Enforced fungal-algal symbioses in alginate spheres

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Abstract

The thallus structure of the lichen symbiosis provides a fungal shelter for the growth of algal partners. The long-living thallus also provides a habitat for other fungi, but experimental studies, which could inform us about the details of their interactions have hardly been conducted. We present a new approach by embedding axenically cultured strains of fungi together with algae in alginate spheres, which allows easy transfer of co-cultures on solid media. As the growth rates of the organisms are differentially triggered by the underlying medium, alginate embedding can help to adjust optimal parameters for stable culture of the combined symbionts. In our experiments, direct contacts between hyphae and algae and the formation of layered structures were observed in a fungus that is living as a commensal in the host lichen without visible symbiotic structures. The growth of primary lichen symbionts cannot be accelerated by alginate embedding so far, but our approach could artificially enforce symbiotic interactions that are not normally observed in nature.

Keywords: co-culture, encapsulation, lichens, mixed growth, photobionts, synthetic symbiosis.

Introduction

Soon after the discovery of lichen phenotypes as a result of the symbiotic interaction of fungi and algae (Schwendener 1869), researchers started to artificially resynthesize lichen thalli by culturing experiments (e.g., Reess 1872; Stahl 1877; Bonnier 1886). Results from these ongoing experimental approaches provided insights in the selectivity of fungal-algal associations, the regeneration of natural phenotypes under artificial conditions, or the ability to synthesize specialized compounds (e.g. Ahmadjian 1973; Honegger 1990;

Yoshimura et al. 1993; Stocker-Wörgötter 2001, 2002). It remained as a challenge that the symbionts of lichens grow slowly and that the resynthesis of thallus structures requires special conditions and substrates (filters, artificial soil), which help to mimic the conditions found in nature.

Recent researches discovered the diversity of other microorganisms in lichen thalli, including bacteria (Grube et al. 2009), algae (Moya et al. 2017) and other fungi (Arnold et al. 2009; Spribille et al. 2016; Muggia et al. 2016). According to multiomics analyses and culture assays, diverse functions are attributed to members of the species-specific bacterial communities (Grube et al. 2015), whereas variation of physiological performances of different algae isolated from single lichen thalli may extend their ecological amplitude (Casano et al. 2011). Additional fungi have diverse effects on their host lichens as well. Lichenicolous fungi, recognized by propagation structures and symptoms they induce on the lichens, occur as parasites or commensals on their hosts (Lawrey and Diederich 2003). The yeast-stage of a lichenicolous basidiomycete is correlated with production of compounds that leads to a color change of the lichen (Spribille et al. 2016). The biological roles of many, seemingly symptomless lichen-inhabiting fungi are little known so far (Arnold et al. 2009; Fernandez-Mendoza et al. 2017). It is not even clear whether fungi isolated from lichens grow actively or just represent trapped spores.

Co-culturing of isolated partners under controlled conditions is an experimental strategy to gain more information about requirements and effects of lichen-inhabiting fungi. Several co-culture systems can be distinguished by type of technology, including microfluidic systems, solid support systems (including Petri dish co-cultures), bioreactor systems or transwell systems (Goers et al. 2014). These systems allow to inoculate the mixed partners together or to let them interact after they were inoculated individually. Irrespective of the technological sophistication, the conditions for sustained co-cultures need to be carefully designed to keep the growth of the symbionts in balance. In this study, we present a new approach for the study of symbiotic interactions of lichen partners in alginate spheres, which are placed on solid media. We used this approach to inoculate multiple combinations of fungal and algal strains, each taken from axenic cultures. We focused here on three types of fungi: one lichen mycobiont, one lichenicolous fungus and three fungi with so far unclear relationships with algae. We combined these fungi with photobionts of the genus *Trebouxia*, a common and widespread type of photobiont in many lichen symbioses. We suggest that our experimental set up is convenient and cost efficient for handling of co-cultures and can be used to enforce symbiotic interactions among non-model organisms.

