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Experimental Lichenology

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1. Introduction

The late 19th and, especially, the early 20th century were marked by the introduction of experimental approaches in various biological disciplines. The methods of accumulative and axenic microorganism cultures were already widely used in microbiology of that period; in animal and plant sciences, attempts were made to grow whole organisms, individual organs, tissues and/or individual cells under controlled laboratory conditions (Vochting, 1892; Harrison, 1907). By the early 20th century, some results had already been achieved in cultivating animal tissues (Krontovsky, 1917 cited in Butenko, 1999), and, in the 1920s, plant and animal cells and tissues (Czech, 1927; Prat, 1927; Gautheret, 1932; White, 1932). An important step in plant tissue cultivation was the discovery of phytohormones and development of specialized cultivating media that allowed inducing, on the one hand, dedifferentiation and callus formation, or, on the other hand, cell differentiation. These achievements helped to solve a number of problems, both theoretical and applied (Street, 1977; Butenko, 1999). With time, the spectrum of organisms introduced in cultures was widening, the principal methods of growing plant cells *in vitro* were developed, and the foundations were laid for microclonal propagation.

The said period was also marked by the formation and development of the notion of symbiosis. The revolutionary works of A.S. Famintsyn (1865) and S. Schwendener (1867) (as cited in Famintsyn, 1907) discovered the dual nature of lichens. The notion of symbiosis was formulated in 1879 by A. de Bary. In the early 20th century, K.S. Mereschkowski established the theory of symbiogenetic origin for the eukaryotic cell and formulated the notion of two "plasms" (Mereschkowski, 1907, 1909).

Symbiosis is currently studied by a special scientific discipline, symbiology, and regarded as a stable super-organism system undergoing balanced growth and characterized by specific interrelations of components, and by unique biochemistry and physiology (Ahmadjian & Paracer, 1986; Paracer & Ahmadjian, 2000).

It is noteworthy that the development of each of the above-mentioned fields of study has not been independent. Constantly intervening with each other, works in all these fields were conducive to the formation of a new branch, already within the new science of symbiology. In the 1990s, this new branch was termed experimental symbiology.

2. Specifics of lichens as experimental systems. Peculiarities of the terminology

Lichens are a classic example of symbiotic associations with multicomponent composition as their principal feature. According to the number of partners forming the thallus, two- and three-component lichens are recognized. The former consist of a fungal component (the mycobiont) and a photosynthetic component (the photobiont). In two-component lichens, the photobiont is represented either with a green alga or a cyanobacterium; in three-component lichens, with both: a green alga in the basal part of the thallus and a cyanobacterium in specialized formations, cephalodia (Rai, 1990, Paracer & Ahmadjian, 2000).

According to the type of localization in the lichen, internal (intra-thallus) and external (surface) cephalodia are recognized. In nature, lichens with internal cephalodia are probably prevalent. Some investigators, e.g., P.A. Genkel and L.A. Yuzhakova (1936) (the history of the question is described in: A.N. Oksner, 1974) suggested that nitrogen-fixing bacteria (such as *Azotobacter* spp.) also constitute an obligatory symbiotic component of lichens. Experimental evidence did not support this view (Krasilnikov, 1949). On the other hand, it is currently believed that bacteria are associated, minor symbionts in the lichen system, participating in the morphogenesis of the thallus (Ahmadjian, 1989).

In addition to morphology, lichens as symbiotic systems demonstrate a number of peculiar biochemical and ecological features. Only occasional findings of the so-called lichen compounds in monocultures of lichen symbionts (in most cases, mycobionts) have been reported (Ahmadjian, 1961, 1967). At the same time, large amounts of phenolic compounds (mainly depsides and depsidones), found almost nowhere else, are present in lichens (Culberson, 1969; Vainshtein, 1982a, 1982b, 1982c). The functions of these compounds are not yet fully known. Various compounds probably play different roles in the vital functions of lichens: some participate in the initiation of symbiotic interactions (Ahmadjian, 1989), some provide for the exchange of nutrients between the symbionts (Vainshtein, 1988), and some are used for adaptation to environmental conditions (e.g., in substrate destruction or in competition: Tolpysheva, 1984a, 1984b, 1985; Vainshtein & Tolpysheva 1992; Manojlovic et al., 2002). Symbiosis helped lichens to become extremely widespread, but they are prevalent in extreme or simply oligotrophic habitats. This probably reflects the fact that lichens are capable of surviving considerable changes of temperature, drying, poor substrates, but at the same time, due to slow growth, it is hard for them to survive competition with higher plants (Paracer & Ahmadjian, 2000).

The multicomponent composition of lichens makes it difficult to use them in biotechnology. Lichens are super-organism multicomponent systems, and we believe that it is necessary to discuss here the terminology used for growing lichens in culture. In English-language literature, the word "culture" is used for laboratory manipulations with lichen thalli and their fragments, but different authors understand this term differently. Taking into account the fact that experimental lichenology developed largely on the basis of approaches borrowed from plant physiology, we believe that it is advisable to define the notion of "culture" more accurately, in the light of the sense this term has in plant physiology, where it means the growing of dedifferentiated parts of an organism on growth media under controlled laboratory conditions (Street, 1977; Butenko, 1999). Lichens have no true tissues,

only plectenchymas, and thus the term "tissues" can be used only in quotation marks. We believe that it is possible, by analogy, to use also a number of other terms, such as explants, "callus cultures", etc.

3. History and classification of the experimental approaches in lichenology

Two principal groups of experimental approaches can be recognized in lichenology (Fig. 1, 2): first, resynthesis of lichens and producing of model systems (developed by V. Ahmadjian, M. Galun), and second, obtaining lichen "tissue cultures" (developed by Y. Yamamoto). The former groups of approaches is based on the dissociation of the initial thallus into components (Fig. 1) and subsequent attempt to resynthesize the initial sample or model it based on similar systems (Ahmadjian, 1973a, 1973b). In the latter case, dedifferentiated biomasses of the lichen are obtained (Fig. 2), using thallus fragments, soredia, or isidia (Yoshimura & Yamamoto, 1991; Yoshimura et al., 1993; Yamamoto et al., 1993).

