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# Two-phase medium – a new approach to microbiological culturing

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## ABSTRACT

We tested a new two-phase culture medium for culturing microorganisms, in particular algae and fungi. In media composed of mixed gel and solid phases of granite, basalt, sandstone, limestone and sodium glass, we observed the growth of selected species inhabiting the boundaries of the gel medium with the solid phase. The species used were *Klebsormidium dissectum* (F.Gay) H.Ettl & Gärtner, *Vischeria polyphem* (Pitschmann) Kryvenda, Rybalka, Wolf & Friedl and *Muriella decolor* Vischer (algae); and *Aspergillus tubingensis* R.Mosseray, *Aureobasidium pullulans* (de Bary) G.Arnaud, *Chaetomium globosum* Kunze and *Penicillium* sp. Link (fungi). In the six-weeks period of parallel cultures, the growth rate of the algae inoculum in two-phase media was twice that observed in the control culture (gel medium). The algae also maintained high viability in the new medium.

**KEY WORDS**
Methodology,
two-phase media,
culture collection,
algae,
fungi,
rocks.

**RÉSUMÉ**

*Milieu à deux phases – une nouvelle approche de la culture microbiologique.*

Nous avons testé un nouveau milieu de culture à deux phases pour la culture de micro-organismes, en particulier des algues et des champignons. Dans des milieux composés de phases mixtes incluant un gel et une phase solide constituée de granite, basalte, grès, calcaire et verre sodique, nous avons observé la croissance d'espèces sélectionnées à l'interface du milieu gélifié et de la phase solide. Les espèces utilisées correspondaient à *Klebsormidium dissectum* (F.Gay) H.Ettl & Gärtner, *Vischeria polyphem* (Pitschmann) Kryvenda, Rybalka, Wolf & Friedl et *Muriella decolor* Vischer pour les algues, et à *Aspergillus tubingensis* R.Mosseray, *Aureobasidium pullulans* (de Bary) G.Arnaud, *Chaetomium globosum* Kunze et *Penicillium* sp. Link pour les champignons. Au cours de la période de six semaines de cultures parallèles, le taux de croissance de l'inoculum d'algues dans les milieux à deux phases était le double de celui observé dans la culture témoin (milieu gel). Les algues ont également maintenu une viabilité élevée dans le nouveau milieu.

**MOTS CLÉS**
Méthodologie, milieux diphasiques, collection de culture, algues, champignons, roches.

## INTRODUCTION

It has been 140 years since Louis Pasteur used broth as a liquid culture medium. Four years later, Robert Koch was the first to use gelatine-solidified media. He demonstrated this new technique at the International Medical Congress in London in 1881 (Zwolska 2013). Agar became the staple ingredient of most media and continues so today, but the richness and diversity of microorganisms intended for in vitro culture have required the development of a large number of different kinds of culture media. Media substrates are classified in many ways. So far, the basic division of biological media into liquid, semi-liquid, gel and solid remains unchanged. These include natural, semi-synthetic and artificial, simple, enriched, selective or differentiating, and special media (Jabłoński 1979; Kunicki-Goldfinger 1998).

The commonly used selective media permit the growth of only a specific group of microorganisms due to the addition of specific chemicals. These media enable significant and relatively rapid progress in studies of particular micro organisms. Through the use of differentiating media, two species of microorganisms can be distinguished and separated from each other. Differentiating media can also be used as selective media for a specific group (e.g. bacteria, algae, fungi); then they are termed 'selective-differentiating' (Bonnet *et al*. 2020).

All of the examples of substrates mentioned above are single-phase systems. These are mixtures of various substances and chemical compounds, usually as liquids, which, with the increase in the content of gelling and solidifying substances and proper temperature selection, alter their viscosity and rheological properties, becoming solids. The monophasic nature of the substrates used to date imposes certain limitations on research on the behaviour of microorganisms occurring at the interface of two phases (e.g. liquid/solid, solid/water vapour). The formation of biofilms adhering to the surface of solid bodies immersed in or washed with a liquid under favourable environmental conditions is a well-known phenomenon. The experimental systems that various researchers have used to study the behaviour of microorganisms at the interface between liquid and solid substrates take the form of unit solutions, often with unique parameters that are not always accepted by other research teams (Rindi & Guiry 2003).

Currently, many tools are available to facilitate culture and observation of multi-species communities developing on multi-component media. A well-known device for studying the formation of bacterial biofilms was developed by Ceri *et al*. (1999), the Calgary Biofilm Device. Domingue *et al*. (1994) developed the Modified Robbins Device in which liquid culture medium is forced through porous membranes by a peristaltic pump. Murga *et al*. (2001) studied biofilm formation using a device that circulates a liquid culture medium through serially connected catheter tubes. According to Preisig & Andersen (2005), Pringsheim used extracts of soil as a supplement in purely mineral media to obtain better growth of microorganisms. He prepared biphasic media using pasteurized soil covered with water. This medium differs from ours by the lack of a stable phase which in our case contains small pieces of stones. So far, none of the proposed methods have been recognized as standard.

These methods cannot be applied in tests that require free access of the cultured microorganisms to air, as in mycological, phycological, lichenological or bryological cultures employing classical petri dishes. This often-encountered situation in field research requires the development of a new microbiological medium: a two-phase liquid-solid medium or one that ensures constant contact with the third gas phase. It is known that microorganisms, often inhabiting heterogeneous boundary surfaces, use the nutritional resources of various types of substrates.

It has long been known that rock substrates can exert effects via their physical characteristics (hardness, roughness, adhesion) and the chemical makeup of the environment, but the relationship between rock types and algal communities is still not precisely determined. Whitford (1956) and Nell (1968) found no major correlation between the presence of algae and the type of rock. However, in Glacier National Park (USA), Parker *et al*. (1973) observed that *Monostroma quaternaarium* (Kützing) Desmazières grew on iron-rich rocks, *Hydrurus foetidus* (Villars) Trevis. grew only on limestone and sandstone

rocks, and *Batrachospermum* Roth was indifferent to the type of rock. The occurrence and role of microorganisms on various substrates in nature has recently been studied by many researchers. Interesting communities of microorganisms that build biofilms of algal-bacterial mats in a very shallow chromium-polluted ditch have been examined (Augustynowicz *et al*. 2021; Ociński *et al*. 2021). These researchers identified as many as 67 taxa of microorganisms in the mats. The development of communities of fungi and bacteria on diverse substrates in nature has also attracted attention in recent years (Chlebicki *et al*. 2005; Drewniak & Skłodowska 2007; Drewniak *et al*. 2007, 2008). Chlebicki *et al*. (2014) noted six species of fungi coexisting with bacterial biofilm on rocks in an abandoned mine. This rock biofilm formed a diverse community with a mosaic distribution of fungal species.