Material and Methods

Selection of the biological materials — Material for the cultures was available at the Cultures of Lichenized and Extremotolerant Organisms (CLEO) collection managed by LM and maintained at the Institute of Plant Sciences Graz. We used cultured strains of the following fungi (Table 1): *Tephromela atra, Lichenothelia calcarea, L. convexa, Saxomyces alpinus* and *Muellerella atricola*. *Tephromela atra* is a lichen mycobiont of worldwide distribution and representative of a swarm of

closely related species with wide ecological spectrum (Muggia et al. 2014a). *Lichenothelia calcarea*, *L. convexa* (Muggia et al. 2013, 2015) and *Saxomyces alpinus* (Selbmann et al. 2014) are three rock-inhabiting fungi in the class Dothideomycetes, which are occasionally found in association with endolithic and epilithic algae in their natural environments. *Muellerella atricola* (Verrucariales, Chaetothyriomycetidae) is a lichenicolous fungus and was isolated from thalli of *Tephromela atra* (Muggia et al. 2015). The *Trebouxia* algal strains isolated from thalli of *Tephromela atra* correspond to *Trebouxia* sp. 1 and *Trebouxia* 'clade IV' (Table 1), as characterized in Muggia et al. (2014b).

Setup of mixed cultures: alginate sphere formation and embedding of symbionts — One gram of alginate was mixed with 150 ml distilled water. The fungal material was carefully ground in sterile ceramic mortars with the alginate-water slurry. Algal colonies were taken with sterile inoculation loops and diluted in 1 ml of water. The suspension of algal cells was added to the slurry containing the ground mycelium. The mixture of fungi and algae was carefully mixed by repeated pipetting. Droplets of the mixture of about 100 µl were then pipetted in a 0.5% solution of calcium nitrate, leading to solidification of the alginate drops into globules of 3-4 mm in diameter (Fig. 1). Up to ten alginate globules containing both symbionts were carefully taken with sterile tweezers and placed in Petri dishes (made of plastic) prepared with different solid media: *Trebouxia* medium (TM, Ahmadjian 1967), Malt Yeast extract medium (MY, Ahmadjian 1967), Lilly and Barnett's medium (LB, Lilly and Barnett 1951), Bold´s basal medium (BBM, Bold 1949; Bishoff and Bold 1963) and water agar. The cultures were sealed with parafilm and stored in a growing chamber at 20 °C, with a light-dark regime of 14:10 hours, a light intensity of 60-100 µMol photons $m^{-2}s^{-1}$ and 60% humidity. Subcultures were set after one year on TM and MY media for fungal-algal combinations which developed stable mixed growth.

Results

Experiments with alginate globules placed on nutrient-containing media increased the growth rates of both, fungi and algae. On BBM medium, algae and fungi grew much slower than on TM, LB and MY media. In the majority of the experiments, both strains of *Trebouxia* grew massively, emerging at the surface of the globules (Fig. 2A-D, G-L), but remaining contained in the globule volume. In one of the cultures on TM, algae covered completely the fungal mycelia after one year and extended on the medium by surrounding the globules (Fig. 2E). The growth of fungi was not significantly accelerated by any of the different media. In contrast, growth of the symbiont colonies was very limited and slow in alginate globules placed on water agar. Algal overgrowth of fungi was primarily observed in the experiments set up with the black fungal strains (i.e. *Saxomyces* and *Lichenothelia*), which are already known for their very slow growth rates

(Ametrano et al. 2017). Alternatively, a rather balanced growth was observed in the globules prepared with the mycobiont *Tephromela atra* (not shown) and the lichenicolous fungus *Muellerella atricola* (Fig. 2J-L). The mixed growth of fungi and algae emerged three months after inoculation and did not show further improvements or alterations within at least ten months thereafter. Irrespective of the medium or participating fungus, we repeatedly observed bleaching of the algal colonies after ten months (Fig. 2B), which then resemble white granular clumps. In other cases, the peripheric algal colonies that escape the alginate spheres (as well as the fungal mycelium) turned yellow, suggesting that their chloroplasts became chromoplasts (Fig. 2C, H, I, L). In internal parts of these cultures, however, the green chloroplasts were preserved (Fig. 2I). After 12-14 months of co-culturing, the alginate globules collapsed and the cultures extended on the surrounding medium.

