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Research Article

RESEARCH ARTICLE

Schizoxylon as an experimental model for studying interkingdom symbiosis

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One sentence summary: A tripartite fungal–algal–bacterial model system based on mixed cultures is established and presented; it offers multiple opportunities for functional studies on interkingdom symbioses.

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ABSTRACT

Experiments to re-synthesise lichens so far focused on co-cultures of fungal and algal partners. However, recent studies have revealed that bacterial communities colonise lichens in a stable and host-specific manner. We were therefore interested in testing how lichenised fungi and algae interact with selected bacteria in an experimental setup. We selected the symbiotic system of *Schizoxylon albescens* and the algal genera *Coccomyxa* and *Trebouxia* as a suitable model. We isolated bacterial strains from the naturally occurring bacterial fraction of freshly collected specimens and established tripartite associations under mixed culture experiments. The bacteria belong to Actinobacteria, Firmicutes and Proteobacteria and corresponded to groups already found associated with fungi including lichens. In mixed cultures with *Coccomyxa*, the fungus formed a characteristic filamentous matrix and tightly contacted the algae; the bacteria distributed in small patches between the algal cells and attached to the cell walls. In mixed cultures with *Trebouxia*, the fungus did not develop the filamentous matrix, but bacterial cells were observed to be tightly adhering to the fungal hyphae. Our experiments show that this tripartite fungal–algal–bacterial model system can be maintained in culture and can offer multiple opportunities for functional studies based on experiments under controlled conditions in the laboratory.

Keywords: algae; bacteria; culture; fungi; scanning electron microscopy; system

INTRODUCTION

Most textbooks describe lichens as a partnership of two organisms, a fungus and an alga. This two-partner concept of the lichen symbiosis was introduced by Schwendener (1869) but was challenged very early, not least by contemporary researchers who could not at first accept the idea that lichens were not a separate group of organisms. Cengia-Sambo (1924) suggested the term 'polysymbiosis' for cases where cyanobacteria are present in addition to the green algal symbionts, now known as tripartite lichen symbioses (Kaasalainen *et al.* 2009; Magain and Sérusiaux 2014). Recently, culture-independent analyses and microscopic approaches revealed that in lichens bacterial communities occur in a stable and host-specific manner (e.g. Cardinale *et al.* 2008; Grube *et al.* 2009; Hodkinson and Lutzoni 2009; Bates *et al.* 2011; Mushegian *et al.* 2011; Hodkinson *et al.* 2012; Wedin *et al.* 2016). While algal and cyanobacterial partners have clear functional assignments concerning their provision of fixed carbon or

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nitrogen to the symbiosis, the role of other bacteria is still unknown. Grube *et al.* (2015) used metagenomic and metaproteomic approaches to highlight potential functions of the bacterial fraction in lichen symbioses. To what extent bacterial functions actually influence the symbiotic community of lichens remains a challenge. Such functional studies require controlled experimental conditions, which can potentially be achieved if the interacting partners can be co-cultured.

Lichen symbionts have been cultured since the 19th century, but due to their slow growth they never became popular as experimental systems. The early attempts to re-synthesise lichens by co-culturing their partners had primarily the intent to test the dual hypothesis of the lichen symbiosis (e.g. Bonnier 1886), which was still very controversial at that time. Re-synthesis experiments of lichens were accomplished repeatedly with success, especially in the second half of the 20th century (e.g. Ahmadjian 1973; Honegger 1990; Yoshimura *et al.* 1993; Stocker-Wörgötter 2001 and references therein), and these experiments also demonstrated the specificity of the algal–fungal relationship to form the characteristic thallus morphology (e.g. Ahmadjian, Russell and Hildreth 1980; Etges *et al.* 2000). Bacterial species were never included in any of these experiments, and if they emerged, they were generally regarded as contaminations. In nature, however, lichen fungi are in contact with bacteria almost continuously. So far, the only reported co-culture of bacteria with lichen mycobionts was provided by Seneviratne and Indrasena (2006). Their experiments showed increased solubilisation of mineral substrate in co-cultures of an isolated lichen fungus with N-fixing *Bradyrhizobium elkanii*. Their images also showed tight attachment of the bacteria onto the fungal hyphae in culture. Alternatively, Lõrincz *et al.* (2010) studied an artificial symbiosis by co-culturing the green algae *Chlamydomonas*, the bacterium *Azotobacter* and the fungus *Alternaria* and demonstrating that the basis of the interdependence is the complementation of photosynthetic CO_2 assimilation and atmospheric nitrogen fixation. We are not aware, though, of any other study which attempted cultivation of lichen fungi, algae and bacteria together. We were therefore interested in testing whether lichenised fungi and algae interact with selected bacteria under culture conditions, to assess the influence of the latter on the development of the re-synthesised thallus.

Here, we present our results from experiments done to investigate if a tripartite system (fungus–algae–bacteria) indeed was possible to establish for experimental symbiosis purposes. We used the lichenised fungus *Schizoxylon albescens*, the associated algae *Coccomyxa*, other lichenised algae of the genus *Trebouxia* and bacterial strains isolated in cultures from the naturally occurring bacterial fraction of freshly collected *Schizoxylon* specimens. *Schizoxylon* belongs to the Stictidaceae, a group of ascomycetes where lichenised species have evolved within clades of closely related, saprotrophic and parasitic species (Baloch, Gilenstam and Wedin 2009, 2010, 2013) and where several species either can live as lichens or as saprotrophs ('optional lichenisation'; Wedin, Döring and Gilenstam 2004). In contrast to other lichens, which require algal partners to develop symbiotic thalli as a prerequisite for fungal sexual reproduction, *Schizoxylon* also grows as saprotroph on dead wood (Wedin, Döring and Gilenstam 2006). Phylogenetic and haplotype analyses have shown that these two morphs of *Schizoxylon* neither are genetically differentiated nor correlate with geographical origin in any of the markers analysed (Muggia *et al.* 2011). In nature, *S. albescens* forms simple lichenised stages with a consortium of algae of the genus *Coccomyxa*. Previous co-culture experiments also indicated that the fungus shows certain selectivity for some of the isolated algal strains and forms lichenisation structures in mixed cultures (Muggia *et al.* 2011). Ongoing analyses on environmental samples revealed a diversity of bacteria in both the lichenised and saprotrophic morphs of *Schizoxylon*.

