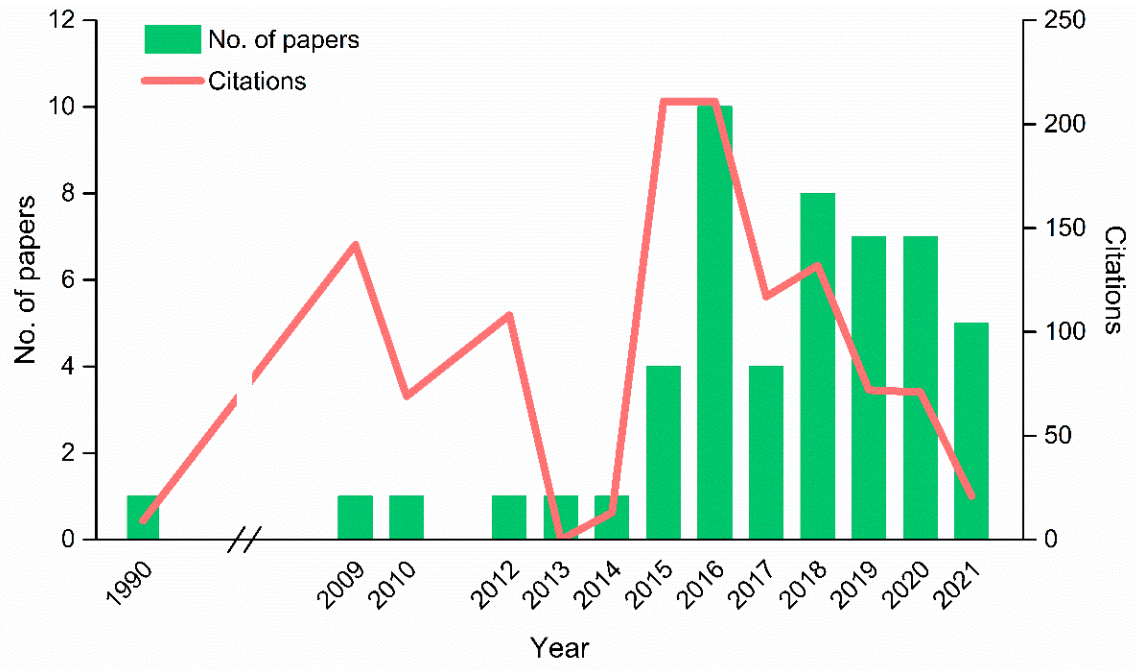

Figure 3. Number of papers related to the lichen microbiome and their citations. Data obtained using the search terms "lichen microbiome" or "lichen microbiota" or "lichen bacterial community" or "lichen microbial" or "lichen bacteria" or "lichen-associated bacteria" in the Web of Science (https://www.webofscience.com/wos/woscc/basic-search) accessed on 13 February 2022. All results were checked to ensure that they were referred to the presence of microorganisms in the lichen symbiosis.

5. Mutualism or Parasitism?

Although lichens are usually considered mutualistic symbioses, many lichen characteristics identify them as controlled parasitic interactions [68,69]. Initial studies in this field deemed lichens as algae parasitized by fungi because they found algal cells in a lichen thallus which were dead or penetrated by fungal haustoria. However, other authors considered lichens as mutualists based on the seemingly healthy and long-lasting

relationship among its partners. Two different experimental approaches have been developed to study selectivity among the lichen partners. The first one is based on in vitro resynthesis of the independently cultured mycobiont with different photobiont species. An alternative approach involves the assessment of the specimens collected in different geographical areas and the identification of the partners present in the lichen thalli [70]. In vitro studies give researchers the opportunity to observe initial stages of the lichenization process. Several developmental stages of lichenization have been described depending on the interacting alga, and timing of these events is variable depending on the species, media, and incubation conditions. The first developmental stage which has been described is the "pre-contact" stage, crucial for the establishment of symbiont recognition mechanisms and biont specificity [71,72], where the partners are in close proximity to share extracellular secretions but not physical contact. In a second phase, the "contact" stage, the two bionts start making physical contact by fungal appressoria (which are flattened hyphal tips that bind to the host cell surface and start a penetration peg), whereas, in a third phase termed the "growth together" stage, the two partners grow together in a network to form cellular masses containing both bionts [73–76].

Attempts of resynthesis in the laboratory starting from the isolated partners have been made with various and inconsistent outcomes [52,68,69,77–86], making the study of lichenization mechanisms hard due to the lack of consistently repeatable results. In vitro re-synthesis experiments showed that the interaction of a mycobiont with its compatible algal partner triggers specific morphological differentiation that is not seen when in contact with incompatible algal partners [68,71,87]. Conversely, parasitic behaviors of the fungus can be observed in interaction with nonlichenized algae or lichenized algae from different lichen species [68,71,87,88]. Switching to parasitism and saprotrophic nutrition is also known in some lichens, like *Ochrolechia frigida* when growing without its alga partner. In algal-free stages, this species seems to be capable of saprotrophic nutrition on mosses, phanerogams and other lichens [89].

In agreement with an optional parasitic or saprotrophic lifestyle of the mycobiont, Munzi et al. recorded for the first time in lichens a high activity of extracellular enzymes able to digest organic matter of different types and that are usually found in mycorrhizal fungi, which also alternate between different lifestyles (unpublished). A common characteristic of axenic reconstitutions is either that they do not progress beyond the soredia or squamule stages or, if they do, the resulting thalli do not resemble closely the corresponding natural lichens in shape, size, and full differentiation. Gene expression studies [72] and specific exudation patterns of lichen photobionts [90] also point to extracellular communication between lichen symbionts without cellular contact [57]. For example, ribitol was capable of overcoming fungal growth arrest [90], fungal lectins induced chemotropism of compatible *Nostoc* cells in cyanolichens [91], and chitinase, a defense enzyme in plants against pathogens, was downregulated in the photobiont during resynthesis stages [87]. Interestingly, the results of proteomic analyses in the thalli of the lichen *Xanthoria parietina* [92] included expression of proteins linked to the signaling compound pathways mentioned above.

Our knowledge about the cytological and biochemical interactions between the symbionts in lichens is still scarce [88]. However, increasing evidence indicates conservation of signaling pathways involved in the establishment of other major symbioses between plants and mutualistic microbes. Both common effectors and genes have been found to be essential for the establishment of rhizobial, Frankia, mycorrhizal and fungal endophytic symbioses, including plant-produced strigolactones, microbial partner-produced chitooligosaccharides (COs) and lipo-chitooligosaccharides (LCOs) [93–99], genes encoding Vapyrin [93,100,101] and several transcription factors [102,103]. Three of these transcription factors (CYCLOPS, NSP1, and NSP2) are well conserved between actinorhizal, legume, non-legume, and mycorrhizal symbioses [96]. It is therefore possible that some or all these factors are also present and play essential roles in lichenization, and their absence or differential presence may relate to re-synthesis failures or establishment of associations with parasitic outcomes.

6. Conclusions and Prospects

A large scientific effort is still needed to achieve the goal of revealing the physiological mechanisms operating in convoluted lichen symbioses and the roles of the various organisms involved in this complex holobiont. As the latest scientific evidence has shown, this research challenge must be addressed by considering lichens as self-sustained and adaptable systems of partnerships, just like us!

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