We could not detect any reliable sign of symbiotic interaction in globules set up with the fungi *Lichenothelia calcarea* and *L. convexa* (Fig. 3A, B). Algal cells were kept tightly together and were surrounded by hyphae only in the spheres with *Saxomyces alpinus* (Fig. 3C). The fungus *Muellerella atricola* (Fig. 3D-I) was observed to produce abundant conidiogenous cell masses (Fig. 1L, Fig. 2E, I), irrespective of the type of medium or the associated photobiont. In the spheres containing *Muellerella atricola* (Fig. 3D-I) and either strains of *Trebouxia* (*Trebouxia* sp. 'clade IV' and sp.1), algal cells and fungal hyphae have arranged in a layer-like structure (Fig. 3D-F). Here, the algal clumps were tightly compacted by the hyphae (Fig. 3H) and direct contact between hyphae and algae could be observed (Fig. 3G). The abundantly produced conidiogenous cell masses were observed to have developed always next to and above the algal colonies (Fig. 3E, I). From the alginate globules of *Muellerella atricola* with *Trebouxia* few, tiny clumps of algae (including both, the yellow and green algae) were taken and subcultured on Petri dishes. All these inocula developed into abundant, green algal colonies with fungal mycelia in between, which in some parts grew around and over the algal cells (Fig. 4A-D).

Discussion

In this paper, we present a simple and flexible approach for experiments in symbiosis research. We make use of the encapsulation of microorganisms in alginate, a technique widely known in cell immobilization biotechnology. This procedure can be used to enforce symbiotic interactions where it has not been observed in nature yet and to test various combinations and starting concentrations of interaction partners. Our example for this enforced symbiosis was the lichenicolous fungus *Muellerella atricola*, which lives as a specific commensal of *Tephromela atra* lichen thalli. No interaction with algae could be observed so far under the microscope using sections of the infected thalli, whereas layer-like structures and algal cells that are tightly bound by fungal hyphae were enforced in alginate spheres. In addition, the interaction led to richly developed mitotic propagules (conidia) by *Muellerella*, which we consider a clear sign of a fitness-enhancing symbiotic effect. The results of our work also show that media composition has a drastic effect on the formation of symbiotic relationships in cultures. Only MY media equally supported the growth of both

fungi and algae in alginate globules, whereas other media did not support growth of symbiotic relations at all.

It is likely that a wider range of fungi has a potential to form sustained interactions with algae. A previous study showed the formation of lichenoid structures on Petri dishes by a black meristematic fungus that was isolated from a lichen (Brunauer et al. 2007). This was not found for other black fungi under the conditions tested in the present study. A latent capacity for fungal-algal mutualism was achieved by niche engineering even for fungi that are normally not considered as typical symbiosis formers, such as baker's yeast *Saccharomyces cerevisiae* (Hom and Murray 2014). Our approach offers the tools for a wider screening of fungi and conditions for their potential in enforced symbiosis.

The new approach could also be used to assess whether bacteria are more tightly involved in symbiotic functions and can be experimentally tested by co-cultivation. Recently, Muggia et al. (2016) focused on the optionally lichenized fungus *Schizoxylon albescens* to test the effect of bacteria in mixed cultures with fungal and algal strains in Petri dishes. In their experiments, no clear effects could be found with the few tested bacteria. Thus, the authors suggested that more strains of lichen-derived bacteria need to be screened for their effect on the lichen symbiosis. The alginate embedding procedure provides a suitable basis for the high number of parallelized experiments required for this purpose.

The mixed cultures established here keep their structure for several months. However, as the sealing with parafilm allows gas exchange, it is not uncommon that the medium, on which alginate globules are placed, dries out and reduces in thickness. We experimented that it is sufficient to add few drops of water on the medium once a while to reduce the desiccation. Alternatively, the globules can be transferred onto a freshly prepared medium to prolong the co-growth of the organisms. No cryo-stock conservation has been prepared yet, but this is doable and should follow an already established protocol of cryo-conservation of culture material (e.g. Dahmen et al. 1983).