These various findings point to the need for experimental media that will allow researchers to observe the development of individual taxa, such as algae and fungi, in a two-phase environment maintained in sterile culture conditions.

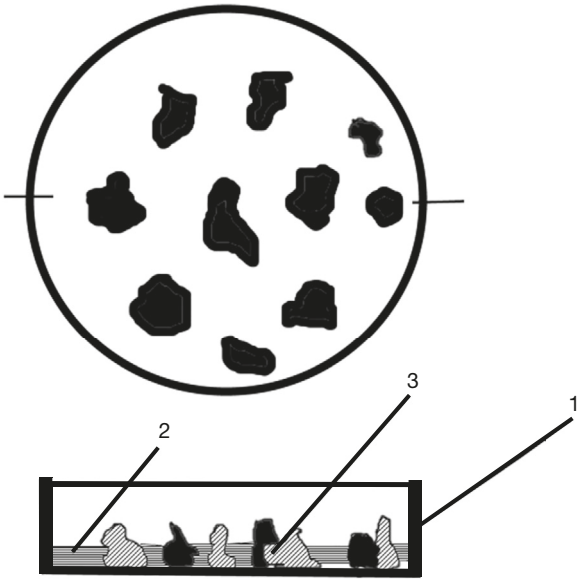


Fig. 1. — Petri dish (**1**) 9 cm in diameter with two-phase gel-solid (**2**, **3**) medium.

## MATERIAL AND METHODS

The microbiological medium we tested and present here is a two-phase medium of gel-solid type following Chlebicki *et al*. (patent pending PL 434076-A1). The medium consists of two components. The first basic component consists of the known monophasic microbial media used for preparation of a liquid phase or a gel. The second component consists of pieces of various types of stone providing solid material that is insoluble in the basic component. Their diameter is closely related to the thickness of the first component layer. The rock fragments used are taken from the fraction that passes through a sieve having mesh diameter equal to three thicknesses of the gel layer (5-10 mm). The material for production of the second component may be biologically inert to the cultured microorganisms (e.g. quartz glass, polystyrene) or active, as in the case of granite, basalt, limestone, etc. Selection of the material depends on the kind of research and the microorganism studied. Here, we focused on algae and microfungi (strains that can be grown on enriched agar media), each requiring the use of an appropriate medium.

The medium was prepared in standard sterile laboratory conditions according to the following protocol: (i) pieces of five sort of material (granite, sandstone, basalt, limestone, and additionally water glass), approx. 7 mm in diameter, were sterilized in dry hot air at 160°C for 60 minutes in a sterilizer (dryer with thermoregulation in the range of 100-200°C); (ii) each type of solid component (rock or glass) was spread separately in sterile plastic petri dishes (9 cm in diameter) on about 25 % of the pan surface; and (iii) the petri dishes with such a prepared solid phase were poured successively with Woods Hole Agar (Nichols 1973) and PDA (potato dextrose agar) for algae (Fig. 1), or with Synthetic Nutrient-Poor Agar (SNA) and PDA for microfungi.

Five different two-phase media were inoculated (using sterile micro-pipettes with disposable dispenser) with: (i) liquid of unialgal cultures (1 ml) of *Muriella decolor* Vischer (KRAM-A,16) (Chlorophyta, Trebouxiophyceae), *Vischeria polyphem* (Pitschmann) Kryvenda, Rybalka, Wolf & Friedl (KRAM-A,17) (Ochrophyta Eustigmatophyceae) and *Klebsormidium dissectum* (F.Gay) H. Ener (KRAM-A, 16) (Charophyta, Klebsormidiophyceae) available in the KRAM culture collection. Each species was separately used in a series of experiments. The cultures (ii) of *Aspergillus tubingensis* (isolated from rocks in Cameroon) and *Aureobasidium pullulans* (isolated from rocks in an anchialine cave on Long Island, Croatia), and preserved on PDA medium in the KRAM culture collection [note: some resistant fungi and other organisms survived inside the rocks after sterilization (*Penicillium* sp., *Chaetomium globosum* and strains of Bacteria)].

After inoculation, the algae and fungi were cultured separately. The algae were cultured in boxes under 185-200 µE m2 $s^{-1}$ light intensity under a 12-hour photoperiod at 21°C in sterile conditions. The fungi were cultured at room temperature under a 12-hour photoperiod. Under the same conditions, tests on the growth of lichens were carried out using only small thallus fragments (Stocker-Wörgötter 1995).

The rocks used in the research originated from Polish deposits (granite and basalt from Lower Silesia, sandstone from the Carpathians, and limestone from the Kraków-Częstochowa Upland). Quality laboratory from commercial quarries of stones for construction industry was a source of granite, sandstones and basalt. Lime stones comes from stone-pit for Portland cement production and also was delivered by quality laboratory of cement producer. To visualize the distribution of algae growth around and on the surface of the stones, we used solarisation, (thermal colour mapping) with a graphic program (Corel Draw X8). The microorganisms used for the experiment were observed with a Nikon Eclipse 600 light microscope with Nomarski phase contrast. Micrographs were taken with a Nikon DS-Fi I camera.

## RESULTS

### Course of development of algae using two-phase media

In the second and third weeks, in all cases, we observed clear development of algal species around the rock fragments (gravel) used in the experiment (Table 1). The growth of algae in control samples (culturing only with Woods Hole fortified agar medium) was significantly slower than in biphasic media using Woods Hole and stones. The growth of algae was denser in two-phase medium, manifested by a clear increase in algal mass at the interface between the nutrient solution and rock (i.e., between phase 1 and phase 2). Algal development significantly differed only in media containing glass. The algae developed on the surface of the gel around the glass, but did not colonize the surface of the glass. In this case, the culture time probably needs to be extended, and a liquid phase should be used. Glass particles are not a good alternative to the other components of the two-phase substrate.

In the fourth week of culture, we noted a further increase in the growth of algae, especially around the rock fragments. The cultures showed complete stabilization, as seen in microscopy of the studied individual taxa.