The aims of our study were: (i) to establish and maintain 'three-partner associations' in culture by growing the *Schizoxylon* fungus together with different algal and bacterial strains, and if successful, using such three-partner mixed cultures for small pilot studies; (ii) to study the morphological organisation of these associations; (iii) to study whether the presence of bacteria induce *Schizoxylon* to develop further symbiotic stages with *Coccomyxa*; (iv) to study whether the presence of bacteria triggers *Schizoxylon* to interact also with *Trebouxia*, a common lichen photobiont.

MATERIALS AND METHODS

Sampling

A total of 20 fresh specimens of the lichenised (15, L1–L10, MW9634–MW9638) and saprotrophic (5, S1–S5) forms of *Schizoxylon albescens* were collected in Sweden (Hälsingland province, Färila parish, south of lake Skålvallssjön, 61°56'24.6" N/15°36'32.6" E, 225 m a.s.l., in deciduous-tree-dominated succession forest on former agricultural land, 15/09/2013, e.g. S. Fernández-Brime and M. Wedin) and were used for bacterial isolation.

The fungus and algae used in the experiments are all stored as fresh cultures or cryostocks in the culture collection of the first author at the University of Graz (numbered as 'LMCCxxxx'). *Schizoxylon* was isolated from the lichenised specimen MW7645 and has been maintained in living cultures since 2008 (Muggia *et al.* 2011; cryostock n. LMCC0016). The two *Coccomyxa* algal strains PL2-1 and MW8233 were also isolated in 2008; they were available from the cryostocks (LMCC0023 and LMCC0038) and were freshly plated 2 months before starting the experiments. Two additional strains of *Trebouxia* photobionts, isolated from the lichen *Tephromela atra* and kept in living cultures, *Trebouxia* 'sp. 1' (LMCC0107) and *Trebouxia* 'TR1' (LMCC0121)—according to the nomenclature followed by Muggia *et al.* (2014)—were used for the mixed culture experiments.

Culture isolations and mixed culture experiments

The fungal and the algal strains were isolated in 2008 as described in Muggia *et al.* (2011). Of the 20 freshly collected samples of *S. albescens*, 10 lichenised (L1–L10) and the five saprotrophic (S1–S5) were selected for the isolation of bacteria. The samples were analysed under the stereo microscope and for five lichenised and the five saprotroph two types of pieces were distinguished and dissected for culture isolations: (A) the pieces containing *Schizoxylon* ascomata together with the algae clumps and a portion of the surrounding substrate in the lichenised one, or containing only the ascomata in the saprotrophic ones, (B) the pieces of the bark (for the lichenised samples) and of the wood (for the saprotrophic samples), without ascomata and algae. In doing this, we aimed at maximizing the isolation of the whole spectrum of culturable bacteria that are associated with the fungus, the fungus and the algae, and the growth substrates (Fig. 1a). The selected pieces were put in 1.5 ml tubes and washed with a 0.9% of NaCl solution by vortexing the tube for 2 min. The solution was plated both undiluted and 1/2 diluted on R2a agar (Reasoner, Blannon and Geldreich 1979) plates, as this medium is the minimal one to isolate universal bacteria (Zachow *et al.*