The number of papers studying lichen-inhabiting fungi is increasing and recent reviews already propose endolichenic fungi as a source for novel drugs (e.g. Kellog and Raja 2017; Singh et al. 2017; Suryanarayanan and Thirunavukkarasu 2017). A recent example was a *Pestalotiopsis* strain isolated from the lichen *Cetraria islandica*, which produced polyketide-terpene hybrid molecules, some of which showed pronounced antifungal effects against the economically important fungal pathogen *Fusarium oxysporum* (Yuan et al. 2017). Although the growth rate of the fungi tested in the present work is modest, we are aware that more fungi like *Pestalotiopsis*, which grow faster under controlled conditions, are also common as co-inhabitants of lichens. It is therefore essential to expand the cultural techniques by which such interesting fungi could be retrieved and studied. Muggia et al. (2017) compared effects of growth media on the diversity of culturable fungi from lichens to extend the number of fungal strains that can be isolated from lichens. The authors however, have not tested the effect of symbiotic partners of lichens to achieve this goal yet. We are convinced that alginate embedding and enforced symbiosis could also here provide interesting tools to further discover the diversity of lichen-associated fungi and their metabolic potentials.

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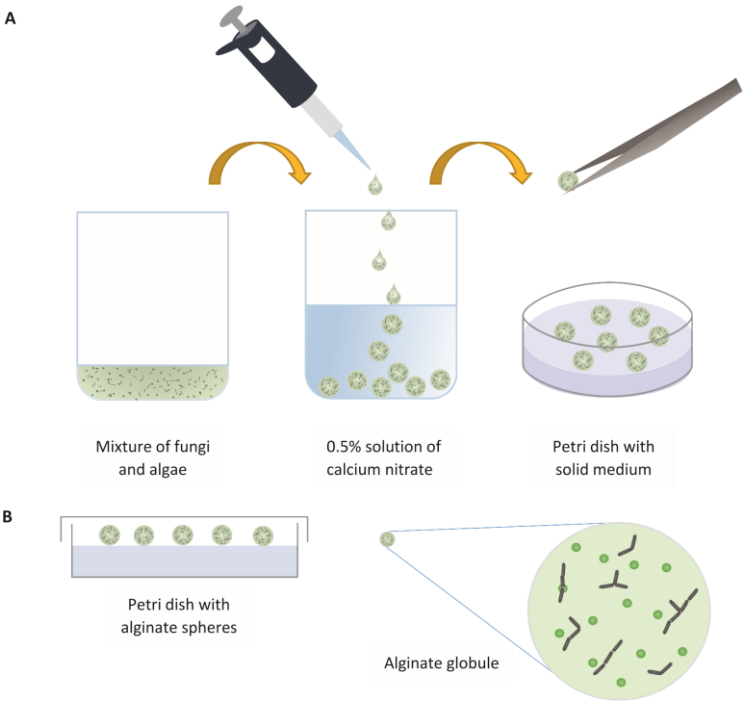

Figure 1. Schematic workflow of the preparation of alginate inclusions for mixed growth of fungal-algal cultures. A) Grinded fungal mycelia and algal cells are mixed in an alginate-water slurry and dropped with a pipette into a solution of calcium nitrate to let the drops solidify into globules; the alginate globules are picked with a forceps and placed on a growth medium into a Petri dish. B) Frontal view of the inoculated

plate and enlarged view of the alginate globules (3-4 mm diameter) containing fragments of fungal mycelia and algal cells.