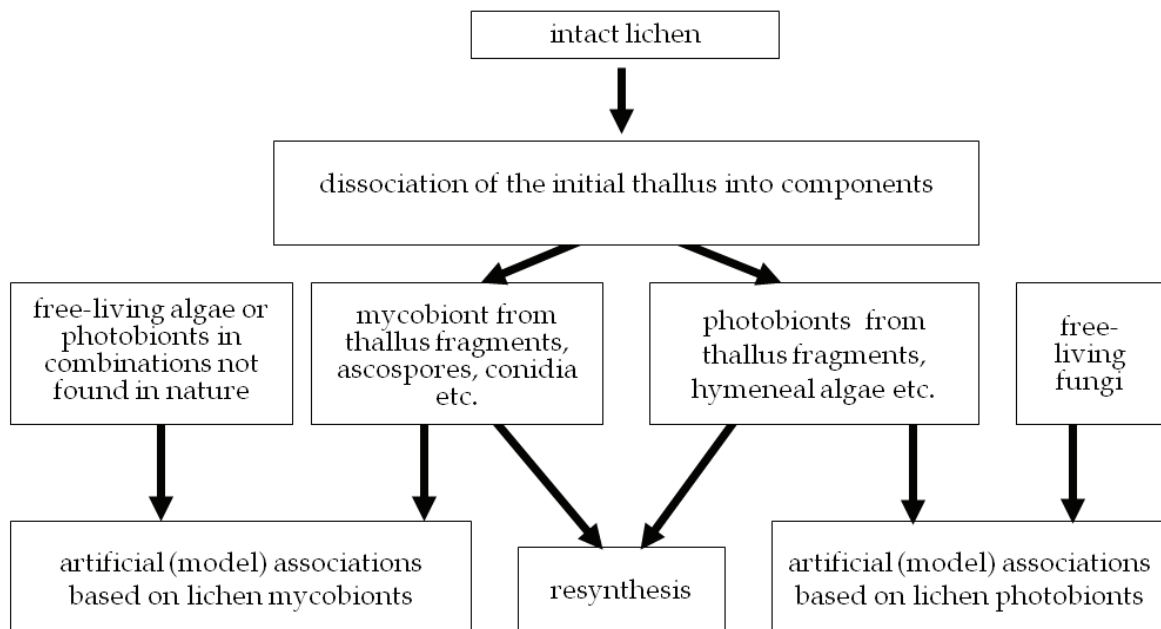


Fig. 1. Main stages of the method of lichen resynthesis.

The former group of approaches reconstructs the general situation emerging in lichens in the course of sexual reproduction, and the latter resembles rather the processes that take place in nature in the course of their vegetative reproduction. For instance, soredia are dedifferentiated parts of the thallus consisting of cells of both symbionts, which makes them very convenient sources for obtaining "tissue cultures". Isidia, morphologically structured thallus fragments, are natural explants of microclonal propagation (Fig. 2). But soredia and isidia are found only in some lichens, which makes obtaining "lichen tissue cultures" from thallus fragments an important approach (Yamamoto et al., 1993; Yoshimura et al., 1993). Interestingly, lichen thalli develop similarly in all cases, independently of the approach used (Ahmadjian, 1973a, 1973b; Yoshimura et al., 1993; Stocker-Worgotter & Turk, 1989).

Attempts to synthesize lichens from separate components (fungi and green algae or cyanobacteria) were made simultaneously with the discovery of the dual nature of lichens by S. Schwendener in 1867. In the same year, A.S. Faminsyn and O.V. Baranetsky, cultivating fragments of lichen thalli, succeeded in describing for the first time the growth of the photobiont outside the thallus. However, almost all attempts to resynthesize a lichen from separate components in the late 19th century were unsuccessful (Bornet, 1873; Bonnier, 1889; critique of these works: Ahmadjian & Jacobs, 1983). The cause of these failures was the lack of both theoretical and experimental foundations laid for such experiments. Successful experiments on lichen construction were performed only three decades later, due to the development of theoretical data on the biology of lichens and laying of methodological foundations for cultivating lichen component monocultures on artificial media.

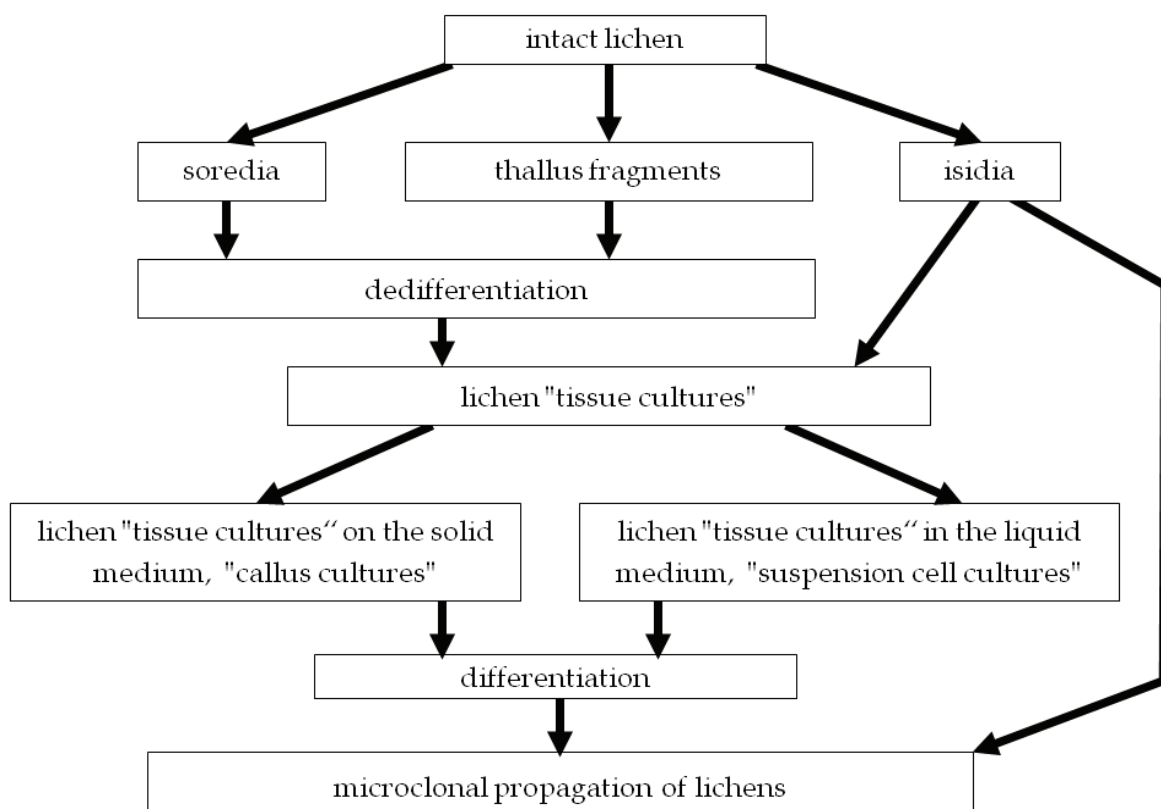


Fig. 2. Main stages of the method of "lichen tissue cultures".

In 1939, E. Thomas was the first to successfully resynthesize the lichen *Cladonia pyxidata* (Paracer, Ahmadjian V., 2000). However, he was unable to reproduce his experiment: none of the 800 subsequent attempts to resynthesize the lichen was successful. The 1950s mark the start of the epoch of experimental lichenology. In different countries, several scientific schools emerged, each of them developing approaches of its own and following its own general direction of studies: in the United States, V. Ahmadjian (resynthesis and construction of artificial associations); in Israel, M. Galun (sensory signal interactions); in Germany, S. Ott and his colleagues (lichen morphogenesis); in Japan, Y. Yamamoto (obtaining and biotechnological application of lichen "tissue cultures"). In the former Soviet Union, methods for isolating photobionts from thalli were developed by E.A. Vainshtein and I.A. Shapiro. During this

period, methods for extracting mycobionts and photobionts from thalli were perfected, appropriate media for cultivating symbiont monocultures were developed, and new substrates for reconstructing lichen thalli were proposed (Ahmadjian, 1973a).