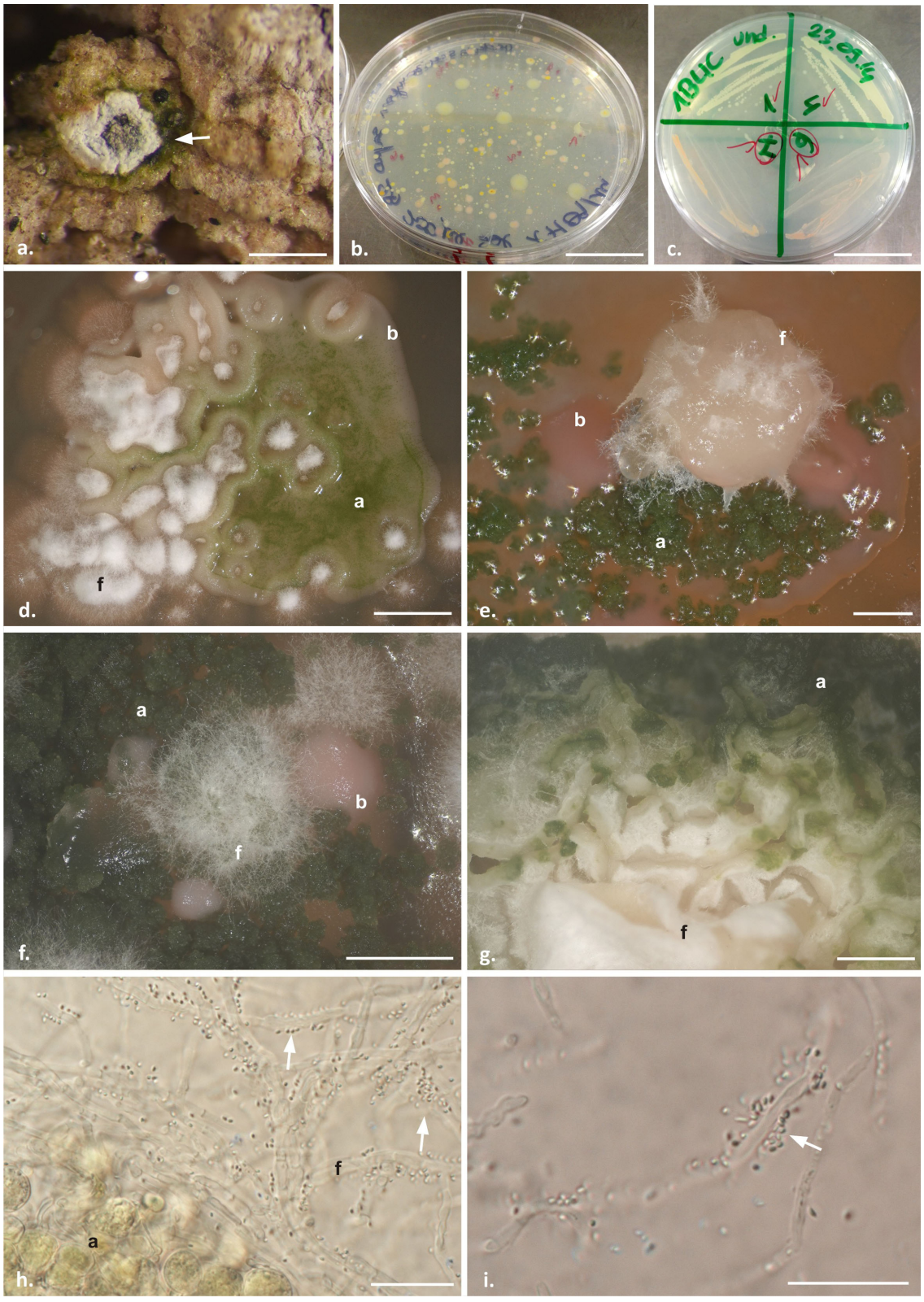

Figure 1. Habit of *S. albescens* in nature and co-cultured with bacteria and algae (a = algae, b = bacteria, f = fungus): **(a)** *Schizoxylon* on *Populus* bark, green algal colonies are visible around the fungal fruiting body (arrow); **(b, c)** bacterial colonies on agar plates; **(d–i)** mixed cultures of *Schizoxylon* with different algae and bacteria strains: **(d)** *Schizoxylon*, *Coccomyxa* PL2-1 and SAP-5B/1w on MY medium; **(e)** *Schizoxylon*, *Trebouxia* sp.1 and LIC-4A/10 on TM medium; **(f)** *Schizoxylon*, *Trebouxia* sp.1 and LIC-4A/10 on MY medium; **(g)** *Schizoxylon*, *Coccomyxa* MW8233 and MW9636.7 on MY medium; **(h, i)** squash preparations mounted in water of *Schizoxylon*, *Trebouxia* sp.1 and LIC-1B/1 on MY medium, bacteria are visibly attached to the fungal hyphae (arrows). Scale bars: a, e = 0.5 mm; b, c = 2.5 cm; d = 4 mm; f, g = 1 mm; h, i = 20 μm.

2013). The plates were incubated at room temperature for 2–4 days for allowing bacteria to grow. Once bacterial colonies grew, they were picked and individually sub-cultured to obtain single-colony isolates. We picked for each plate, a maximum of 12 different bacterial colonies, distinguishing them according to their different colour (Fig. 1b and c), in order to include a broad diversity of the strains.

The remaining five specimens of lichenised *Schizoxylon* (MW9634–MW9638) were used to isolate bacteria directly from the algal clumps surrounding the ascomata of the fungus (Fig. 1a). Algal clumps were picked with a sterile needle and inoculated on Bold basal medium (BBM; Bold 1949). All the different bacteria that grew out of the inocula were further individually picked and sub-cultured to obtain single-colony isolates.

All the successfully grown single-colony isolates were prepared for cryostock storage and molecular analyses for their identification. Cryostock preparation consisted in inoculating a single-cell colony in 500 μl liquid R2a medium, adding the same volume of 40% glycerol and storing them in tubes at –80°C.

Mixed cultures of multiple combinations of *Schizoxylon*, algae and bacteria were set on five different growth media: malt yeast (MY; Ahmadjian 1967), *Trebouxia* medium (TM; Ahmadjian 1967), Sabouraud agar (SAB; Sigma), Lilly and Barnett's medium (Lilly and Barnett 1951) and water-agar medium (a medium without nutrients solidified only with agar). The fungus was ground in water in a sterile mortar with a pestle and fragments were pipetted on the medium. Algae and bacteria were suspended in 1 ml water and for each mixed culture 200 μl were pipetted on the medium on the same spot of the fungus. The mixed cultures were set as follows: the same fungus was combined with each of the four algae strains (two *Coccomyxa*, PL2-1 and MW8233, and two *Trebouxia*, 'sp.1' and 'TR1'). Each fungal–algal combination (four in total) was subsequently combined with a total of 14 different bacterial strains. These were randomly taken from those both isolated from the fragments of the lichenised (four strains: LIC-1B/1, LIC-4A/10, LIC-4A/11, LIC-4B/12) and the saprotrophic (four strains: SAP-4A/2, SAP-5B/2, SAP-5B/1r, SAP-5B/1w) specimens, either from the type A or type B pieces, and those isolated directly from the algal clumps (six strains: MW9636.1, MW9636.2, MW9636.5–.8). A total of 280 plates were therefore prepared. No replicate on the same medium was performed for any of the fungus–algae–bacteria combination.

Scanning electron microscopy analyses

The mixed cultures that successfully developed the intertwined growth of the three organisms were selected for electron microscopy analyses. A piece of about 1 cm^2 was taken from each culture and fixed with a glutaraldehyde (2%) cacodylate 0.2 M pH 7.4 buffer for 2 h at room temperature. The fragments were washed three times with the cacodylate 0.1 M pH 7.4 buffer and dehydrated with an ethanol series consisting of three fast washes with EtOH 30%, three fast washes with EtOH 50%, two washes of 5 min with EtOH 70%, two washes of 12 min with EtOH 90% and two final washes of 15 min with EtOH 100%. The samples were then dried at the CO_2 critical point and gold sputtered. For each sample, both vertical sections and upper surfaces were arranged on the stubs.