Granite particles proved to be a very good component of two-phase media for all the algae studied (Fig. 2), but the *Klebsormidium dissectum* (F.Gay) H.Ettl & Gärtner strain developed particularly well in all types of two-phase media. The increase of *K. dissectum* mass was highest at the interface of the two-phase medium components (Fig. 3).

Microscopy plainly showed that in two-phase culture the morphology of the species was better preserved, in a state usually found only in the natural environment. *Klebsormidium* P.C.Silva, Mattox & W.H.Blackw. maintained on liquid or gel media alone showed altered morphology: most often, only small single cells that did not form thread-like filaments or else clumped into shapeless masses. The biphasic culture substrate provides conditions most similar to those in nature. In our experiment, we observed clear linear arrangements of *Klebsormidium* cells on the surface of the rock particles and then also on the surface of the gel phase. The linear arrangement of algal cells was maintained despite their high concentration at the interface between the rock and the gel substrate (Fig. 4).

Used as solid phase, basalt is poorly accessible not only to the fungi indicated below, but even to such expansive algae as *Klebsormidium dissectum*. Besides the rock types, we also tested pieces of glass. They were not inhabited by the algae and did not affect the development of algal communities on the surface of the gel substrate.

### Course of development of fungi using two-phase media

We tested the suitability of the two-phase media for culturing microorganisms other than algae, in particular fungi. Of the rock used as solid phase in the media, both limestone and basalt have uniform structure and lack distinct cracks; sandstone and granite are of diverse composition and have cracks in the areas of contact between their different minerals. The cracks create microniches for the growth of saxicolous fungi.

Fungi grew very slowly on the two-phase media applied. After one month, we noted the occurrence of nonsporulating fungi and bacteria on limestone (marl) fragments (Fig. 5). The bacterial biofilm surrounding those particles reached 3 mm thickness (at surface of medium). *Chaetomium globosum* Kunze appeared on granite after two months.

Granite in two-phase medium stimulated the growth of *Aspergillus tubingensis* R.Mosseray, *Aureobasidium pullulans* (de Bary) G.Arnaud, *Penicillium* sp. and *Chaetomium globosum* (Fig. 6). The fungi grew near and directly on the granite. *Aspergillus tubingensis* colonies developed only on the shaded side of the rock particles. Basalt formed a distinct inhibition halo surrounding the rock particles. For sandstone and limestone, such a halo was not formed, but fungal growth was weak, and for glass no influence on the growth of fungi was noted.

SNA medium with rock operates selectively, enabling the growth of lithobionts and decreasing the growth of fast-growing fungi that require rich media such as PDA.

### Development of lichens and mosses using two-phase media

Attempts to culture single species of lichens (e.g. *Physcia adscendens* (Th.Fr.) H.Olivier, *P. tenella* (Scop.) DC., *Xanthoria parietina* (L.), Beltr.) on our two-phase media were unsuccessful. Accidentally we found that mosses grew successfully instead of lichens cultures; their spores probably occurred in the samples, and also could have developed from introduced fragments of thalli. After two weeks, we observed green filaments on the contact surface between the rock and the gel substrate (Fig. 7).

### Halo formation around rock particles

The presence of solid macroscopic fragments in the gel medium clearly influenced the organisms' development and their distribution in the form of halos of various shapes. Presumably, this interaction resulted from the simultaneous operation of several physical and chemical phenomena.

Microorganisms cultured in gel medium form a colloidal system, so the nanometer-thick layer comprising the rock/gel interface should promote adhesion of the growing microorganisms to the surface of the fragments regardless of the chemical structure of the fragments. This process is manifested in the formation of bacterial and fungal biofilms. We assume that this interaction operates to some extent in unicellular algae suspended freely in liquid/gel medium and thus forming a colloidal system. Operating in the conditions described by the DLVO theory (named after Boris Derjaguin, Lev Landau, Evert Verwey and Theodoor Overbeek), in biofilm formation the first colonies of microorganisms adhere to the surface initially through weak reversible adhesion via van der Waals and electric double layer forces (Carniello *et al.* 2018). When microorganisms closely approach solid surfaces, at 2 nm to 50 nm distance, they are within the range of van der Waals, hydrophobic, ionic and electrostatic interaction forces. This

