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Nivicolous myxomycetes in agar culture: some results and open problems

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Summary

A method to cultivate nivicolous species of myxomycetes in agar is proposed. Sixty three specimens of nivicolous myxomycetes representing 10 taxa (9 species and 1 variety) from 5 genera and 3 families were tested for ability to germinate in agar plates at room temperature. Germination occurred in 28 specimens of 6 species. In some samples microplasmodia started to appear at the same conditions, but in most samples further developmental stages were observed only when cultures were kept at +2°C. Two species (*Lepidoderma chailletii* and *Physarum nivale*) showed plasmodia formation, but we did not manage to cause sporulation. Partial sequences of the SSU gene obtained from cultured amoebae of two *L. chailletii* and one *P. nivale* specimens were identical to those obtained from their spores and comparison with GenBank sequences proved species identity. Some data about tolerance of *L. chailletii* amoebae to low temperatures were obtained that could be helpful in understanding the ecology of nivicolous myxomycetes.

Key words: Amoebozoa, agar culture, germination, Myxogastria, nivicolous myxomycetes, temperature tolerance

Introduction

The plasmodial slime molds are a monophyletic group of terrestrial amoeboid protists that produce aerial spore-bearing structures (sporocarps). These fructifications were found in most terrestrial ecosystems (Stephenson et al., 2008), and trophic stages appear to be abundant in soil (Urich et al., 2008). In ecological terms they are phagotrophic bacterivores, helping to maintain the balance that exists in nature between bacterial and fungal decay (Stephenson et al., 2011). Approximately 940 species of myxomycetes have been described based on the currently applied morphological species concept (Lado, 2005–2014), which are placed in five taxonomic orders plus genus *Ceratiomyxa* as a sister group.

The nivicolous myxomycetes constitute a distinct ecological group of myxomycetes that form fruiting bodies at the edge of melting snowbanks in spring. Most nivicolous taxa are members of the orders Stemonitales and Physarales, forming

a monophyletic clade characterized by melanine-containing (dark) spores (Fiore-Donno et al., 2012). The group includes about 100 taxa that are found in habitats in subalpine and alpine landscapes, in boreal lowland forests and in forest belt of low-altitude mountains (Ronikier and Ronikier, 2009; Novozhilov et al., 2013). As one can conclude from the steady association of their fructifications with snow banks, the life cycle of nivicolous myxomycetes requires specific microclimate conditions with stable temperatures around 0 °C, as they occur under a thick snow cover. In literature several reports on moist chamber cultures of nivicolous myxomycetes are available (Marx, 1998; Dulger et al., 2007; Ronikier et al., 2010), but we could find only two mentions about cultivation of nivicolous species in agar culture: from spore to spore (Kowalski, 1971) and from spores to myxamoebae (Wikmark et al., 2007), but details of these experiments were not published.

This paper represents the first published study of nivicolous myxomycetes in agar culture. The objectives were: 1) to ascertain the ability of nivicolous myxomycetes to germinate from spores under laboratory conditions as found for most other ecological groups of myxomycetes (Haskins and Wrigley de Basanta, 2008); 2) to attempt a cultivation of some nivicolous species from spore to spore; 3) to gather data on the ability of their trophic stages to tolerate low temperatures.

Material and methods

Specimens

Sixty three specimens of sporocarps of nivicolous myxomycetes representing 10 taxa (9 species and 1 variety) from 5 genera and 3 families were used in this study (see list of specimens below). Determination of the specimens was made by the collectors as described in (Novozhilov et al., 2013). Nomenclature follows Lado (2005–2014), except for cases were Poulain et al. (2011) applied a more differentiated morphospecies concept; such cases are mentioned in the annotated species list of nivicolous myxomycetes in the northwestern Caucasus (Novozhilov et al., 2013). Voucher specimens are deposited in the mycological herbarium of the Komarov Botanical Institute, Laboratory of Systematics and Geography of Fungi (LE) and in the private herbarium of the third author (sc) deposited at the Botanical State Collection Munich. The study was conducted with specimens collected in 2011–2013, for this reason the age of sporocarps used for agar cultures ranged between 6 and 36 months.