Molecular analyses of the bacterial strains

Bacterial colonies were first transferred to microcentrifuge tubes containing 200 μl of ddH_2O and then samples were spun for 10 min at 7500 rpm in order to precipitate the bacterial cells. Once the supernatant was discarded, the DNA was extracted using the KingFisher Cell and Tissue DNA Kit (Thermo Fisher Scientific, Vantaa, Finland) or the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturers' instructions. The primers F515 and R806 were used to amplify part of the III and the IV 16S variable regions (Chakravorty *et al.* 2007; Caporaso *et al.* 2011). We sequenced this fragment in order to incorporate the data obtained from these strains into a broader study using MiSeq Illumina data targeting the same fragment (Fernández-Brime *et al.* in preparation). The PCR was performed using Illustra Hot Start Mix RTG (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's instructions and the following PCR cycle was applied: 5 min at 95 °C followed by 37 cycles, each of 45 s at 95°C, 45 s at 50°C, 90 s at 72°C and a final extension of 10 min at 72°C. PCR products were then purified with the enzymatic method Exo-sap-IT© (USB Corporation) and the sequencing of both strands was performed with the Big Dye Terminator technology kit v3.1 (ABI PRISM, USA) using the same PCR primers. Sequences fragments were edited and assembled using Sequencer v.4.9 (Gene Codes Corp., Ann Arbor, MI). Primers were truncated at this point. The dataset was processed in QIIME v.1.9.1 (Caporaso *et al.* 2010) with the script for closed-reference operational taxonomic unit (OTU) picking in order to pick OTUs with a 0.97 similarity threshold, using as reference the Greengenes core set. Taxonomy was assigned with the uclust classifier accepting a maximum of three hits and a minimum similarity of 0.90.

RESULTS

Culture isolations and mixed culture experiments

A total of 121 bacterial colonies isolated from the five lichenised and the five saprotrophic samples and a total of 18 bacterial colonies isolated from the algal clumps were successfully sub-cultured and prepared for cryostock storage and molecular sequence identification. Of the 139 colonies, 14 (8 out of the 121 and 6 out of the 18) were arbitrarily taken for the mixed culture experiments and were identified by sequencing (see below). After 2 months, two-thirds of the mixed cultures were discarded due to overgrowth of the bacteria or failed growth. Mixed cultures failed to grow on 95% of the water-agar plates; on the remaining water-agar plates, algae and bacteria grew inconspicuously and very slow, whereas the fungus did not grow at all. These cultures were excluded from further analyses. About 20% of the mixed culture experiments developed successfully and eight of them were selected for scanning electron microscopy (SEM) analyses after 6 months of co-growth (Table 1).

In mixed culture with *Coccomyxa*, the fungus developed within a homogeneous mass formed by the intermixed growth of algal and bacterial cells (Fig. 1d and g). In mixed culture with *Trebouxia*, algae and bacteria formed well-delimited clumps of tightly connected cells. The fungal hyphae either grew among the algal and the bacterial colonies (Fig. 1f) or they built a more compact mycelial mass with filamentous hyphae in contact with bacterial and algal cells (Fig. 1e). In squash preparations mounted in water of *Schizoxylon* grown with *Trebouxia* sp.1 and the bacterial strain LIC-1B/1 on MY medium, it was possible to observe bacteria attached to the fungal hyphae (Fig. 1h and i).

In axenic culture, a developing mycelium can first be observed c. 2 months after plating the *Schizoxylon* inoculum, growing at a rate of 0.3 mm/month on MY medium. When the fungus was co-cultured with algae and bacteria, mycelium growth was initiated considerably earlier, c. 1 month after

Table 1. Combination of fungus-algae-bacteria mixed cultures used for SEM analyses. LIC: coming from lichenised sample, SAP: coming from saprotroph sample.

SEM sample no.	Fungus	Algae strain	Bacteria strain	Growth medium	Age of mixed culture (months)
1	*S. albescens* MW7645	*Trebouxia* 'sp. 1'	LIC-4A/10	MY	6
2	"	*Trebouxia* 'sp. 1'	LIC-4B/12	MY	6
3	"	*Trebouxia* 'sp. 1'	LIC-1B/1	SAB	6
4	"	*Coccomyxa* PL2-1	MW9636.7	MY	6
5	"	*Trebouxia* 'TR1'	MW9636.5	TM	6
6	"	*Coccomyxa* MW8233	MW9636.7	MY	6
7	"	*Coccomyxa* PL2-1	MW9636.1	TM	6
8	"	*Coccomyxa* MW8233	SAP-5B/2	MY	8

inoculation, but grew at a similar rate. No difference in growth morphology, growth rate or fitness of the mixed cultures was observed amongst the bacterial strains.

SEM analyses

The mixed cultures which succeeded in growth and were analysed by SEM, were set with bacteria (Actinobacteria, Firmicutes and Gammaproteobacteria, as it follows) isolated from the lichenised samples, both from the pieces of type (A) and directly from the algal clumps. In mixed cultures with the two *Coccomyxa* strains, the hyphae were in tight contact with the algae (Fig. 2a and b) and the fungus formed a characteristic filamentous matrix (Fig. 2c) concordant with the results in Muggia *et al.* (2011). This filamentous matrix enclosed the *Coccomyxa* cells and appeared locally well developed covering the algal layer entirely (Fig. 2e). Bacteria were detected in small, irregular patches between the algal cells and attached to the cell walls (Fig. 2d). In some parts of the mixed cultures the fungus overgrew and algal cells were scattered in low amount between the hyphae (Fig. 2f). In mixed cultures with *Trebouxia*, the fungus did not develop any filamentous matrix. The hyphae spread among the algal colonies, entangling single cells or algal clumps. The observed clumped growth of *Trebouxia* is well known and it is due to the clonal growth by autospores inside the parental gelatinous sheet (Fig. 3b and d). In these mixed cultures, bacterial cells were observed to be tightly adhering to the fungal hyphae (Fig. 3a–e), and distributed according to the branching of the hyphae, also where the three organisms grew in close contact. We seldom observed bacteria adhering to *Trebouxia* cells.