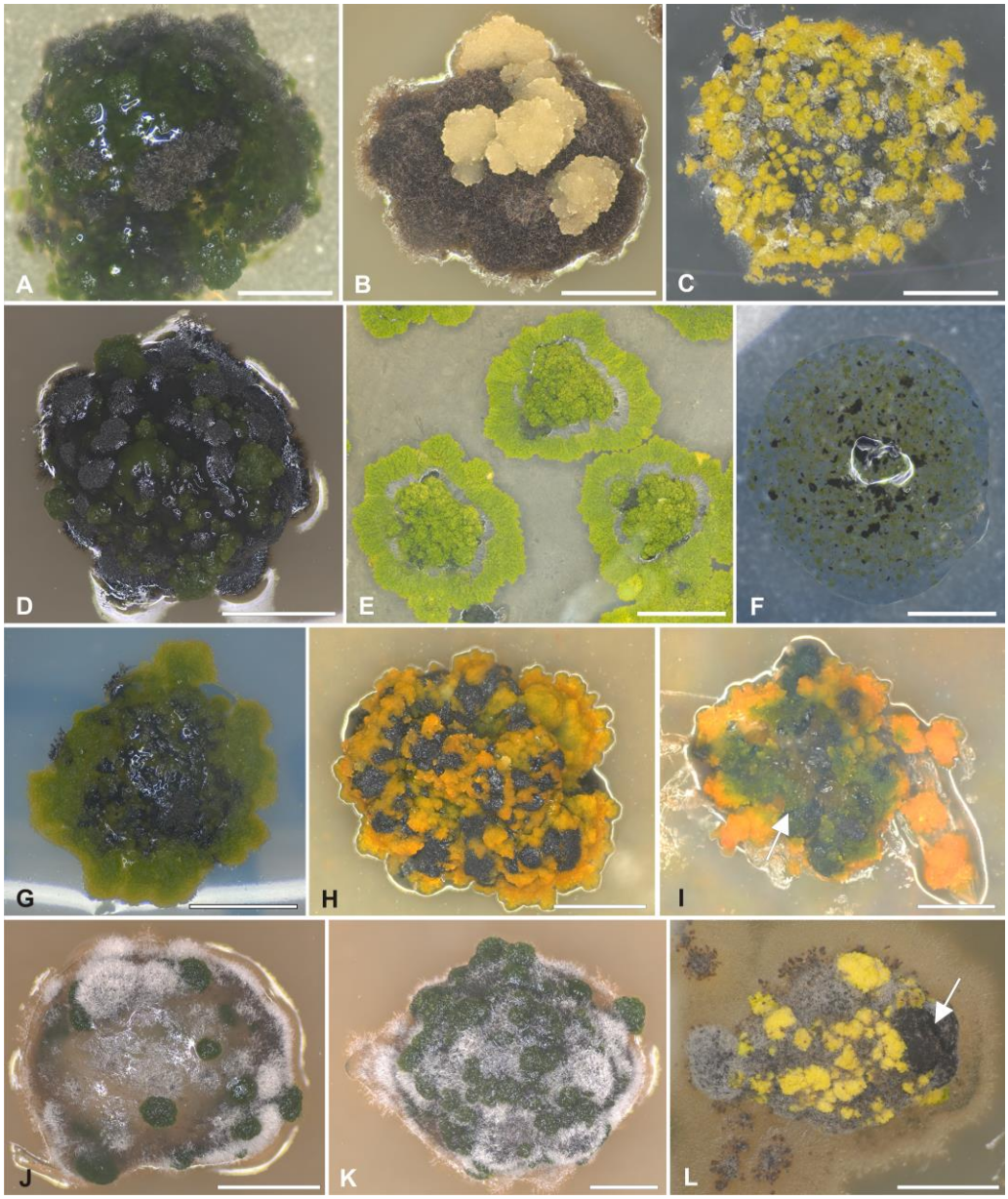

Figure 2. Alginate spheres formed in calcium nitrate in different stages of development. For each photograph the information is reported as follows: name of the fungus (ID number) and name of the algae (ID number) as reported in Table 1, age of the culture, medium on which the alginate spheres were placed. A) *Lichenothelia calcarea* (L1852) with *Trebouxia* sp.1 (L1379), three months after inoculation, on TM; B, C) *L. calcarea* (L1852) with *Trebouxia* sp.1 (L1379), one year after inoculation on TM (B) and on water agar (C); D, E) *Saxomyces alpinus* (CCFEE 5470) and *Trebouxia* sp.1 (L1379), three months on MY (D) and one year on TM (E) after inoculation; F) *Saxomyces alpinus* (CCFEE 5470) and *Trebouxia* sp.'clade IV' (L1661), immediately after inoculation on BBM; G) *Saxomyces alpinus* (CCFEE 5470) and *Trebouxia* 'clade

IV' (L1661) three months after inoculation, on BBM; H, I) *Saxomyces alpinus* (CCFEE 5470) and *Trebouxia* sp. 'clade IV' (L1661) one year after inoculation on MY; J-L) *Muellerella atricola* (L1993) and *Trebouxia* sp.1 (L1379) three months (J), six months (K) and one year (L) after inoculation on MY. In some alginate globules after one year the photobionts have either bleached (B) or the chloroplasts of the cells of the outer layers have turned into chromoplasts as the cells presented a yellow coloration (C, H, I, L); the more internal layers of the algae preserved the original green state (arrow in I). L) The fungus *Muellerella atricola* produces conidiogenous masses (arrow) also in co-growth with the photobionts (arrow). Scale bars: A, C, F, K) 1 mm; B, D, G-J, L) 2 mm; E) 4 mm.