List of 63 specimens used to set up agar cultures. Abbreviations: TR – Russia, Karachaevo-Cherkesian Republic, Northern Caucasus, Teberda State Biosphere Reserve; LEN – Russia, Leningrad oblast, Karelian isthmus, Vaskelovo settlement; KH – Russia, Murmansk oblast, Kola Peninsula, Kirovsk, Polar-Alpine Botanical Garden-Institute; GAP – Germany, Bavaria, Wettersteingebirge, Garmisch-Partenkirchen.

Diderma niveum (Rostaf.) T. Macbr. – TR, leg. Yu. Novozhilov, M. Schnittler, LE 285748. LEN, leg. Yu. Novozhilov et al, LE: 285958, 285959.

Diderma peyerimhoffii (Maire and Pinoy) H. Neubert, Nowotny and K. Baumann (= *Lepidoderma peyerimhoffii* Maire and Pinoy) – TR, leg. Yu. Novozhilov, M. Schnittler, D. Erastova, LE: 285135, 285215, 285854.

Lamproderma ovoideum Meyl. – TR, leg. Yu. Novozhilov, M. Schnittler, LE: 285772, 285828, 285871, 285879.

Lamproderma spinulosporum Mar. Mey., Nowotny and Poulain. – TR, leg. Yu. Novozhilov, M. Schnittler, D. Erastova, LE: 285932, 285933. LEN, leg. Yu. Novozhilov et al., LE 285953.

Lepidoderma carestianum (Rabenh.) Rostaf. – TR, leg. Yu. Novozhilov, M. Schnittler, D. Erastova, LE: 285229, 285869, 285870.

Lepidoderma chailletii Rostaf. – French Alps, leg. M. Meyer, LE 284732. TR, leg. Yu. Novozhilov, M. Schnittler, D. Erastova, E. Heinrich, LE: 285143, 285156, 285716, 296701, 296753, 296754. LEN, leg. Yu. Novozhilov et al., LE: 285952, 285955, 285957. KH, leg. Yu. Novozhilov, D. Erastova, LE: 289759, 289760, 289762, 289763, 289764, 289765, 289766, 289767, 289768, 289795, 297153, 297213, 297215. GAP, leg. M. Schnittler, Yu. Novozhilov et al., LE: 296529, 296574, sc 26422.

Meriderma carestiae (Ces. and De Not.) Mar. Mey. and Poulain – TR, leg. Yu. Novozhilov, M. Schnittler, LE: 285695, 285922. LEN, leg. Yu. Novozhilov et al., LE 285964.

Meriderma carestiae* var. *retisporum ad int. (Poulain et al., 2011) – LEN, leg. Yu. Novozhilov et al., LE 285947.

Physarum albescens Ellis ex T. Macbr. – KH, leg. Yu. Novozhilov, D. Erastova, LE: 297158, 297050, 297118, 297145, 297147, 297151, 297165, 297168, 297178, 297180, 297194.

Physarum nivale (Meyl.) Mar. Mey. and Poulain – TR, leg. Yu. Novozhilov, M. Schnittler, D. Erastova, LE: 285190, 285194, 285195, 285196, 285265, 285267.

Agar cultures

Spores from mature sporocarps taken from field collections were sown on 1.5% water agar at pH 7.0. Sporocarps were crushed and spores released over the agar surface in 60 mm diameter sterile plastic Petri dishes using alcohol flamed forceps; 1 ml of sterile distilled water was added to each plate. Parafilm was used to seal the dishes. Cultures were kept at room temperature (20–23 °C) in diffused light. After spore germination cultures were transferred to 5% water agar, then to carrot agar (Indira, 1969) or to SM agar (1.5 % agar on SM broth, Sussman, 1966). Bacteria sown accidentally together with spores were used as a food source. Crushed autoclave-sterilized oat flakes were used as an additional food for plasmodia (Haskins and Wrigley de Basanta, 2008). Some cultures were kept over different periods in a refrigerator at +2 °C in the dark.