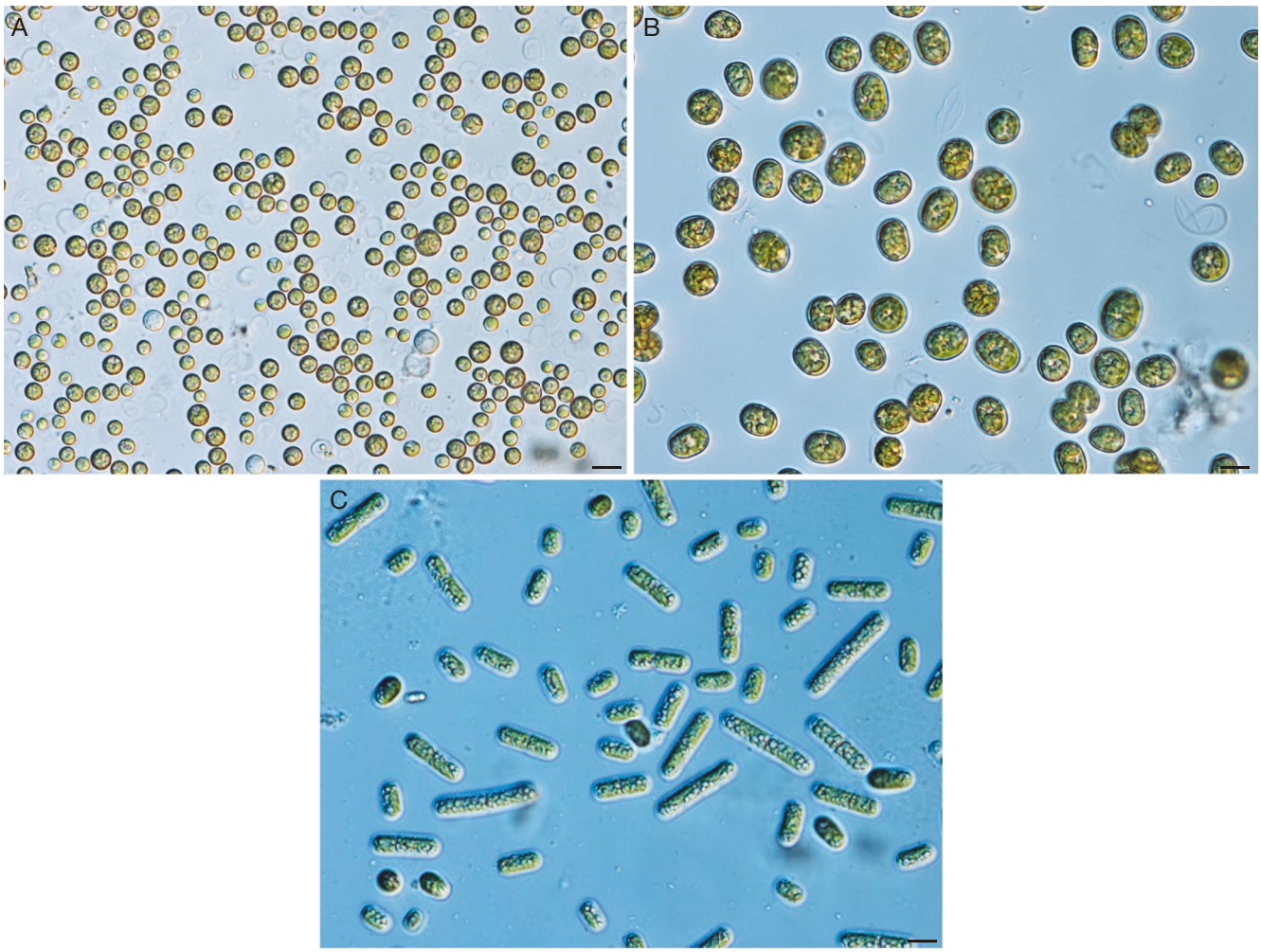


FIG. 2. — Algal species growing on two-phase media with granite: **A**, *Muriella decolor* Vischer; **B**, *Vischeria polyphem* (Pitschmann) Kryvenda, Rybalka, Wolf & Friedl; **C**, *Klebsormidium dissectum* (F.Gay) H.Ettl & Gärtner. Scale bars: 10 µm.

TABLE 1. — Course of development of algal strains on two-phase media during three weeks (I, II, III). The evaluation used an estimated organoleptic scale: **0**, none; **1**, very poor; **2**, poor; **3**, good; **4**, very good.

| Taxon | Granite | | | Basalt | | | Limestone | | | Sandstone | | | Glass | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | I | II | III | I | II | III | I | II | III | I | II | III | I | II | III |
| *Klebsormidium dissectum* (terrestrial semiaerophyte) | 2 | 3 | 4 | 0 | 2 | 3 | 1 | 3 | 3 | 1 | 2 | 3 | 0 | 1 | 2 |
| *Vischeria polyphem* (terrestrial semiaerophyte) | 0 | 2 | 4 | 0 | 1 | 2 | 1 | 2 | 3 | 0 | 1 | 2 | | – | |
| *Muriella decolor* (freshwater) | 1 | 2 | 3 | 0 | 0 | 2 | 0 | 1 | 2 | 0 | 2 | 2 | | – | |

mechanism must be responsible for the interaction with the rock and with the chemically inert glass immersed in a colloidal suspension with the microorganisms.

In the case of rock particles, an additional phenomenon should be considered. All the rock materials used are subject to faster or slower dissolution in a water environment, accompanied by diffusion of mineral compounds into the liquid medium. Due to the ionic nature of these compounds, the pH of the liquid/gel phase changes. In the case of acidic rocks containing more than 65 % silica, such as granite, the medium pH will decrease, and in the case of basic rocks, such as limestone, the pH will increase. At the same time, the addition of minerals beneficial to a given species stimulates the growth of cultured microorganisms in the immediate vicinity of the rock particles. The abundance of substances added to the culture medium from the rock material is illustrated by the average composition of granite, based on analyses of 2485 samples (Blatt & Tracy 1996): SiO2 72.04 wt.%, Al2O3 14.42 wt.%, K2O 4.12 wt.%, Na2O 3.69 wt.%, CaO 1.82 wt.%, FeO 1.68 wt.%, Fe2O3 1.22 wt.%, MgO 0.71 wt.%, TiO2 0.30 wt.%, P2O5 0.12 wt.% and MnO 0.05 wt.%.