Lichenologists became considerably more interested in resynthesizing the thalli of two-component lichens thanks to the classic studies of Ahmadjian (1961, 1967, 1973a, 1973b, 1990). His contribution to this area is hard to overestimate: over 40% of publications on lichen thalli resynthesis were produced by him alone or in collaboration with others. He developed the method for extracting and cultivating the mycobiont from apothecia. Thanks to Ahmadjian's work, thallus resynthesis of some lichen species became common practice. The principal advantages and disadvantages of the methods he proposed are now clear, but while the advantages are currently widely used in experimental studies, the disadvantages have not yet been overcome, restricting the area where this approach is applied (Ahmadjian, 1973b, 1990).

Integrating the first group of approaches and methods used for obtaining plant tissue cultures, Y. Yamamoto (1985) founded another branch of experimental lichenology: cultivation of ground lichen thallus fragments on artificial growth media (Fig. 2). One of the most important advantages of this approach was using vegetative parts of the thallus, which made the approach equally applicable to lichens with different modes of reproduction. In addition, Yamamoto's method allowed obtaining, under controlled conditions and on a tight timetable, a biological system consisting of both components and displaying a number of properties typical of intact lichens (Yoshimura et al., 1993; Yamamoto et al., 1993). Taking into account the fact that under particular conditions for cultivating such systems, thalli morphologically and anatomically similar to the natural ones are formed (Yoshimura & Yamamoto, 1991; Yoshimura et al., 1993), this approach can be used for reproducing lichens *in vitro*.

The interest towards these studies can be illustrated by the fact that the number of lichen species cultivated *in vitro* increased from two in 1985 to 193 in 1993, and the collection of mycobiont and photobiont cultures and "tissue cultures" by that time comprised over 400 lines (Yamamoto et al., 1993) and 400 species (Yamamoto et al., 1995). These included lichens from different climatic and geographical zones, growing in Canada, England, Finland, Israel, Japan, Antarctica, and Malaysia.

4. Lichen resynthesis and producing artificial (model) associations

The method of lichen resynthesis includes the following stages: (1) dissociation of the natural lichen into components; (2) obtaining monocultures of the mycobiont and photobiont; (3) mixed cultivation of the mycobiont and photobiont; and (4) producing a stable morphogenetic association (Fig. 1).

4.1 Lichens used in attempts of resynthesis

Although works on the experimental resynthesis of lichens are in progress since over 70 years ago, the number of species of lichens resynthesized to date remains not too high (Table 1). Experiments were successful with far from all systems, and those lichen species

that produced more or less developed thalli in resynthesis experiments, such as *Cladonia cristatella* (Ahmadjian & Jacobs, 1983) and *Xanthoria parietina* (Bubrick et al., 1985; Galun, 1989), were repeatedly used subsequently for solving particular lichenological problems (Fig. 3). Most resynthesized species are crustose two-component forms, and only one, *Stereocaulon vulcani*, is three-component, which probably reflects the complexity of such systems (Table 1).

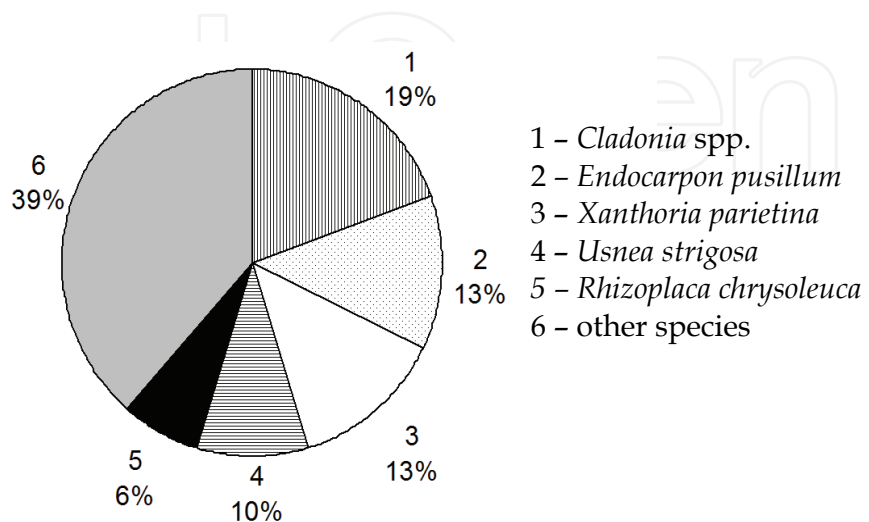


Fig. 3. Lichen species used in attempts of resynthesis

Most attempts to reconstruct thalli were made on lichens with green algal photobiont (Fig. 4). Among lichens with cyanobacterial photobiont, successful resynthesis was achieved only in members of the genus *Peltigera*; attempts to resynthesize *Leptogium issatschenkovi* were unsuccessful (Ahmadjian, 1989). Among the almost 30 genera of green algae occurring as photobionts in lichens (Oksner, 1974; Ahmadjian & Paracer, 1986), members of only 10 were used in resynthesis experiments; in 60% of the studies, species of the genera *Trebouxia*, *Desmococcus* (= *Protococcus*) and *Chlorella* were used (Fig. 4).

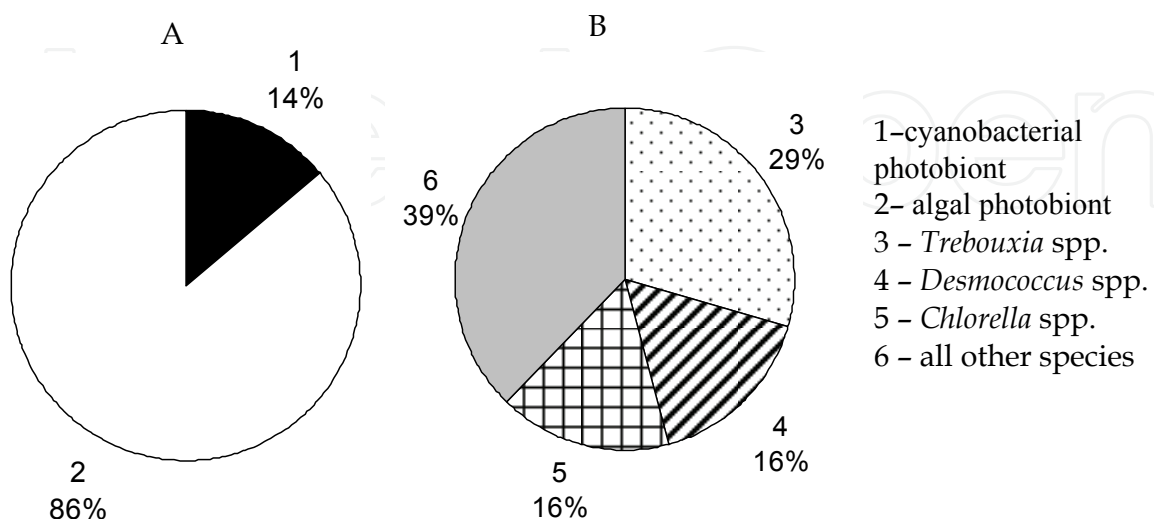
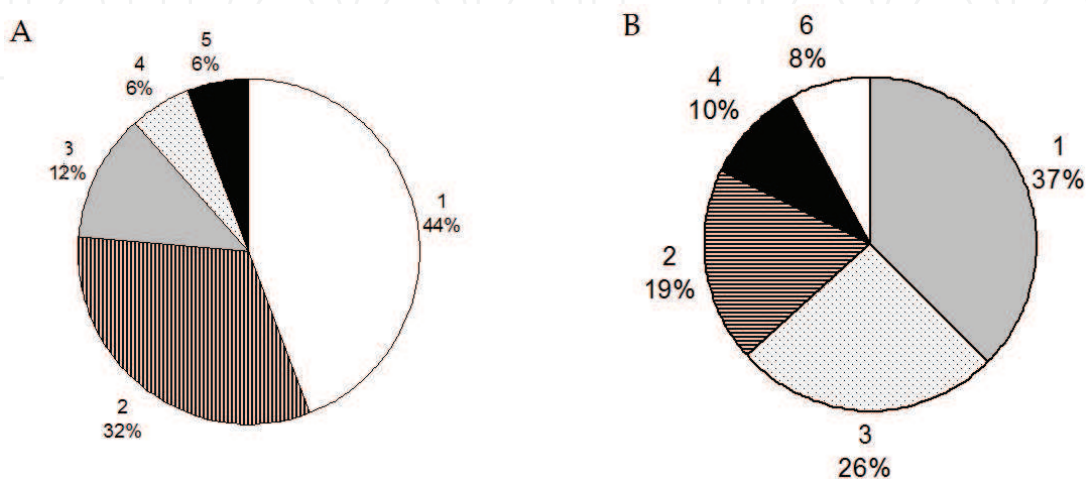