Microscopy

All microscopic observations were made with a Zeiss Axio Imager A1 light microscope equipped with AxioCam MRc5 digital camera, UV-lamp mbq 52ac and filter for DAPI. Agar plates were inverted on the stage of the microscope and cultures observed through the bottom with a 4× or 10× objective and a 10× ocular.

Photomicrographs of amoebae and microplasmodia were obtained using total preparations with DAPI-stained ethanol-fixed cells, fluorescent microscopy and light microscopy with differential interference contrast (DIC).

Temperature tolerance test

Three isolates of *Lepidoderma chailletii* were used to check the tolerance of myxamoebae to low temperatures. Six agar cultures (SM agar) of each isolate at exponential growth phase initially kept at +2 °C were used in a series of experiments:

1. One plate was kept at +2 °C in a refrigerator for 7 days.

2. One plate was kept in a centrifuge K24 (Janetzki, GDR) without a rotor with the temperature set up at 0 °C for 3 d.

3. One plate was kept at 0 °C for 24 hrs, then it was slowly refrigerated to –5 °C in 6 hrs in the centrifuge K24 (Janetzki, GDR) without a rotor. All amoebae encysted to that moment, and the plate was transferred to a freezer at –11 °C for 4 d. Afterwards the culture was kept 4 d at +2 °C.

4. One plate was treated like in experiment 3, except that the temperature in the freezer was –18 °C.

5. One plate was quickly frozen by transferring directly from +2 °C to –11 °C for 20 hrs and then back to +2 °C for 4 d.

6. One plate was kept at room temperature (22–24 °C) for 7 d.

In all experiments cultures were kept in the dark. After treatments cultures were observed under the light microscope with 10X objective, for each plate the amount and percentage of active, encysted and lysed amoebae were counted in three microscopic fields each showing from 50 to 150 amoebae or their remnants.

DNA extraction from spores

Procedure of DNA extraction from spores was performed according to Novozhilov et al. (2013). Three or four adjacent sporocarps of a specimen from field collection were carefully transferred to a 2 ml safe-lock Eppendorf tube, refrigerated to -80 °C with a sterilized metallic bead (5 mm diameter) and then disrupted using a ball mill (Retsch MM301, 1 min, 30 Hz). DNA was extracted using the DNeasy plant mini-kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

DNA extraction from amoebae

A small agar block (approximately 3x3 mm) bearing a dense group of amoebae was cut out from a culture plate with sterile scalpel and transferred to a 2 ml safe-lock Eppendorf tube. 25 µl of 1% Brij 58 aqueous solution was added, the tube was vortexed and left at 65 °C for 30 min. This solution was used directly for PCR without DNA purification.

DNA amplification and sequencing

The first part of the SSU rRNA gene (552 bp) was amplified using forward primer S1 (aacctggttgatcctgcc, Fiore-Donno, Meyer et al., 2008) and reverse primer SU19R (tgtcctctaattgttactcga). PCR was performed with a total volume of 25 µl in 40 cycles (30 s at 95 °C, 30 s at 52 °C, 1 min at 72 °C). Sequencing was carried out at an ABI 3130 sequencer in the Institute of Botany and Landscape Ecology, Ernst-Moritz-Arndt University in Greifswald (Germany). Obtained sequences were manually checked and automatically aligned using BioEdit 7.0.9.0. All new sequences reported in this paper have been deposited in the GenBank/EMBL database under accession numbers KJ676863 and KJ676864.

Results

Cultures

In plates with spores of *Lamproderma spinulosporum, Lepidoderma carestianum, Meriderma carestiae* and *M. carestiae* var. *retisporum* ad int. germination did not occur (see Table 1 for specimen information). In one of three plates with *Diderma peyerimhoffii* and one of three plates with *Diderma niveum* kept at room temperature a few amoebae and microcysts were found 5 and 11 days, respectively, after spores were placed on agar (Fig. 1, A). There was not a considerable increase of amoebal density within the following days, and approximately two weeks after germination all amoebae were encysted. Further placement of cultures at +2 °C did not cause any changes.

In three of four plates sown with *Lamproderma ovoideum* spores after 6–9 d microcysts and a small amount of amoebae were found (Fig. 1, B). The number of amoebae did not grow, and after 10 d all of them were found encysted. After 5 d at +2 °C no cysts could be found (apparently they decayed).