In the culture set with the saprotrophic strain SAP-5B/2 (which was not identified due to the bad quality of the sequence), we did not observed mixed growth of the three organisms (not shown).

DNA analyses of bacterial identity

From the 14 bacterial strains used for the mixed culture experiments, PCR products were not obtained for the strain MW9636.6 and sequences of two strains (MW9636.5 and SAP-5B/2) were excluded from further analyses due to low quality. The remaining 11 sequenced strains were processed in QIIME and their putative identities are summarised in Table 2. A total of eight OTUs were recognised within the phyla Actinobacteria (Geodermatophilaceae and Microbacteriaceae), Firmicutes, Alphaproteobacteria (Sphingomonadaceae) and Gammaproteobacteria (Xanthomonadaceae). The identity of the strains was assessed according to the hits agreeing with the lowest taxonomic level: one strain, SAP-4A/2, was identified at the species level as *Luteibacter rhizovicinus*. Six strains were classified to the genus level, MW9636.1 as *Acinetobacter*; LIC-1B/1 as *Bacillus*; LIC-4A/11 and MW9636.7 as *Frigoribacterium*; MW9636.2 and MW9636.8 as *Sphingomonas*, and four to the family level, LIC-4B/12, SAP-5B/1r and SAP-5B/1w in Microbacteriaceae and LIC-4A/10 in Geodermatophilaceae.

DISCUSSION

A model for interkingdom microbial symbioses

Lichen symbioses represent suitable subjects for studying interaction among distantly related organisms (Berg 2015). Axenic cultures of lichen mycobionts have earlier been established to study patterns of secondary metabolite production under different controlled conditions (e.g. Brunauer *et al.* 2007; Fazio *et al.* 2009; Stocker-Wörgötter and Elix 2009; Fazio, Adler and Maier 2014). Yet, only few co-cultivation experiments with photobionts (green algae) and attempts of re-synthesis of lichen thalli have been reported in recent times (e.g. Stocker-Wörgötter 1995; Joneson and Lutzoni 2009; Guzow-Krzeminska and Stocker-Wörgötter 2013), probably due to the slow growth of lichen symbionts. Thus, *in vitro* re-synthesis experiments of lichen thalli have focused on the first stages of thallus formation, which are highly dependent on the compatibility of the fungal and the algal symbionts (Ahmadjian, Jacob and Russell 1978; Schaper and Ott 2003, Joneson and Lutzoni 2009; Guzow-Krzeminska and Stocker-Wörgötter 2013). Compatible partners are commonly first characterised by molecular analyses and later co-growth experiments are set up (Muggia *et al.* 2011; Guzow-Krzeminska and Stocker-Wörgötter 2013). In most cases, however, the development of more differentiated thallus structures, such as lobes or fungal fruiting bodies, has hardly ever been reported on solid agar media. For this purpose, the use of selected grow substrates, such as sterilised soil, and controlled growth conditions proved to be triggering (Bubrick and Galun 1986; Stocker-Wörgötter and Türk, 1991, 1993; Stocker-Wörgötter 2001). Re-synthesis experiments of lichens starting from their aposymbiontic partners is in general challenging, as the experimental system has to be optimised for each case, usually by simulating some ecological factors as close as possible by culture conditions. Modified media (Guzow-Krzeminska and Stocker-Wörgötter 2013), adjusted light-dark regimes and temperature can be applied to balance the joint growth of the different organisms, which indeed favour different conditions (Muggia, pers. comm.).

Co-culture experiments of lichens have hitherto only considered two partners. Because non-photosynthetic bacteria have been increasingly recognised to be regularly present in lichen