Fig. 4. Proportion of resynthesized lichens with different photobionts: A, proportion of cyanolichens (1) and phycolichens (2); B, different genera of green algae

Lichen name	Photobiont genera	Source
<i>Acarospora fuscata</i>	<i>Trebouxia</i>	Cited by: Oksner, 1974
<i>Buelia</i> spp.	<i>Trebouxia</i>	Cited by: Oksner, 1974
<i>Bacidia bagliettoana</i>	<i>Chlorella</i>	Bornet, 1873
<i>Caloplaca decipiens</i>	<i>Chlorella</i>	Cited by: Oksner, 1974
<i>Cladonia cristatella</i>	<i>Trebouxia</i> or <i>Chlorella</i>	Remmer, Ahmadjian, Livdahl, 1986 – cited by: Ahmadjian & Paracer, 1986 ; Ahmadjian, 1967
<i>C. furcata</i>	<i>Trebouxia</i> or <i>Chlorella</i>	Jahns, 1978 – cited by: Ahmadjian & Paracer, 1986
<i>C. pyxidata</i>	<i>Trebouxia</i> or <i>Chlorella</i>	Thomas, 1939 – cited by: Oksner, 1974
<i>Collema limosum</i>	<i>Nostoc</i>	Rees, 1871 – cited by: Oksner, 1974
<i>Dermatocarpon miniatum</i>	<i>Hyalococcus</i> or <i>Protococcus</i>	Stocker-Worgotter & Turk, 1989
<i>Endocarpon pusillum</i>	<i>Myrmecia</i>	Bertsch, Butin, 1967; Stahl, 1877 – cited by: Stocker-Worgotter & Turk, 1988
<i>Graphidaceae</i> g. sp.	<i>Trentepohlia</i>	Herriset, 1946 – cited by: Oksner, 1974
<i>Heppia echinulata</i>	<i>Scytonema</i> , cyanobacterium	Marton, Galun, 1976 – cited by: Ahmadjian & Paracer, 1986
<i>Huilia albocaeerolenscens</i>	green alga	Cited by: Ahmadjian & Paracer, 1986
<i>Lecanora</i> spp.	<i>Protococcus</i>	Ahadjian, Russell, Hildreth, 1980; Culberson, Ahmadjian, 1980 – cited by: Ahmadjian & Paracer, 1986
<i>Lecidia</i> spp.	<i>Trebouxia</i> , <i>Pseudochlorella</i> , <i>Cocobotrys</i> or <i>Chlorosarcinopsis</i>	Cited by: Ahmadjian & Paracer, 1986
<i>Lepraria</i> spp.	<i>Stichococcus</i> or <i>Chlorella</i>	Cited by: Ahmadjian & Paracer, 1986
<i>Leptogium issatschenkovi</i>	<i>Nostoc</i>	Danilov, 1929 – cited by: Oksner, 1974
<i>Peltigeria canina</i>	<i>Nostoc</i>	Ahadjian, 1989
<i>Physia stellaris</i>	<i>Trebouxia</i>	Bonnier, 1888
<i>Rhizoplaca chrysoleuca</i>	<i>Protococcus</i>	Cited by: Ahmadjian & Paracer, 1986
<i>Rinodina sophodes</i>	green alga	Bonnier, 1888
<i>Sarcogyna</i> spp.	<i>Myrmecia</i>	Cited by: Ahmadjian & Paracer, 1986
<i>Stereocaulon vulcani</i>	<i>Trebouxia</i> + cyanobacterium	Cited by: Ahmadjian & Paracer, 1986
<i>Staurothele rugulosa</i>	<i>Protococcus</i>	Stahl, 1877 – cited by: Stocker-Worgotter & Turk, 1988
<i>Usnea strigosa</i>	green alga	Ahadjian & Jacobs, 1983, Ahmadjian & Jacobs, 1985
<i>Verrucaria macrostoma</i>	<i>Cocobotrys</i> or <i>Protococcus</i>	Stocker-Worgotter & Turk, 1989
<i>Xanthoria parietina</i>	<i>Trebouxia</i>	Bornet, 1873; Bonnier, 1889 – cited by: Oksner, 1974

Table 1. Lichen species used in attempts of synthesis

The proportion of such studies performed on photobionts from the genus *Trebouxia* is especially high. This is explained by the fact that 90% of lichen species have photobionts belonging to this genus (Oksner, 1974). However, using photobionts of the genus *Tribouxia* in experiments on lichen thallus resynthesis (Ahmadjian, 1973a) did not guarantee success; this is why in 75% of experiments photobionts of other genera were used. In their natural environment, all resynthesized lichen species form sexual reproductive organs (apothecia or perithecia), and most form asexual reproductive organs as well: conidia, pycnoconidia, etc. (Fig. 5A).



A: 1, sexual reproduction with apothecia; 2, asexual reproduction (with conidia etc.); 3, vegetative reproduction with soredia; 4, vegetative reproduction with isidia; 5, sexual reproduction with perithecia.

B: 1, sexual reproduction with apothecia; 2, vegetative reproduction with soredia; 3, asexual reproduction (with conidia etc.); 4, vegetative reproduction with isidia; 5, sterile species (reproduction lacking or not observed).

Fig. 5. Proportion of different modes of reproduction in resynthesized (A) and cultivated (B) lichens.

Vegetative reproduction structures, soredia and isidia, are found only in three of the reconstructed lichen species. Experimental resynthesis requires a culture of the mycobiont, and Ahmadjian (1973a) proposed using spores for extracting it from the naturally growing lichen. This is probably why lichens with mainly vegetative mode of reproduction were seldom used in such *in vitro* experiments.