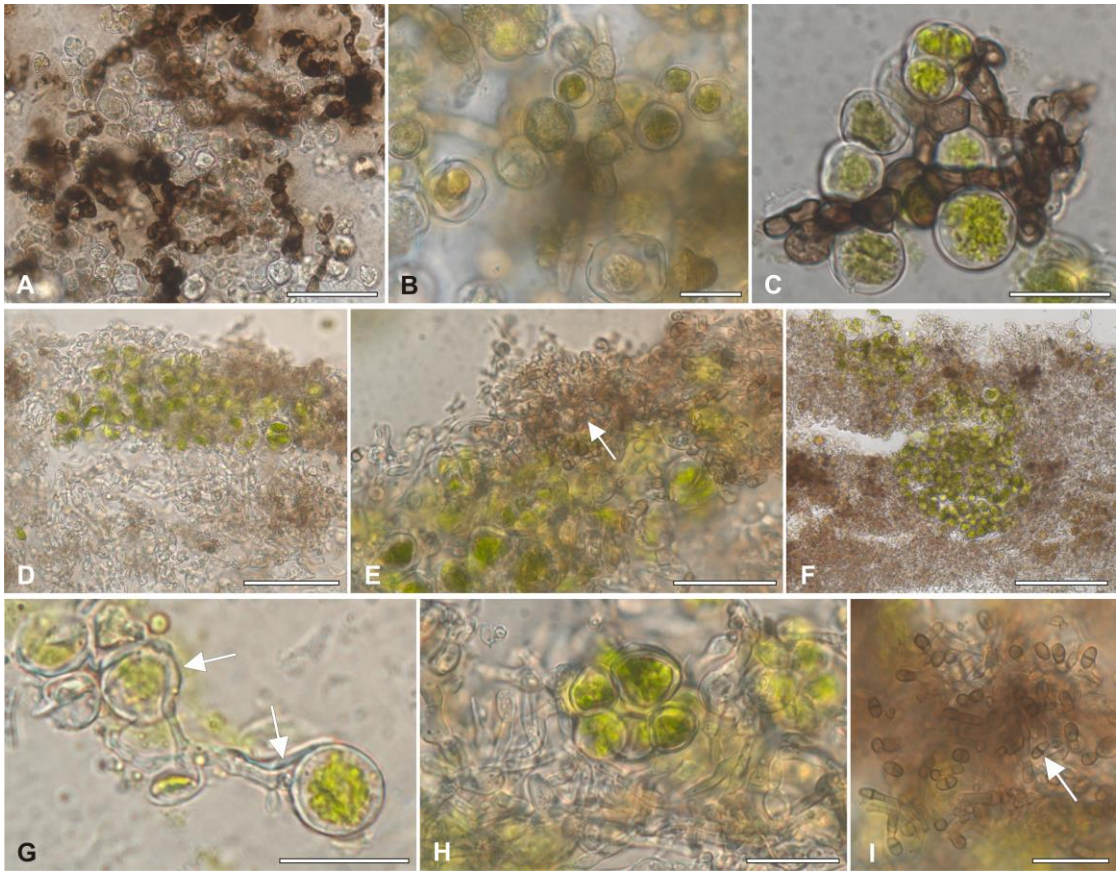

Figure 3. Microphotographs of sections of alginate spheres after one year of fungal-algal co-growth. For each photograph the information is reported as follow: name of the fungus (ID number) and name of the algae (ID number) as reported in Table 1, medium on which the alginate globules were placed. A) *Lichenothelia calcarea* (L1852) and *Trebouxia* sp. 'clade IV' (L1661) on MY; B) *L. convexa* (L1835) and *Trebouxia* sp.1 (L1379) on MY; C) *Saxomyces alpinus* (CCFEE 5470) and *Trebouxia* sp. 'clade IV' (L1661) on BBM; D-I) *Muellerella atricola* (L1993) and *Trebouxia* sp.1 (L1379) on MY. D, F, H) Only in the alginate spheres containing *Muellerella atricola* and *Trebouxia* sp.1 algal cells and fungal hyphae have arranged in a layer-like structure; algal clumps are tightly compacted by the hyphae and direct contact

between hyphae and algal cells are observed (G, arrows). E, I) *Muellerella atricola* abundantly produces conidiogenous cell masses and conidia (arrow). Scale bars: B, C, E, G-I) 20 μm; A, D) 50 μm; F) 100 μm.