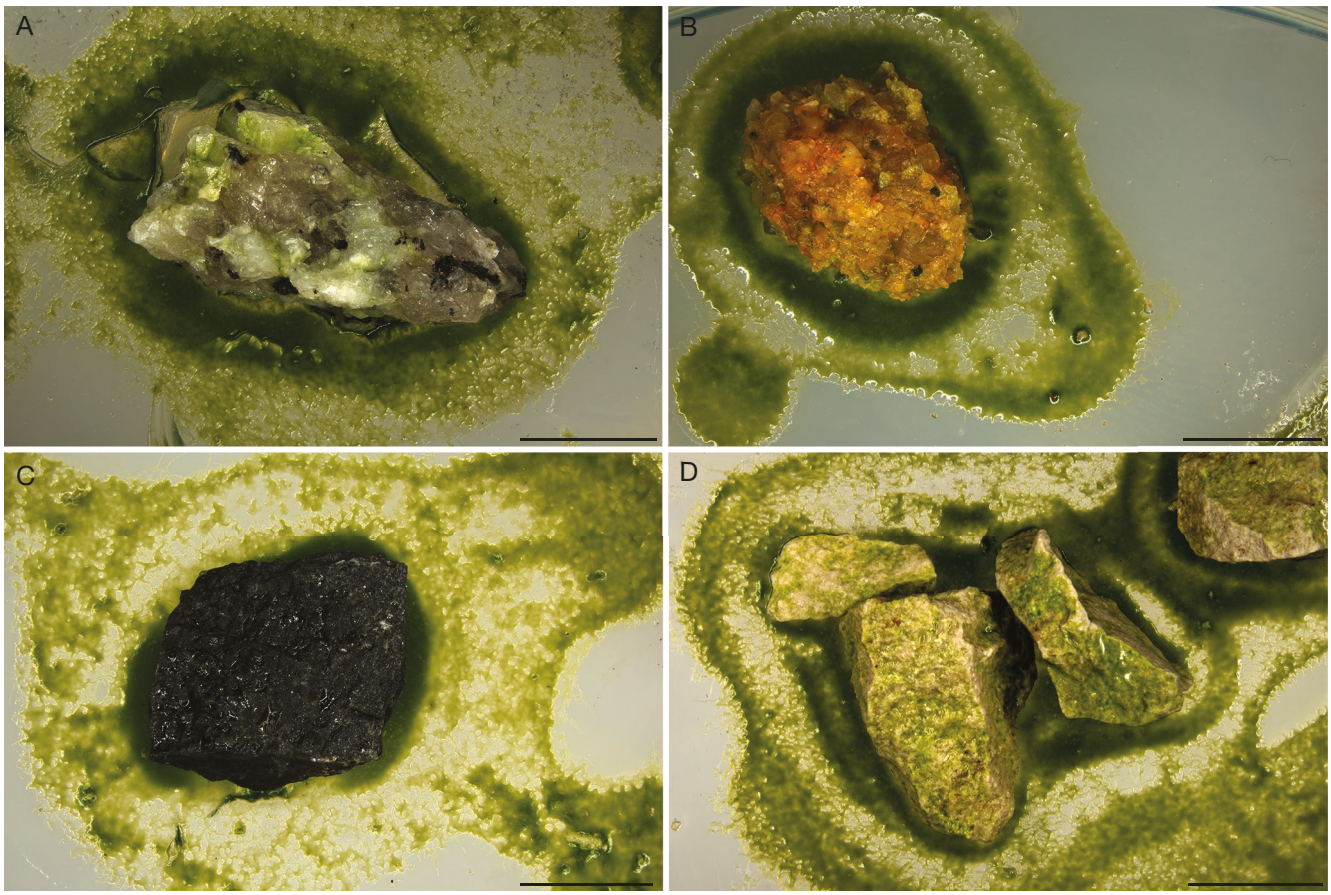


Fig. 3. — Development of *Klebsormidium dissectum* (F.Gay) H.Ettl & Gärtner after 28 days of culture on two-phase media containing rock: **A**, granite; **B**, sandstone; **C**, basalt; **D**, limestone. Scale bars: 5 mm.

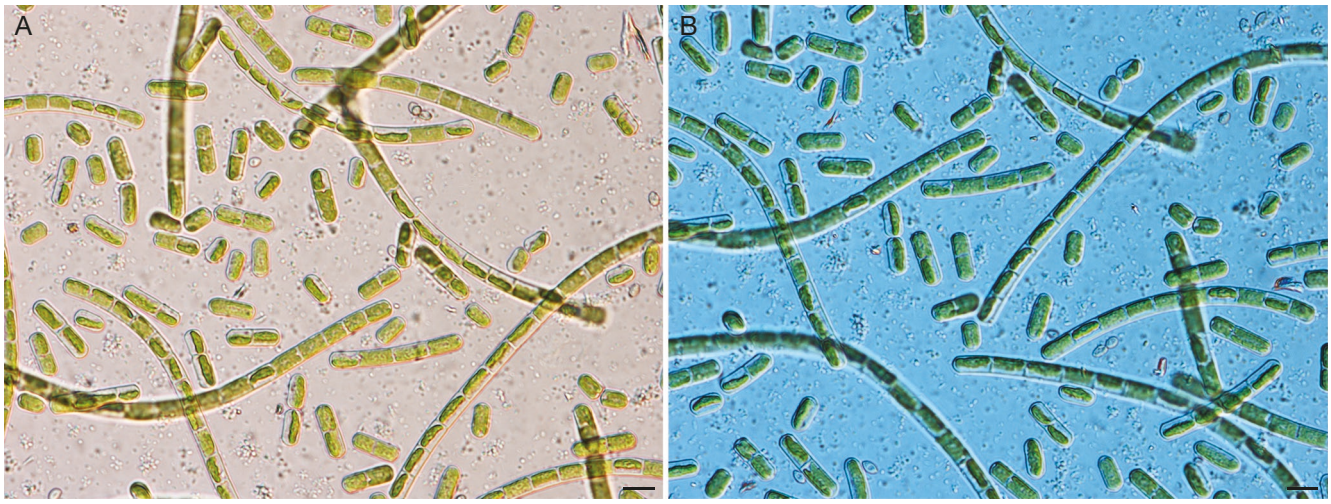


Fig. 4. — *Klebsormidium dissectum* (F.Gay) H.Ettl & Gärtner. Thread-like (filamentous) clusters and single cells developed in two-phase media: **A**, containing limestone; **B**, containing granite. Scale bars: 10 µm.

As solids are only partially immersed in the gel medium, we have to consider other phenomena related to the absorption of individual rock fragments and the capillary transport of moisture to the surfaces protruding above the liquid/gel phase. Accelerated evaporation of water from the surface of the rocks leads to changes in the concentration of nutrients in the immediate vicinity of the particles. Average water absorption is as follows: granite 0.47 wt.%, basalt 0.52 wt.%, sandstone 3.41 wt.% and limestone 3.87 wt.% (Bromowicz & Figarska-Warchoł 2010). To document this, we performed thermal imaging of