It has been found that successful resynthesis of a lichen thallus from mycobiont and photobiont monocultures requires experimental conditions imitating the natural environment: the substrate and medium should be poor, so that the lichen does not dissociate into components, and neither the mycobiont or photobiont get any advantages for growth at any stage of development. To induce thallus differentiation in a mixed culture, alternating drying and moistening periods should be imposed, and the drying of biomass should be gradual (Ahmadjian, 1973b).

4.2 Producing artificial (model) associations

Work on the resynthesis of lichens and producing model association has laid the foundation for solving a number of basic problems of the current lichenology. One of these problems is

the study of selectivity and specificity of symbiont interaction in lichens. For solving this problem in the course of lichen thallus resynthesis, it is possible to cultivate the mycobiont in pairs with free-living algae or photobionts in combinations not found in nature. In 50% of such experiments, the model systems revealed the lichenization of photobiont cells: the initial contact between the partners of the association took place, and pre-thalli were formed (Tables 2, 3). The same mycobiont showed both positive and negative results with algae of different species of the same genus or with photobionts extracted from the same lichen. The cause of this selective interaction of partners in pairs has not yet been revealed; however, several factors affecting the success of this process were determined, the most important of them being the source of the isolated photobiont and its symbiotrophics (Ahmadjian & Jacobs, 1983).

Lichen from which mycobiont was extracted	Natural photobiont	Potential photobiont	Source of photobiont	Result
<i>Cladonia cristatella</i>	<i>Trebouxia ereci</i>	<i>Friedmannia israeliensis</i>	free-living	+
		<i>Myrmecia</i> sp.	not specified	-
		<i>Nostoc</i> sp.	cycads	-
		<i>Pleurastrum terrestre</i>	free-living	-
		<i>Pseudochlorella</i> sp.	not specified	-
		<i>Pseudotrebouxia</i> sp.	<i>Xanthoria parietina</i>	-
		<i>Trebouxia</i> sp.	<i>Pilophoron</i> sp.	+
		<i>Trebouxia</i> sp.	<i>Stereocaulon</i> sp.	+
		<i>Trebouxia</i> sp.	<i>Gymnoderma</i> sp.	+
		<i>Trebouxia</i> sp.	<i>Lecidia</i> sp.	+
		<i>Trebouxia</i> sp.	<i>Lepraria</i> sp.	+
		<i>Trebouxia</i> sp.	<i>Parmelia</i> sp.	-
		<i>Trebouxia</i> sp.	<i>Xanthoria aureola</i>	-
<i>Trebouxia italiana</i>	<i>Xanthoria parietina</i>	+		
<i>Endocarpon pusillum</i>	<i>Myrmecia biatorellae</i>	<i>Protococcus staurothelis</i>	<i>Staurothele regulosa</i>	-
<i>Glyphis lepida</i>	<i>Trentepohlia</i>	<i>Trentepohlia</i> sp.	<i>Pyrenula nitidula</i>	+
<i>Phaeographina fulganata</i>	<i>Trentepohlia</i>	<i>Trentepohlia</i> sp.	<i>Pyrenula nitidula</i>	+
<i>Staurothele regulosa</i>	<i>Protococcus staurothelis</i>	<i>Myrmecia biatorellae</i>	<i>Endocarpon pusillum</i>	-

Table 2. Model associations based on the mycobiont. Note: +, synthesis continued to prethallus; -, attempts of synthesis were unsuccessful

For instance, species of the genus *Trebouxia* competent for the mycobiont *C. cristatella* were, with one exception, derived from lichens taxonomically close to the family Cladoniaceae, while attempts to synthesize thalli with photobionts of the same genus extracted from lichens of distantly related families were unsuccessful. Interestingly, the mycobiont

C. cristatella formed prethallus also with the free-living alga *Friedmannia israeliensis* (Ahmadjian & Jacobs, 1983). The synthesis, however, did not go any further, and, as in all the other cases with constructing lichen-based model systems, the mycobiont killed the algal cells. Unfortunately, these experiments were not continued. It is very likely that some changes in media and/or cultivating conditions would produce a new lichen species: after all, in experiments with compatible cultures of natural symbionts parasitism of the mycobiont on photobiont is also observed under certain conditions.

Potential photobiont	Source of photobiont	Potential mycobiont	Result
<i>Friedmannia israeliensis</i>	free-living	<i>Cladonia cristatella</i>	+
<i>Myrmecia</i> sp.	not specified	<i>Cladonia cristatella</i>	-
<i>Myrmecia biatorellae</i>	<i>Endocarpon pusillum</i>	<i>Staurothele regulosa</i>	-
<i>Nostoc</i> sp.	cycads	<i>Cladonia cristatella</i>	-
<i>Pleurastrum terrestre</i>	free-living	<i>Cladonia cristatella</i>	-
<i>Protococcus staurothelii</i>	<i>Staurothele regulosa</i>	<i>Endocarpon pusillum</i>	-
<i>Pseudochlorella</i> sp.	not specified	<i>Cladonia cristatella</i>	-
<i>Pseudotreboxia</i> sp.	<i>Xanthoria parietina</i>	<i>Cladonia cristatella</i>	-
<i>Trebouxia</i> sp.	<i>Pilophoron</i> sp.	<i>Cladonia cristatella</i>	+
	<i>Stereocaulon</i> sp.	<i>Cladonia cristatella</i>	+
	<i>Gymnoderma</i> sp.	<i>Cladonia cristatella</i>	+
	<i>Lecidia</i> sp.	<i>Cladonia cristatella</i>	+
	<i>Lepraria</i> sp.	<i>Cladonia cristatella</i>	+
	<i>Parmelia</i> sp.	<i>Cladonia cristatella</i>	-
	<i>Xanthoria aureola</i>	<i>Cladonia cristatella</i>	-
<i>Trebouxia italiana</i>	<i>Xanthoria parietina</i>	<i>Cladonia cristatella</i>	+
<i>Trentepohlia</i> sp.	<i>Pyrenula nitidula</i>	<i>Glyphis lepida</i>	+
<i>Trentepohlia</i> sp.	<i>Pyrenula nitidula</i>	<i>Phaeographina fulganata</i>	+

Table 3. Model associations based on the photobiont. Note: +, synthesis continued to prethallus; -, attempts of synthesis were unsuccessful

On the other hand, photobionts isolated from lichen *X. parietina* – *Pseudotreboxia* sp. and *Trebouxia* sp. – interacted differently with the mycobiont *C. cristatella*: the mycobiont quickly *Pseudotreboxia* sp., but formed prethallus with *Trebouxia* sp. (Table 2). It has been found that some photobionts (*Trebouxia* spp., *Trentepohlia* spp.) and mycobionts (*Graphidaceae* g. spp.) are better than others (e.g., *Myrmecia* spp.) at forming artificial associations. All the attempts to synthesize model associations using the photobiont *Trentepohlia* sp. and mycobionts extracted from lichens of the family *Graphidaceae* were successful. This was probably determined, on the one hand, by the greater or smaller resistance of the alga to parasitism and by the degree of mycobiont aggression, and, on the other hand, by the proximity of the potential photobiont in a number of characters (type of vegetative reproduction, etc.) to the natural photobiont. In addition, there are more and less specialized and symbiotrophic genera or species of photobionts and mycobionts.