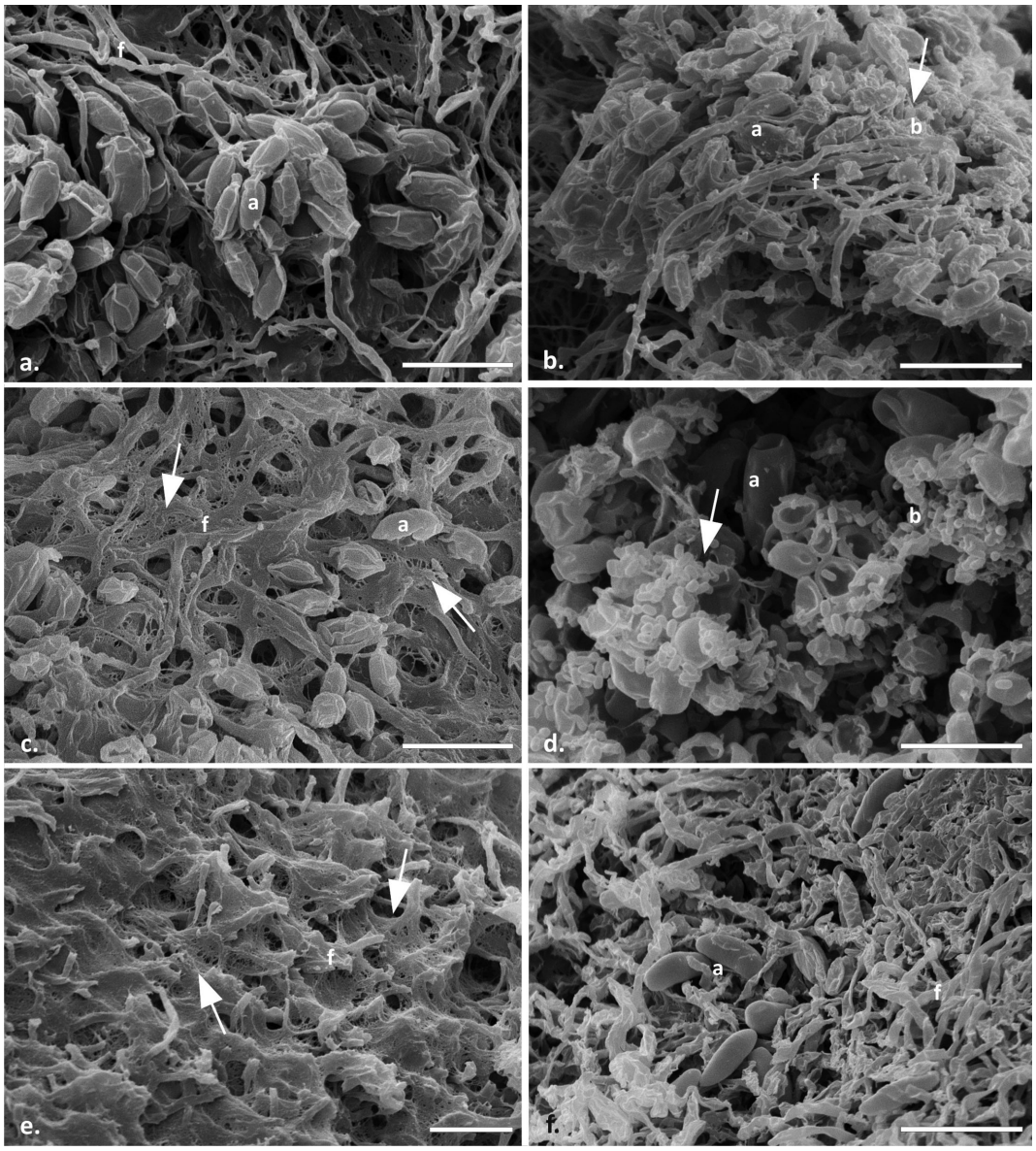

Figure 2. SEM microphotograph of *S. albescens* co-cultured with *Coccomyxa* algae and bacterial strains (a = algae, b = bacteria, f = fungus). (**a**, **b**) Algal cells intertwined by fungal hyphae. (b, **d**) Bacterial cells are spread among fungal hyphae and algal cells, but are locally distributed only in some parts of the mixed cultures (arrow). (**c**, **e**) The filamentous matrix formed by the fungus when co-grown with *Coccomyxa* algae is visible (arrow). (a, b) *Schizoxylon*, *Coccomyxa* PL2-1 and MW9636.7 on MY medium; (c, d) *Schizoxylon*, *Coccomyxa* MW8233 and MW9636.7 on MY medium; (e, **f**) *Schizoxylon*, *Coccomyxa* PL2-1 and MW9636.1 on TM medium. Scale bars: a–c, f = 10 μm; d = 6 μm; e = 15 μm.

symbioses, it is tempting to include these in symbiotic re-synthesis experiments as well (Grube *et al.* 2009, 2015; Aschenbrenner *et al.* 2014). Bacteria are localised in lichen thalli by *in situ* hybridisation, which also show different spatial arrangements at different ages of the thalli (Cardinale *et al.* 2008, 2012). Generally, only a minor fraction of the lichen-associated bacteria can successfully be isolated by axenic cultures and further characterised for their biological potential without the hosting lichen (Cernava *et al.* 2015; Erlacher *et al.* 2015). Previous studies of lichen-associated bacteria have focused on well-structured lichen thalli of model species (e.g. *Lobaria pulmonaria*), which develop 3D structures. However, the mycobionts of these model lichens grow slowly in axenic culture and do not offer suitable systems for long-term and repeatable culture experiments. Further, the use of these species for re-synthesis experiments would first require axenic cultures of all individual symbionts, which is not always straightforward.

Schizoxylon albescens, which is facultatively lichenised and able to either associate with *Coccomyxa* or to live as a saprotroph, was thoroughly investigated some years ago. We earlier proposed this fungus as a suitable model species for studies of the lichen symbiosis (Muggia *et al.* 2011). The availability of axenic cultures of both the fungus and the associated algae and the successful isolation of a bacterial fraction associated with the apothecia and the algal clumps enable us to set up pilot experiments with mixed cultures including three partners. *Schizoxylon* grows faster than other lichen mycobionts in culture, and this reduces the risk of being overgrown by faster growing algae. As the system *Schizoxylon–Coccomyxa* was available, we used it as a reference to test whether the addition of bacteria as third