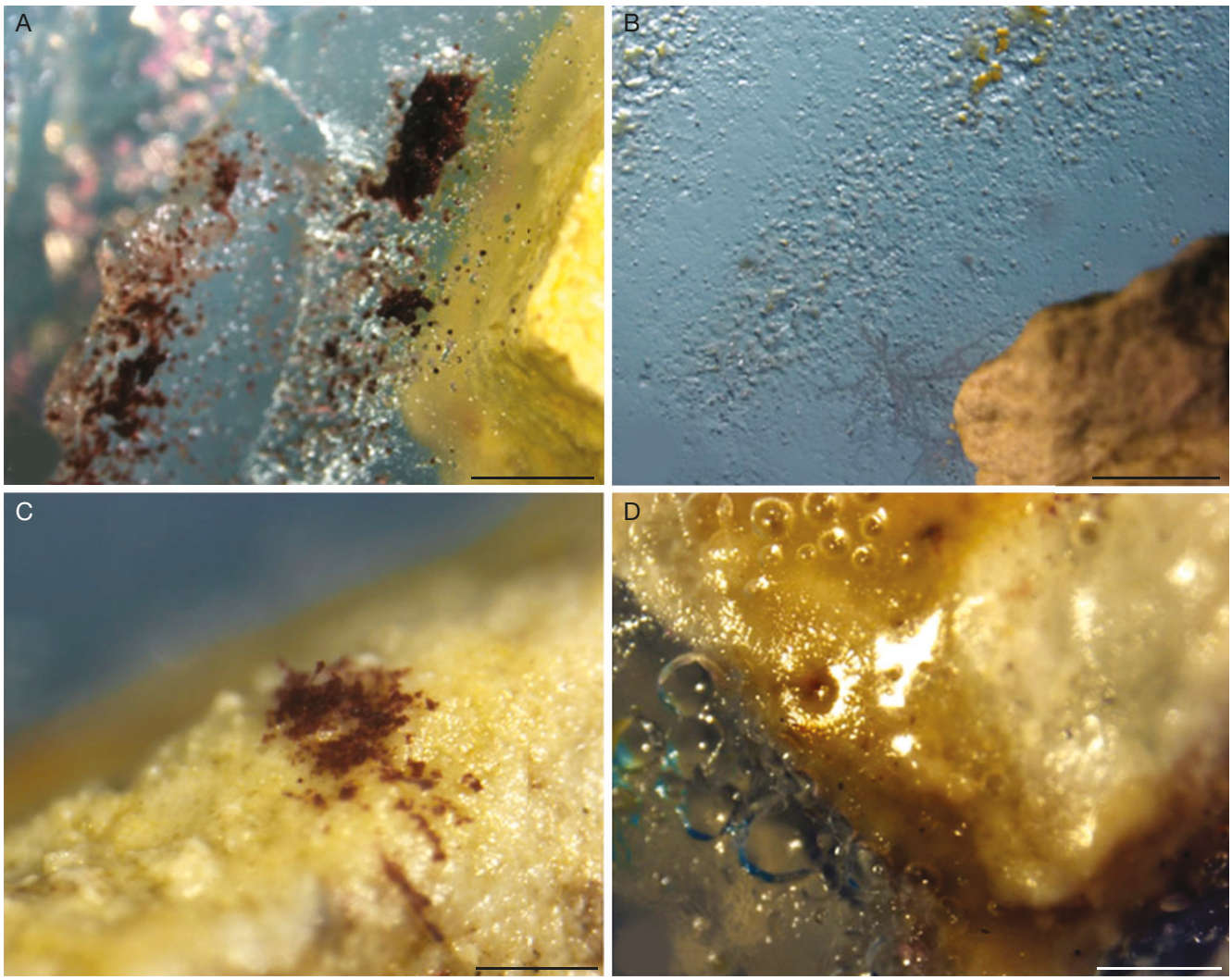


Fig. 5. — Microorganism development on two-phase medium containing limestone (marl): **A**, *Aspergillus tubingensis* R.Mosseray formed on surface of gel phase; **B**, *A. tubingensis* on limestone (solid phase); **C**, **D**, bacterial biofilms formed on surface of medium near limestone. Scale bars: 500 µm.

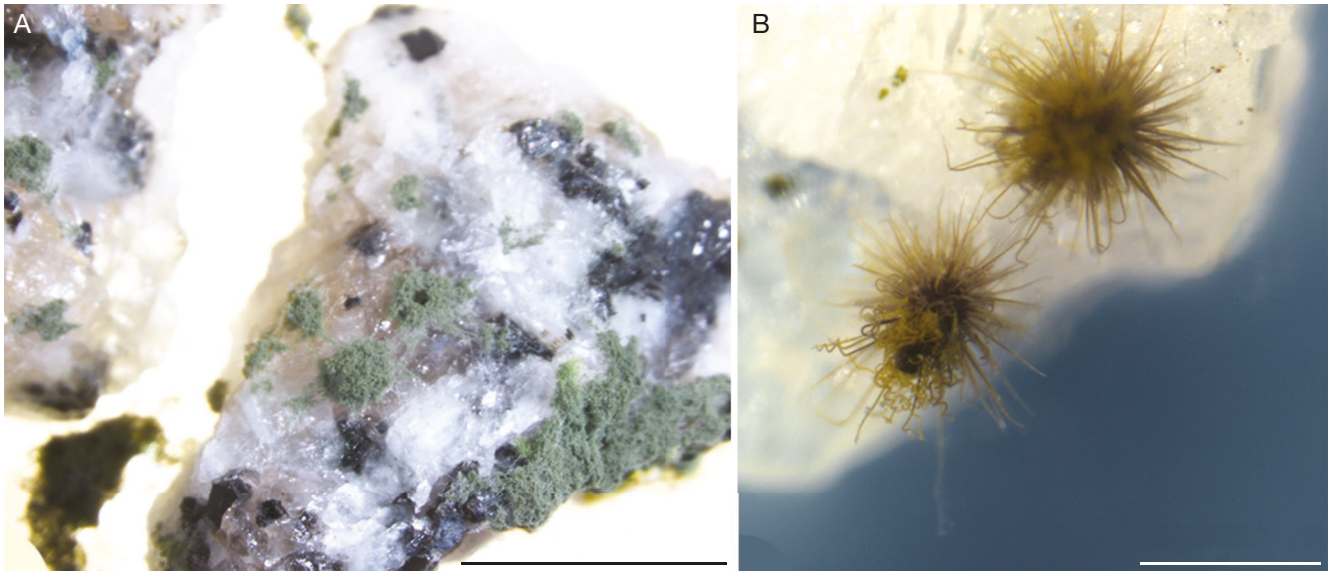


Fig. 6. — Fungal development on two-phase medium containing granite: **A**, *Penicillium* sp.; **B**, *Chaetomium globusom* Kunze. Scale bars: A, 1 cm; B, 200 µm.