Unfortunately, there are no data in the literature on model associations based on lichen photobionts and free-living fungi (Table 3). Meanwhile, we believe that this problem is

very interesting, since the photobiont, intensely influenced by the mycobiont in the lichen, has developed a number of protective adaptations. These adaptations may be conducive to success in producing model associations of the photobiont and free-living symbiotic fungi.

Another problem is the construction of artificial three-component associations. For solving this problem, a third component, a cyanobiont, can be added to the resynthesized lichen to study the process of cephalodia formation (Ahmadjian, 1989; Ahmadjian & Jacobs, 1983; Ahmadjian & Paracer, 1986). Attempts to model three-component lichens have been made by adding strains of the symbiotrophic and free-living *Nostoc* sp. to two-component lichens with a green alga (e.g., to *C. cristatella*). In all such experiments, the mycobiont parasitized the cyanobiont, and the formation of cephalodia was not observed.

A large field for work in experimental lichenology is opened by the opportunity to obtain protoplasts of the symbionts. The methodology of this procedure for a lichen mycobiont grown from a spore was developed by V. Ahmadjian (1991). Kinoshito (Yamamoto et al., 1993; Kinoshito et al., 2001) modified this procedure to develop a method independent of the way of mycobiont extraction, making it possible to obtain mycobiont protoplasts from vegetative parts of the thallus (experiments on obtaining mycobiont protoplasts were performed only with members of the genus *Cladonia*). Using isolated protoplasts makes it possible to model intracellular interactions of the symbionts. Interestingly, a similar enigmatic organism is known in nature, the fungus *Geosiphon pyriforme*, which hosts inside the cell the cyanobacterium *Nostoc punctatum* (Wolf, Schüßler, 2005).

5. "Tissue cultures" of lichen

The field of experimental lichenology has been developed especially to obtain "callus tissue cultures" of lichens. Currently, lichens cultivated on solid growth media include 52 genera from 22 families, represented mainly by fruticose forms (52%), somewhat fewer foliose forms (36%), and only 12% crustose forms. The vast majority of the species maintained in "callus cultures" are two-component lichens with green algal photobionts. Attempts to introduce three-component lichens to a "callus culture" result in their dissociation into components and formation of "chimeric" forms (see below).

The ratio of different modes of reproduction among cultivated lichens differs from that among reconstructed ones, and there is no pronounced prevalence of forms with sexual or asexual reproduction (necessary for extracting the mycobiont according to the first group of experimental approaches). On the contrary, there is quite a high proportion of lichens with vegetative reproductive structures, soredia and isidia: 36% together, the same as the proportion of lichens with apothecia (37%). Furthermore, the universality of the approach allows cultivating fragments of lichens that form apothecia extremely rarely (Fig. 5B).

5.1 Problems emerging in the course of lichen cultivation

Among difficulties of this experimental approach, the high proportion of explants infected with accompanying microorganisms and the low proportion of thallus fragment germination can be named. In the case of *Usnea rubescens*, growth was observed only in 27%

of the inoculated thallus fragments; in *Peltigera praetextata*, it was observed in 0.4–1.2% cases (maximum and minimum values are given). These parameters are influenced by a number of factors, such as species, habitat, area of the lichen's thallus, number of symbionts (Yoshimura et al., 1993; Yoshimura & Yamamoto, 1991; Smirnov & Lobakova, 2007). The best results are obtained by using soredia of two-component epiphytic fruticose lichens. One possible explanation of this is the fact that 1 g of wet lichen weight contains up to 10^{10} microorganism cells, while in soredia their number is considerably lower (Krasilnikov, 1949); thus, using specialized vegetative reproduction structures (both isidia and soredia) is probably quite promising.

Yoshimura et al. (1993) note that attempts to cultivate fragments of the cyanolichen *P. praetextata* on growth media result in higher levels of infection by contaminant bacteria and myxomycetes, compared to attempts to cultivate explants of lichens with green algal symbionts. This is usually explained in the literature by the fact that symbiotic cyanobacteria have strong, complexly organized surface structures (polysaccharide sheaths), serving as habitats for associated bacteria, complicating the process of obtaining sterile cyanolichen explants (Yoshimura & Yamamoto, 1991; Gusev & Mineeva, 1992). However, in our experiments, in spite of the higher affection level actually observed in fragments of cyanolichens cultivated on growth media, compared to other kinds of lichens, cyanobiont isolates extracted from cyanolichen thallus fragments contained fewer accompanying bacteria than green algal isolates from the same lichens (Smirnov & Lobakova, 2007).

One drawback of the method for obtaining lichen "tissue cultures" described in the literature is the total lack of any primary sterilization of explants. Although some studies refer to unsuccessful attempts to sterilize thallus fragments with mercuric chloride (Ahmadjian, 1989), our data (Smirnov & Lobakova, 2007) demonstrate the efficiency of consistent complex usage of "mild" sterilizing agents (such as hydrogen peroxide, alcohol, chlorhexidine).

In our opinion, works aimed by obtaining "suspension cell cultures" of lichens are especially promising. Experiments in this branch of lichenology are rare and fragmentary, while maintaining symbiont monocultures in liquid media are currently a common practice (Ahmadjian & Jacobs, 1983). In "suspension cultures" of lichens, thalli are not formed; this is why in most studies aimed at obtaining structured lichen thalli, even if thallus fragments were inoculated into liquid growth media, the experiments were never completed, because provisional results did not comply with the initial aims. On the other hand, obtaining "suspension cultures" of lichens is of considerable interest for biotechnology, since methods based on "suspension cultures" are easier to introduce into the industry. Thus, this mode of growth will probably allow obtaining large amounts of dedifferentiated cell biomass and using it in biotechnology as sources of lichen compounds.

A promising approach for obtaining both "suspension cultures" and "callus cultures" of lichens involves using "nurse cultures" (Smirnov & Lobakova, 2008) and conditioning (processing) the cultivating media with metabolites of associated organisms. Data available in the literature give evidence that the initiation of symbiont growth (especially of the

mycobiont) requires compounds secreted by the photobiont or associated bacteria. It has been found that the development of the mycobiont was quicker and more intense if (1) non-sterile tree bark was used as the substrate; (2) the cultivating medium was conditioned by metabolites of the photobiont or bacteria; (3) the spores were infected by bacteria (Ahmadjian, 1989; Yolando et al., 2002; Smirnov, 2006). Our results show that conditioning the media with simple metabolites (after sterilization) is inefficient, compared to using native metabolites (dialysis cultivation).

5.2 Study of lichen morphogenesis

A special place among experimental works in lichenology is occupied by the branch involving the study of lichen thallus morphogenesis and revealing the factors influencing this process. A number of studies have addressed the problem of inducing morphogenesis in "callus cultures" of soredia, both in the laboratory, and in the natural environment (Stocker-Worgotter & Turk, 1988; Stocker-Worgotter & Turk, 1989; Yoshimura & Yamamoto, 1991; Armaleo, 1991; Yoshimura et al., 1993). Comparison of natural lichen thalli with those obtained by inducing morphogenesis in *in vitro* systems demonstrates their anatomical and morphological similarity; the same layers are formed: upper cortex (in some species, lower cortex), photobiont layer, medulla. One drawback of this approach is the fact that non-homogeneous material, e.g. in the shape and size of scales, is often formed in the laboratory, probably because of the heterogeneity of various thallus parts caused by the parasexual process (Stocker-Worgotter & Turk, 1988) or by somatic variation (Street, 1977; Butenko, 1999).