In 16 of 26 plates sown with spores of *Lepidoderma chailletii* Rostaf. germination occurred within 3–4 d at room temperature. Populations of amoebae and flagellate cells grew rapidly in all cultures. In two isolates microplasmodia about 20–50 µm long appeared within 5–6 d, for most other isolates this took 1–6 weeks. Microplasmodia were identified as being at least twice bigger and usually more vacuolated than other amoebae. Observations of DAPI-stained cells with fluorescent microscopy confirmed appropriateness of these criterions (Fig. 1, C, D). In five cultures microplasmodia were never observed. In about two weeks after germination, amoebae and microplasmodia in most plates started to encyst although cultures were transferred to fresh media (carrot agar). If plates were placed in a refrigerator at +2 °C, in 2–3 d excystment occurred and amount of amoebae and microplasmodia increased. Subsequently, in 10 isolates within different periods ranging from 1 week to 4 months small rounded plasmodia of 50–100 µm diameter started to appear. They were densely surrounded by amoebae and were feeding on them; ingested amoebae in food vacuoles could be seen (Fig. 1, E, F). Plasmodia showed slow irregular streaming of cytoplasm without migration over the agar surface. They grew quickly, changed to an elongated shape and started to migrate; within the next two days some plasmodia became reticulate, 19 d later some of them were 900 µm long (Fig. 1, G, H). Plasmodia refused to consume oat flakes, but were probably feeding on agar forming deep hollows in agar surfaces (Fig. 1, I). Cultures were maintained for about six months at +2 °C with periodical transfers to fresh medium, in some isolates white plasmodia up to 3 cm length developed, but no fruiting bodies appeared. The presence of a thin layer of water was necessary for plasmodia, otherwise they sclerotized. Plasmodia in plates that were transferred to room temperature sclerotized in 1–2 d, but amoebae continued to live about a week without encysting. Fast short-term temperature changes during microscopic observations (from +2 °C to about +20 °C in 30 min and back to +2 °C) showed no visible effect on amoebae and plasmodia.

Table 1. Life cycle stages obtained for each species. Numbers indicate the percentage of cultures where the respective life stage was observed and the days after the start of the culture, when this stage was observed for the first time.

Species	Ger	Mic	Pls
Diderma niveum (n=3)	33%/11d	0%/–	0%/–
Diderma peyerimhoffii (n=3)	33%/5d	0%/–	0%/–
Lamproderma ovoideum (n=4)	75%/8d	0%/–	0%/–
Lamproderma spinulisporum (n=3)	0%/–	0%/–	0%/–
Lepidoderma carestianum (n=3)	0%/–	0%/–	0%/–
Lepidoderma chailletii (n=26)	61%/4d	46%/22d	38%/41d
Meriderma carestiae (n=3)	0%/–	0%/–	0%/–
Meriderma carestiae var. *retisporum (n=1)*	0%/–	0%/–	0%/–
Physarum albescens (n=11)	45%/7d	0%/–	0%/–
Physarum nivale (n=6)	33%/6d	16%/8d	16%/75d

Abbreviations: Ger – spore germination (+22–24 °C), Mic – microplasmodium (+2 °C), Pls – plasmodium (+2 °C).

In addition, an attempt was made to germinate spores of three *L. chailletii* specimens (LE 296753, LE 296754, LE 297153) at +2 °C on 1.5% water agar. Germination occured in all specimens, but only after 9–10 d (instead of 4 d at RT).

Spores of *Physarum albescens* germinated in 5 of 11 plates kept at room temperature after 5–11 d. Cultures were placed at +2 °C right after germination and were maintained for only one month, and during this period, the amount of amoebae and flagellated cells grew very slowly in all isolates except LE 297151. A small proportion of amoebae encysted, but no other developmental stages were seen.