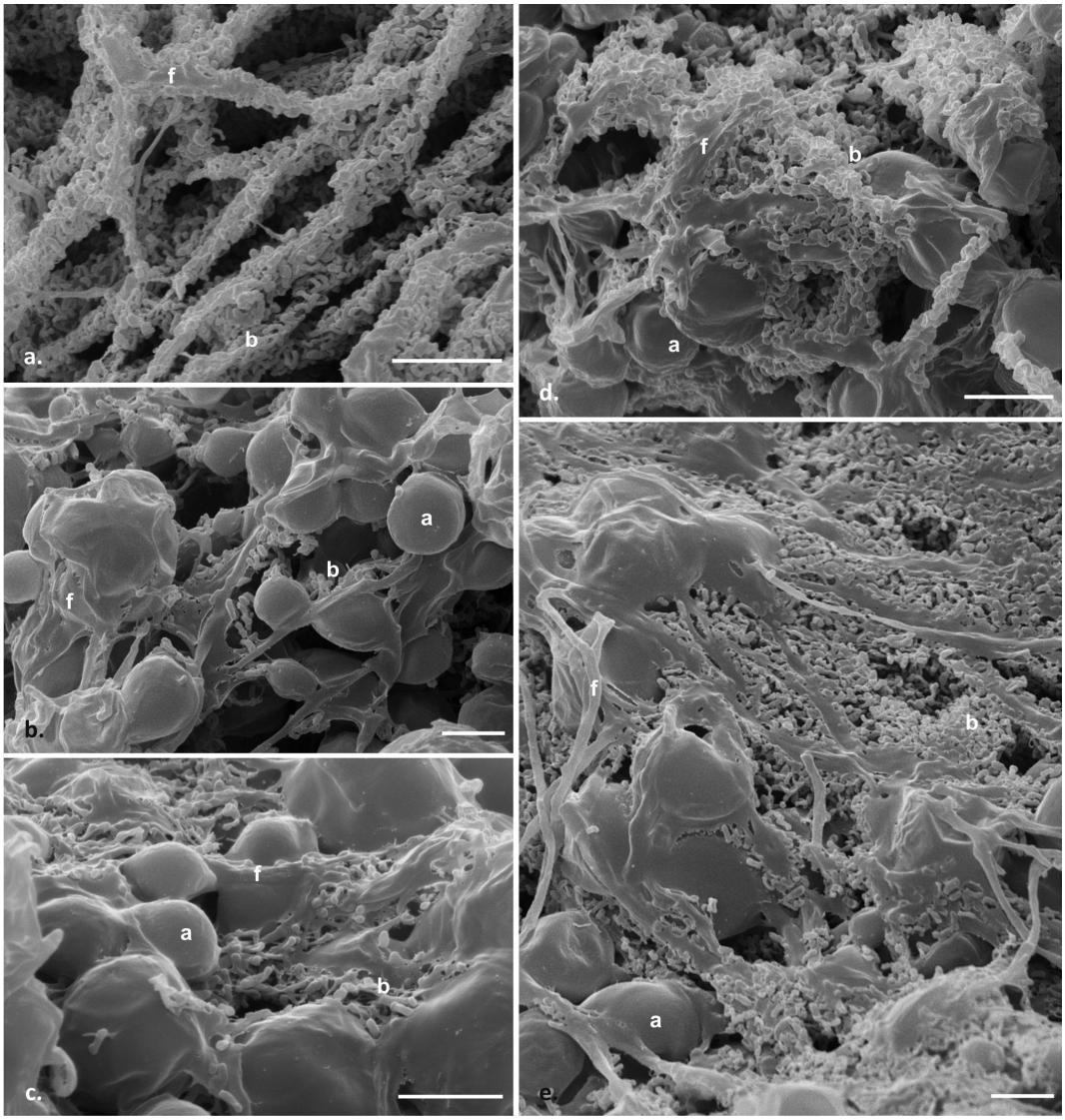

Figure 3. SEM microphotograph of *S. albescens* co-cultured with *Trebouxia* algae and bacterial strains (a = algae, b = bacteria, f = fungus): **(a–c)** *Schizoxylon*, *Trebouxia* sp. 1 and LIC-4A/10 on MY medium; **(d, e)** *Schizoxylon*, *Trebouxia* sp. 1 and LIC-4A/12 on MY medium. Bacteria are tightly adhering to the fungal hyphae (A, D) or spread among algal cells and fungal hyphae (**b**, c, e). Scale bars: a–e = 6 μm.

partner group would induce changes during the joint growth. We clearly find faster initiation of growth of fungal inocula during co-cultivation with symbionts (algae and bacteria in this case). This phenomenon indicates that a symbiotic effect in the co-cultures could be expressed by more rapid initiation of growth, rather than by elevated growth rates. However, we still need to find out whether the effect is due to co-cultivation with algae together with bacteria, or due to one of these alone. Here we focused on the morphological development of the mixed cultures in the presence of different bacterial strains and whether the tested bacteria preferentially grow in certain parts of the cultures to give rise to more complex symbiotic architectures. We were also interested to test whether the presence of bacteria would induce or facilitate fungal association with another photobiont than *Coccomyxa*. Nevertheless, we are aware that the isolated bacteria were arbitrarily selected for the mixed culture experiments and represent only a subset of the whole bacterial community associated with *Schizoxylon*. Strains that by chance were not selected so far or that are not possible to isolate and grow in axenic culture may of course also play a significant role during the lichenisation phases.

Plasticity of the fungal–algal–bacterial association in mixed cultures

In accordance to previous observations (Muggia *et al.* 2011), our analyses showed that *S. albescens*, when cultured with *Coccomyxa*, forms a characteristic filamentous matrix and tightly adheres to algal cells. We reproduced this phenomenon and the formation of initial fungal–algal symbiotic stages (Ahmadjian, Jacob and Russell 1978; Galun 1988; Joneson and Lutzoni 2009). In contrast to the initial experiments by Muggia *et al.* (2011), this time *Schizoxylon* was also able to grow with and contact *Trebouxia* algae. However, no filamentous matrix was observed in the *Schizoxylon–Trebouxia* cultures and further analyses are needed to demonstrate if the fungus forms haustoria with this photobiont, as commonly observed in lichen fungi associated with *Trebouxia* (Honegger 1984). Bacterial cells are

Table 2. Taxonomic assignment of the sequenced strains used in the mixed culture experiments. Letters in the 'Taxonomic assignment' column correspond to the taxonomic rank as follows: k: kingdom, p: phylum, c: class, o: order, f: family, g: genus, s: species. Strain isolation IDs summarise the following information: LIC: coming from lichenised sample, SAP: coming from saprotroph sample, A: from fragments with apothecia, B: from fragments with substrate only. Number in brackets corresponds to the hits that agree at the last taxonomic level assigned (1.0 = 3/3, all three hits agree; 0.67 = 2/3, two out of three hits agree).