Lichens with different modes of reproduction (sexual, asexual and vegetative) under laboratory conditions with morphogenesis induction undergo the same stages of development (lag phase, arachnoid phase, prethallus and thallus: Ahmadjian, 1973a, 1973b; Ahmadjian & Jacobs, 1983; Stocker-Worgotter & Turk, 1989; Yoshimura et al., 1993) as in nature (Ott, 1988). The only difference is the duration of particular stages, depending on the type of the explant and conditions of its cultivation. In *P. didactyla*, thallus develops quicker than in other species (especially at the final stages). It has been found (Stocker-Worgotter & Turk, 1988), that using soredia as explants is conducive to the quick (2–4 times quicker) formation of thallus *in vitro*; however, rates of morphogenesis as high as in nature have not yet been achieved under laboratory conditions.

The experimental approaches in lichenology described here are currently used for solving a number of basic problems like those persistent in biotechnology. The former approaches include studying the ecological and morphological plasticity of lichens and revealing differentiation factors of thalli and the share of each partner in the formation of the unique super-organism system.

In this respect it is especially interesting to study the development of the "tissue cultures" of three-component lichens, such as *Peltigera aphthosa*, with a green alga as the photobiont and a cyanobiont in cephalodia. In "tissue cultures" of *P. aphthosa*, explants often formed a homoiomerous cyanolichen, and the green alga was expelled from the association and remained in the culture as free-living colonies. The drying of the system increased the number of green algal colonies, and they were included into the composition of non-

differentiated mixed aggregates. The slightly raised and drying areas of the homoiomerous cyanothallus became colourless (the cyanobiont disappeared) and were gradually colonized by the green alga, while in other areas, which preserved contact with the substrate, cyanobacteria were preserved. These areas resembled primordia of new green lobes; however, no further development was observed. Due to the difficulties of moisture control, normal thalli did not form in the experiments; the formation of cephalodial primordia was, nevertheless, observed.

The phenomenon described provides an experimental confirmation of the idea that mycobionts can include several morphotypes (as analysis of their DNA has also shown), and/or the formation of chimeric lichens is possible. While the existence of such chimeras was earlier considered unproven, now the reality of this phenomenon has been confirmed both by some field studies (for review, see: Plyusnin, 2002) and by laboratory experiments.

Morphogenetic "tissue cultures" of lichens are convenient experimental models for the study of this phenomenon. The results of using them allow us to state that the formation of a particular morphotype or chimeric lichen depends on moisture. For instance, these results allow suggesting that the cyanobacterial morphotype is more widespread than has been believed earlier and unidentified species of the genus *Peltigera* with cyanobacteria often represent one of the morphotypes of three-component lichens (Yoshimura et al., 1993).

It can be assumed that experimental approaches will also play an important role in the molecular biology of cyanolichens: they will allow studying the exchange of genes, inferred by some authors, between the symbionts by means of plasmids in the course of morphogenesis (Ahmadjian, 1991). Reviewing the data available in the literature has shown that for studying the early stages of lichen thallus morphogenesis, it is better to use methods of resynthesis, while for the study of specificity and selectivity of interactions between components of this symbiosis, as well as of different stages of thallus differentiation, the "tissue culture" and morphogenesis induction methods are more suitable.

Dedifferentiated mixed cellular aggregates of a "callus culture" of lichens can be used in the study of the genetic control over symbionts in the course of the formation of a balanced super-organism system (Yamamoto et al., 1993; Yoshimura et al., 1993).

5.3 The biotechnological potential of lichen "tissue cultures"

Using experimental approaches is promising also for producing from lichens their unique secondary metabolites, the lichen compounds. The biosynthesis of lichen compounds in "tissue cultures" is usually no different from that in the natural thallus in the composition of depsides, tridepsides, and depsidones; triterpenoid compounds are, however, a more labile class of substances, and in "callus cultures" of lichens they often disappear (Table 4).

In most cases, the concentration of lichen compounds in a "culture" is considerably lower than in a natural thallus: the content of the usnic acid in *Usnea rubescens* is 0.9% in the natural state and 0.162% in a "callus culture", i.e., five times higher; in *Ramalina yasudae*, it is even 100 times higher (Yamamoto et al., 1985). But since "tissue cultures" of some lichen

species grow considerably quicker (their biomass increases at least by a factor of 5 over 14 weeks), using the Yamamoto method for industrial production of lichen compounds (Yamamoto et al., 1985; Yamamoto et al., 1993) is very promising. Importantly, using "tissue cultures" of lichens, we can decrease the number of lichens that are removed from their natural environment, and extremely slowly regenerating in nature.

Class of compounds	Compounds	<i>Usnea strigosa</i>		<i>Usnea rubescens</i>		<i>Ramalina yasudae</i>		<i>Peltigera pruinosa</i>		<i>Peltigera aphthosa</i>	
		t	r	t	c	t	c	t	c	t	c
depsides and depsidones	globin acid	+	-								
	connorstictic acid	-	+								
	cryptostictic acid	-	+								
	methyl lecanorate			-	-	-	-	-	-	+	+
	norstictic acid	+	+								
	protocetraric acid			+	+	+	+	-	-	-	-
	salazanic acid			+	-	+	-	-	-	-	-
	usnic acid	+	+	+	+	+	+	-	-	-	-
	fumaroprotocetraric acid	+	-								
evernic acid			+	-	+	-	-	-	-	-	
tridepsides	methyl gyrophorate			-	-	-	-	+	+	+	+
	tenuiorin			-	-	-	-	+	+	+	+
triterpenoids	dolichorrhizin			-	-	-	-	+	-	-	-
	zeorin			-	-	-	-	+	-	-	-
	phlebeic acid			-	-	-	-	-	-	+	-

Table 4. Comparison of lichen compound production by "tissue cultures", resynthesized thalli, and natural thalli, from: Ahmadjian & Jacobs, 1983; Yamamoto et al., 1985; Yoshimura & Yamamoto, 1991. Note: +, compound present; -, compound not found; t, compound extract from natural thallus; c, from resynthesized thallus; c, from "tissue culture".

The expediency of using lichen "tissue cultures" for obtaining biologically active compounds is also supported by the fact that their methanol and acetone extracts demonstrate a levels of superoxide dismutase activity, and have antibacterial (against Gram-positive bacteria: Fig. 6) and antiviral (when EBV test system is used: Fig. 7) effects (Yamamoto et al., 1993; Yamamoto et al., 1995).

The degrees of antibacterial and antiviral activities strongly vary between different lichens, even among species of the same genus (Fig. 7). In most cases, the inhibitory action of extracts of natural thalli is higher than that of "tissue culture" extracts; there are, however, some exceptions: laboratory extracts of *Cladia aggregata* and *Evernia prunastri* displayed higher levels of activity than extracts of their natural thalli. Interestingly, "tissue cultures" of lichens of the genera *Cetraria*, *Evernia* and *Cladonia*, the extracts of which demonstrated considerable levels of antiviral activity, had no antibacterial effect.