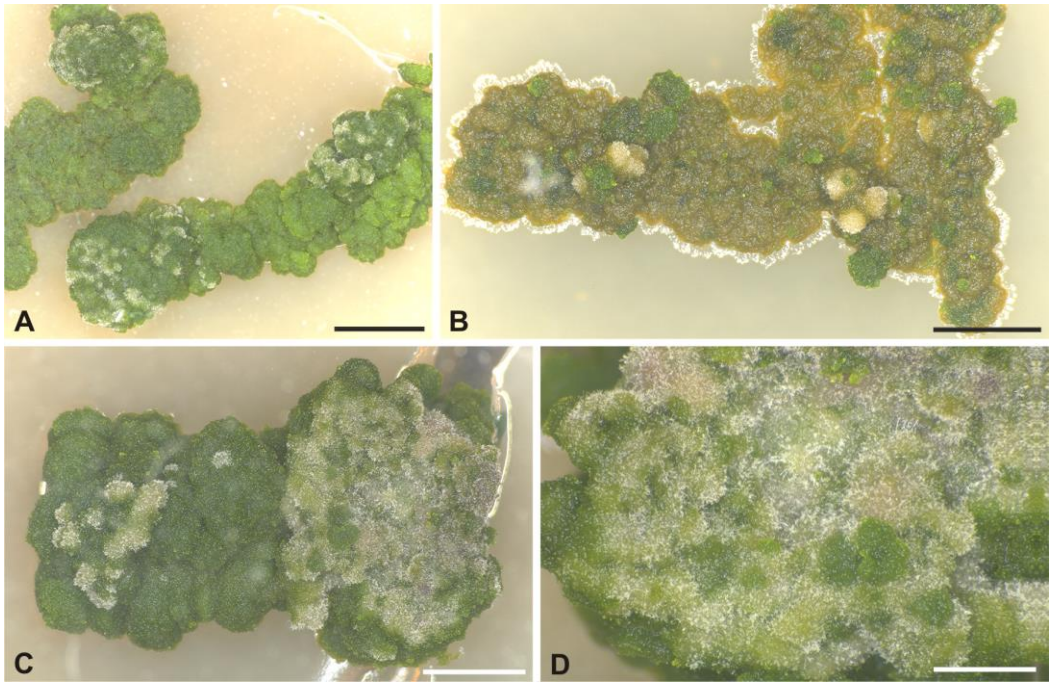

Figure 4. Two months old subcultures set from the initial alginate inoculum of *Muellerella atricola* (L1993) and *Trebouxia* sp.1 (L1379). A, B) Algae spread on the medium developed abundant, green colonies in which the fungus developed its mycelium. C, D) The fungus grows out of and in between the algal clumps and spreads the hyphae over the algal colonies. Scale bars: A) 4 mm; B, C) 2 mm; D) 1mm.

Table 1. Combinations of fungal and algal strains used to prepare the alginate spheres.

	Fungus	**Trebouxia**
1	*Lichenothelia calcarea* L1852	L1101 = *Trebouxia* sp. 1 (=L1379)
2	*Lichenothelia calcarea* L1852	L1414 = *Trebouxia* 'clade IV' (=L1661)
3	*Lichenothelia convexa* L1835	L1101 = T*rebouxia* sp. 1 (=L1379)
4	*Lichenothelia convexa* L1835	L1414 = *Trebouxia* 'clade IV' (=L1661)
7	*Muellerella atricola* L1993	L1101 = *Trebouxia* sp. 1(=L1379)

8	*Muellerella atricola* L1993	L1414 = *Trebouxia* 'clade IV' (=L1661)
9	*Saxomyces alpinus* CFEE 5470	L1414 = *Trebouxia* 'clade IV' (=L1661)
10	*Saxomyces alpinus* CFEE 5470	L1101 = *Trebouxia* sp. 1(=L1379)
11	*Tephromela atra* L1425	L1101 = *Trebouxia* sp. 1 (=L1379)
12	*Tephromela atra* L1425	L1414 = *Trebouxia* 'clade IV' (=L1661)