OTU	Strain isolation ID	Taxonomic assignment
1	LIC-1B/1	k_Bacteria; p_Firmicutes; c_Bacilli; o_Bacillales; f_Bacillaceae; g_*Bacillus* (1.00)
2	LIC-4A/10	k_Bacteria; p_Actinobacteria; c_Actinobacteria; o_Actinomycetales; f_Geodermatophilaceae (1.00)
3	LIC_4A/11 MW9636.7	k_Bacteria; p_Actinobacteria; c_Actinobacteria; o_Actinomycetales; f_Microbacteriaceae; g_*Frigoribacterium* (0.67)
4	LIC-4B/12	k_Bacteria; p_Actinobacteria; c_Actinobacteria; o_Actinomycetales; f_Microbacteriaceae (1.00)
5	MW9636.1	k_Bacteria; p_Proteobacteria; c_Gammaproteobacteria; o_Pseudomonadales; f_Moraxellaceae; g_*Acinetobacter* (1.00)
6	MW9636.2 MW9636.8	k_Bacteria; p_Proteobacteria; c_Alphaproteobacteria; o_Sphingomonadales; f_Sphingomonadaceae; g_*Sphingomonas* (1.00)
7	SAP-4A/2	k_Bacteria; p_Proteobacteria; c_Gammaproteobacteria; o_Xanthomonadales; f_Xanthomonadaceae; g_*Luteibacter*; s_*rhizovicinus* (0.67)
8	SAP-5B/1r SAP-5B/1w	k_Bacteria (100); p_Actinobacteria (100); c_Actinobacteria (100); o_Actinomycetales (100); f_Microbacteriaceae (100).

distributed irregularly in the *Schizoxylon–Coccomyxa* mixed cultures, but only in the *Schizoxylon–Trebouxia* cultures are they pronouncedly attached to the fungal hyphae. The successfully grown mixed cultures that were analysed by SEM were set with bacteria isolated from the lichenised samples, both from the pieces type (A) and directly from the algal clumps. In the culture set with the saprotrophic strain SAP-5B/2 (so far unidentified due to bad quality of the sequence), we did not observe mixed growth of the three organisms (not shown). Further, mixed culture experiments should therefore be set with additional bacterial strains isolated from the lichenised pieces (A) to confirm these first results. While we have not observed differences in the *Schizoxylon–Coccomyxa* cultures, which could have been induced by the addition of bacteria, future analyses will address whether the tight fungal–bacteria interaction triggers the capacity of *Schizoxylon* to associate with *Trebouxia*. Further, the tripartite associations were grown on different media, of which only MY, SAB and TM worked properly. These should be utilised in future experiments to test different *Schizoxylon–Coccomyxa/Trebouxia*-bacteria associations and to standardise the mixed cultures.

Among the bacteria we isolated from *Schizoxylon*, we have identified genera known to be associated with fungi and which also belong to groups already found in lichen microbiota (Cardinale, Puglia and Grube 2006; Cardinale, Grube and Berg 2011; Parrot *et al.* 2015; Grube *et al.* 2016; Aschenbrenner *et al.* 2016). Several strains were identified as Actinobacteria and assigned to the family Microbacteriaceae, a large group that is widespread in various terrestrial and aquatic environments. Within the Gammaproteobacteria, which include N_2-fixers, one strain was assigned to *Acinetobacter* and another was identified as *Luteibacter rhizovicinus*, which has earlier been isolated from *Cladonia coniocraea* and *Peltigera membranacea* (Cardinale, Puglia and Grube 2006; Sigurbjörnsdóttir, Andresson and Vilhelmsson 2015). In the mixed cultures, bacterial colonies grow only in small patches on the filamentous matrix or are attached to the hyphae, sometimes covering the hyphae completely, and rarely to the algal cell walls. Even though organisms may develop different phenotypes when inoculated on different media, we cannot correlate the various detected growth patterns to a certain medium, or to the age of the culture. Medium variations, which may affect the interaction patterns, need to be assessed by extended culture experiments. This may also include liquid cultures, which could represent a feasible approach either for easier propagation of the symbiotic cultures or for maintenance of long-term cultures for time series analyses.

Outlooks of multipartite experimental model systems

Co-culture and mixed culture approaches are increasingly applied to test working hypotheses for transcriptomics and metabolomics analyses, to explore biosynthetic potentials, and to test the latent capacity to form symbiosis (Goers, Freemont and Polizzi 2014; Hom and Murray 2014; Hays *et al.* 2015). Because co-culture approaches require extensive optimisation to subsequent analyses, the application of an established model system for further exploration of symbiotic interactions, e.g. by omics approaches, is essential. The main result from our study is that we have now successfully established a *Schizoxylon*–algal–bacterial system, which offers further opportunities to study lichenisation *in vitro*. As *Schizoxylon* facultatively forms lichenised associations in nature, it represents a useful subject for transcriptomic studies comparing the symbiotic and saprotrophic life styles. Furthermore, a culture system, which allows the recombination of multiple symbiotic partners, is useful for exploring specificity patterns, symbiotic robustness and, eventually, the establishment of artificial symbioses (Hays *et al.* 2015). In the case of the *Schizoxylon*–algal–bacterial system, further stabilisation of the mixed cultures could indeed be performed by modified media or by introducing periodic disturbance or variation in hydratic conditions, which would promote tighter interactions between the symbionts. While bacteria did not influence the growth rate directly according to the present results, they might play a role for the integrity of the symbiosis under the varying, natural conditions, or for establishing the symbiotic phenotype. With our approach we were able to keep mixed cultures of members from different kingdoms in balance, and we are now able to explore the lichen symbiosis and its relation with associated bacteria using controlled experiments.

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