In plates with *Physarum nivale* germination occurred in 2 of 6 isolates (Fig. 1, J). In one of them, all amoebae encysted immediately after

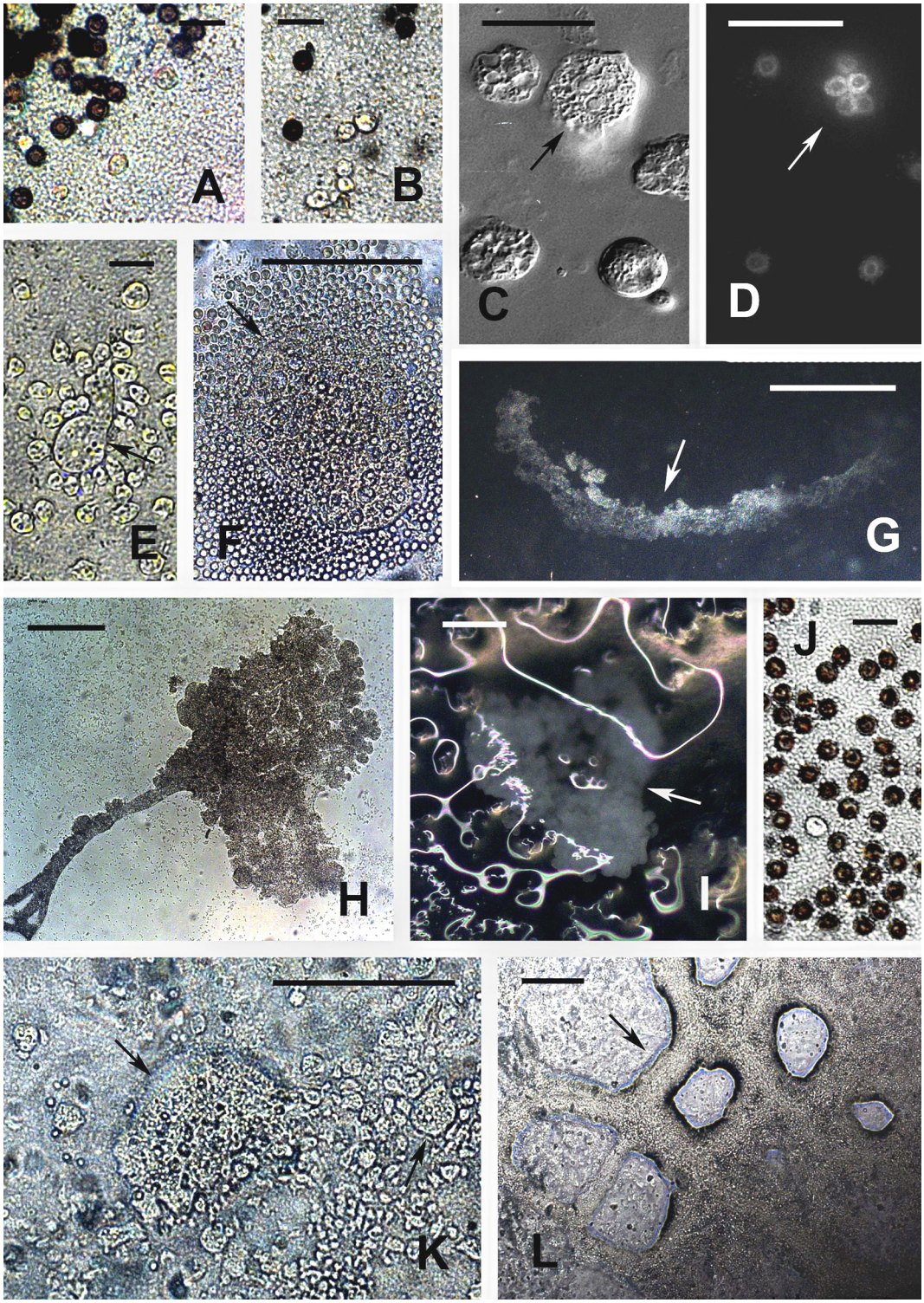

Fig. 1. Photomicrographs of different trophic stages of nivicolous myxomycetes in agar culture. A, B – myxamoebae hatched from spores of *Diderma niveum* and *Lamproderma ovoideum*, respectively; C, D – DAPI-stained amoebae and microplasmodia of *Lepidoderma chailletii* (DIC and fluorescent microscopy, respectively); E, F, G, H – different stages of *L. chailletii* plasmodium development; I – plasmodium of *L. chailletii* inside the pit that it has formed in the agar surface; J – myxamoebae of *Physarum nivale* among spores; K, L – young plasmodia and veins of big plasmodium of *P. nivale*, respectively. *Arrows* indicate plasmodia at different stages of development. Scale bars: A, B, E, J – 25 μm; C, D – 20 μm; F, H, I, K, L – 200 μm; G – 1000 μm.