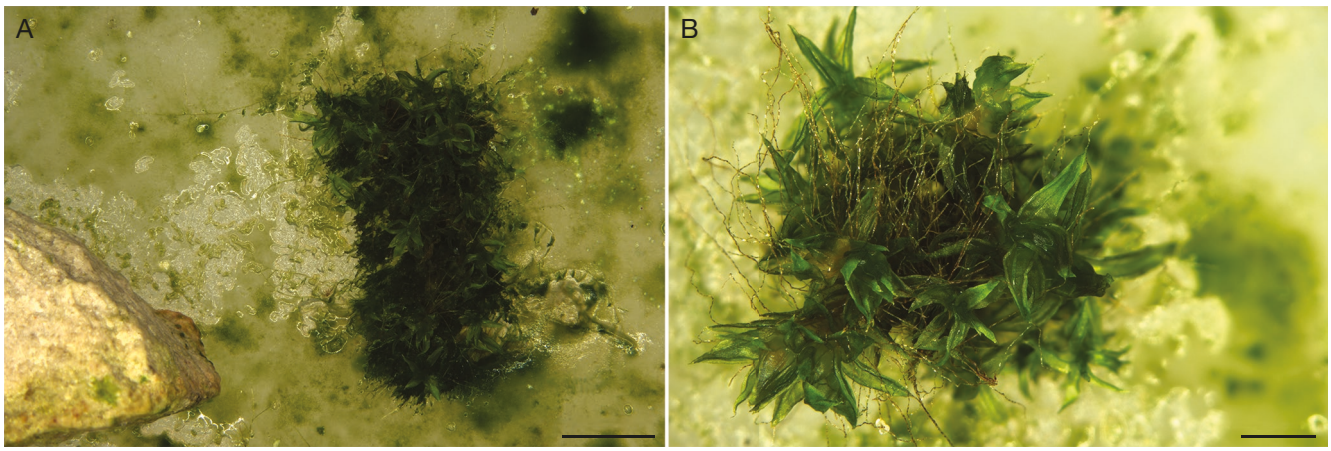


Fig. 7. — Mosses developed on two-phase media within: **A**, 2 weeks; **B**, 4 weeks. Scale bars: A, 2 mm; B, 1000 µm.

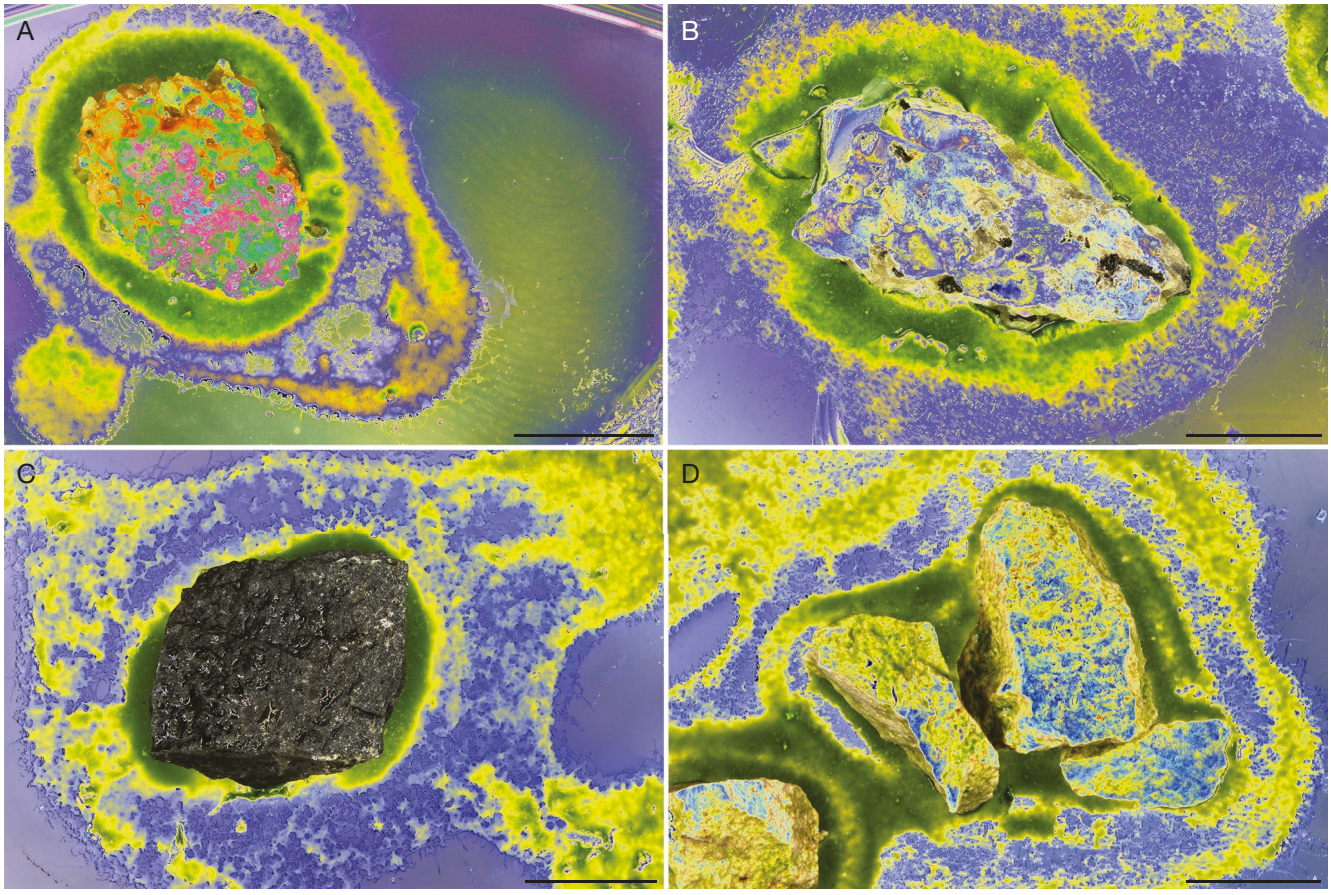


Fig. 8. — Halos formed around rock fragments in two-phase media: **A**, medium with granite; **B**, medium with sandstone; **C**, medium with basalt; **D**, medium with limestone (thermal mapping images made with CorelDraw). Scale bars: 5 mm.

the rock particle surfaces and the substrate in which they were embedded. Thermal mapping (Fig. 8) visualized the shape and distribution of the halo formed around the solid elements of the medium, and indicated that the development of *Klebsormidium dissectum* (Fig. 8) was best within the boundaries of granite and sandstone, whose components diffused to the entire substrate. The range of the halo in the medium with basalt particles was limited to the immediate vicinity of the rock (visible only in the adjacent zone). The halo and diffusion phenomena in limestone and sandstone were similar; interestingly, the surface of sandstone particles was colonized by *K. dissectum*, *Penicillium* sp. and *Aspergillus tubingensis*.