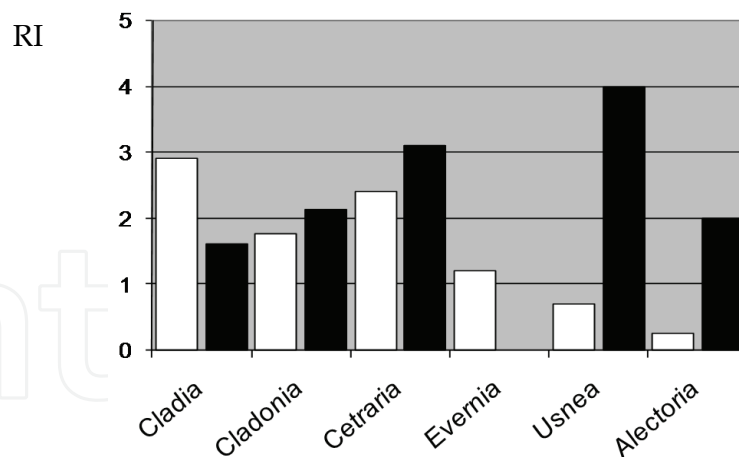


Fig. 6. Antiviral activity of extracts from thalli and "tissue cultures" of lichens (on the base: Yamamoto et al., 1993, 1995). EBV test system was used. RI, ratio of CV in experiments with particular lichen extract and CV in control samples; CV (cell viability), percentage of surviving cells 48 hours after the start of the experiment.

On the other hand, "tissue cultures" of lichens of the genera *Usnea*, *Umbilicaria* and *Ramalina*, which strongly inhibited the growth of Gram-positive bacteria, poorly inhibited viral growth in a EBV test system (Fig. 7). One exception was the "tissue culture" of *Cladonia aggregata*, which demonstrated considerable activity in both cases.

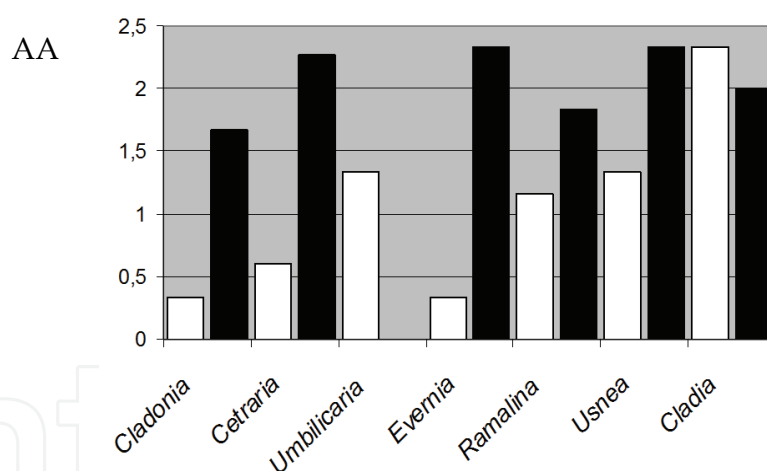


Fig. 7. Antibacterial effect of extracts from thalli and "tissue cultures" of lichens (on the base: Yamamoto et al., 1993). Antibacterial activity (AA) is given in relative units. Tests were performed on the species *Propionibacterium acnes*, *Staphylococcus aureus*, *Bacillus subtilis*.

Interestingly, the concentration of lichen compounds in reconstructed lichen thalli is often higher than in nature; Ahmadjian and Jacobs (1985) explain this by the more favourable conditions for lichen development formed in the course of resynthesis. It is noteworthy that producing artificial associations, with symbiont combinations not found in nature, can be used as a promising source of new antibiotic compounds. The possibility of this application is demonstrated by the two novel compounds, not typical of this species in nature, found in the thallus of *Usnea strigosa* in the course of resynthesis (Table 4). The biotechnological application

of this approach for producing lichen compounds is currently restricted by the low rate of the system's growth, surmountable in the future by optimizing cultivation methods.

A special place among the problems of current lichenology is occupied by the conservation of rare lichen species and their re-introduction into the natural environment. The above-described experimental approaches can be used, among other purposes, for solving these problems. Methods of rare species gene pool conservation in collections and cryobanks are well-developed for higher plants (Street, 1977; Butenko, 1999). Some authors (Tolpysheva, 1998) believe that it would be useful to apply this experience to lichens as well.

6. Conclusion

Among experimental approaches in lichenology, two groups of methods can be recognized: lichen resynthesis and cultivation. The former approach helped to find the answers to many questions of lichen biology, but currently it faces a number of insoluble problems (e.g., the failure of attempts to produce mature spores in sporocarps), due to which the number of studies on lichen reconstruction has considerably decreased (Ahmadjian, 1990). The latter approach is promising for introducing lichens into the field of biotechnological developments. However, this is largely hindered by the low yield of lichen biomass in the course of cultivation. Two principal causes of this can be named: the considerable level of infection with fungi and bacteria (Yamamoto et al., 2004) and the insufficiently quick growth of the culture of the lichen itself. The solution to the problem of "explant" infection with contaminant species may be found in surface sterilization of lichens, similar to that used in plant physiology (Smirnov & Lobakova, 2007). The solution to the problem of culture growth acceleration may be found in conditioning the media with secondary metabolites of various origins. The analysed literature contained no mentions of using "nurse cultures", a method widely used in plant physiology, considerably increasing the rate of growth in cultures (Street, 1977; Butenko, 1999; Butenko et al., 1987). At the same time, a number of authors have shown that secondary metabolites, both of associated fungi and algae, extracted from lichens (Vainshtein, 1988), and of accompanying fungi and algae (Ahmadjian, 1989), can accelerate growth in cultures of isolated symbionts, both mycobionts and phycobionts. Another way of accelerating the growth of cultures, both of the symbionts and of the lichen as a whole, may be found in using suspension cultures. Conditioning of media and suspension cultures can also be useful in the first group of experimental approaches, especially in producing model associations based on lichen photobionts (according to the literature, in most cases it was the mycobiont that served as the basis for novel associations).

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Biotechnology is the scientific field of studying and applying the most efficient methods and techniques to get useful end-products for the human society by using viable micro-organisms, cells, and tissues of plants or animals, or even certain functional components of their organisms, that are grown in fully controlled conditions to maximize their specific metabolism inside fully automatic bioreactors. It is very important to make the specific difference between biotechnology as a distinct science of getting valuable products from molecules, cells or tissues of viable organisms, and any other applications of bioprocesses that are based on using the whole living plants or animals in different fields of human activities such as bioremediation, environmental protection, organic agriculture, or industrial exploitation of natural resources. The volume *Advances in Applied Biotechnology* is a scientific book containing recent advances of selected research works that are ongoing in certain biotechnological applications. Fourteen chapters divided in four sections related to the newest biotechnological achievements in environmental protection, medicine and health care, biopharmaceutical producing, molecular genetics, and tissue engineering are presented.

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