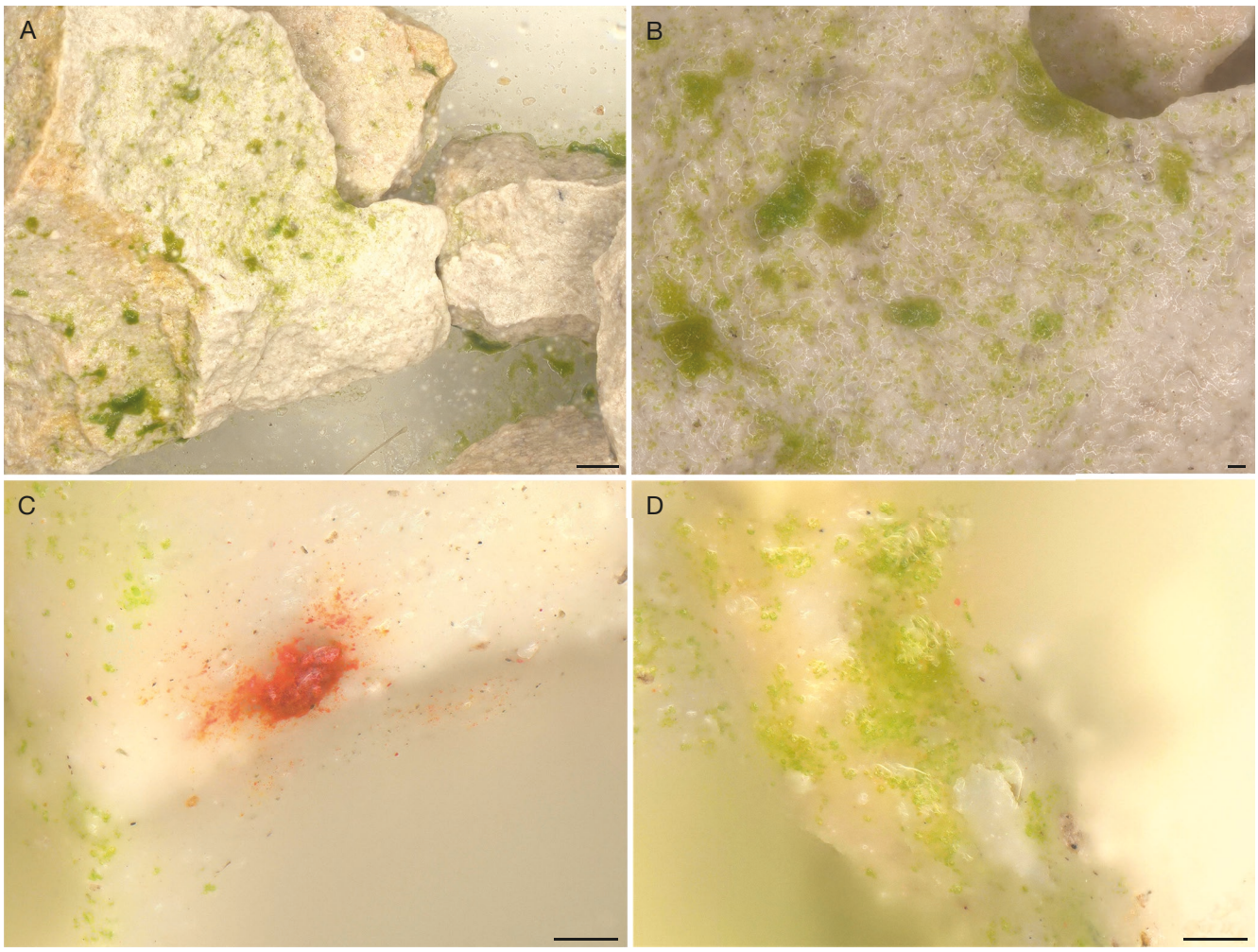


Fig. 9. — Colonization of rock fragments in two-phase medium: **A**, *Klebsormidium dissectum* (F.Gay) H.Ettl & Gärtner with sandstone; **B**, with granite; **C**, *Trentepohlia umbrina* (Kütz.) Bornet with limeston; **D**, *Vischeria polyphem* (Pitschmann) Kryvenda, Rybalka, Wolf & Friedl with limesone. Scale bars: A, 1000 µm; B-D, 100 µm.

## DISCUSSION AND CONCLUSIONS

Our results are in line with findings from Duffer & Doris (1966), who noted that algae growing in the natural environment developed better on granite surfaces than on limestone and sandstone. Kawecka (1980), however, reported finding almost no differences in the structure of algae communities inhabiting high-mountain stream beds built of limestone and granite rock. Blinn *et al*. (1980) showed experimentally that average algal biomass was almost two times higher on sandstone than on basalt or limestone surfaces.

The ecology of microorganisms depends on such factors as whether the substrate is dead or alive, its surface structure and degree of stability, the chemical composition of sediments, the metabolites secreted by organisms, the geological properties of the rock, etc. The type of substrate is an important factor determining the species composition, distribution and structure of communities of algae (Hynes 1972; Whitton 1975; Round 1981; Kawecka & Eloranta 1994) and fungi, especially lithobionts (Staley *et al*. 1981; Sterflinger & Krumbein 1997; Sterflinger 2000).

As we used pieces of various rocks as the second phase of our culture media, our results can be related to findings from algal and fungal communities inhabiting rocks in natural conditions, where they are found in aquatic and terrestrial habitats and also in the margins between those environments. By analogy, they can be said to inhabit sites similar to the interface between the liquid and solid phases of our medium. In two-phase media, the colonization processes and the course of development of microorganisms (e.g. algae) on rock microhabitats can be observed easily (Fig. 9).

Our observations show that the two-phase media we developed are suitable for research on the formation of biological mats by bacteria, fungi and algae (also mosses and lichens) via colonization of hard surfaces with limited water availability. This experimental model can help researchers respond to a hypothesis discussed in the last fifteen years, that light and water were necessary to create life on earth but that they must have occurred in very low amounts – because an excess of light and water can lead to decomposition of DNA and other key molecules (Marshall 2020).

During about twelve months of experiments and observations, we have found that with proper selection of the components for two-phase media the following can be accomplished:

– good culture conditions can be maintained, with microelement flow from the solid phase to the primary (gel) medium;

– specific microhabitats can be created to sustain particular species in conditions similar to natural ones;

– researchers can observe the development of a given species on two different substrates in parallel;

– research on the autecology of species in laboratory conditions can be greatly facilitated;

– experimental research done in parallel on pure cultures of microorganisms can be accelerated and expanded.

It should be emphasized that a properly selected second (solid) phase, that is, one that matches the natural habitat preferences of the studied organisms, may serve as a selection barrier that will prevent the development of organisms other than those being cultured for study.

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