germination, transfer to carrot agar and addition of water did not provoke them to excyst. In the other isolate, considerable numbers of amoebae hatched but most encysted almost immediately. The number of amoebae grew rapidly and three days after germination, microplasmodia started to appear (Fig. 1, K). A week later this culture was placed at +2 °C and excystment of all encysted amoebae occurred. After 2 months of storage, hyaline plasmodia, approximately 200–2000 μm in diameter were found actively moving over the agar surface, some of them were reticulate (Fig. 1, L). Plasmodia ingested amoebae and bacteria; after addition of sterilized oat flakes size of plasmodia increased quickly, they became white and some of them reached 3 cm in diameter. Cultures with plasmodia were maintained for 8 months with periodical transfer to the fresh media and addition of oat flakes. Like in *L. chailletii*, plasmodia sclerotized in absence of the thin water layer. Fruiting bodies were not obtained. At room temperature plasmodia sclerotized in 1–2 d and returned to their active form at +2 °C.

Temperature tolerance

Results of temperature experiments are presented in Table 2 and in a graphic form in Fig. 2. Amoebae grow best and stay active at 2 °C; some of them start to encyst near 0 °C and at +22–24 °C. Apparently fast freezing to –11 °C kills all amoebae and we could not find any of them alive. On the contrary, some amount of amoebae stay alive after frost events if they have enough time to encyst.

Sequences

Since the morphology of vegetative stages does not permit to identify isolates to species level we used molecular markers to ascertain the identity of the cultures. Two *L. chailletii* specimens (LE 285955, LE 285716) and one *P. nivale* specimen (LE 285196) were used to obtain partial SSU rRNA gene sequences from amoebae in cultures and from spores used to set up these cultures. In all specimens sequences from spores and from amoebae were identical. The sequence of *P. nivale* LE 285196 differs in a single nucleotide from the sole *P. nivale* partial SSU sequence in GenBank (DQ903680). The sequence of *L. chailletii* LE 285716 is identical to both *L. chailletii* sequences available in GenBank (JQ812617 and JQ900774) and differs again in one nucleotide from LE 285955. Therefore, sequencing data confirm the species identity of all three cultured specimens.

Table 2. Proportions (%) of active (a), encysted (c) and lysed (l) amoebae of *L. chailletii* after treatment with different temperature regimes (Temp., °C), followed by a growth period at 2 °C for four days (+22–24 °C – without growth period).

Temp.,°C	Amoebal stage	LE 296753	LE 296754	LE 297153
+22–24	a	73	66	90
	c	27	34	10
	l	0	0	0
+2	a	100	100	100
	c	0	0	0
	l	0	0	0
0	a	34	95	64
	c	66	5	36
	l	0	0	0
–11	a	0	31	0
	c	70	22	10
	l	30	47	90
–18	a	0	0	41
	c	60	45	30
	l	40	55	29
–11 (fast freezing)	a	0	0	0
	c	5	5	0
	l	95	95	100

Discussion

The present study demonstrates that at least some species of nivicolous myxomycetes can germinate and form plasmodia in culture. A significant proportion of isolates from *Diderma niveum, D. peyerimhoffii, Lepidoderma chailletii, Physarum albescens* and *P. nivale* (all Physarales) germinated at laboratory conditions standard for most non-nivicolous myxomycetes. Some germination was recorded for *Lamproderma ovoideum*, but all other tested members of the Stemonitales (*Lamproderma spinulosporum, L. carestianum, Meriderma carestiae* and *M. carestiae* var. *retisporum*) failed to germinate. Apparently, these species need more specific conditions for spore germination. Besides this, we had two hypotheses concerning specimens that did not germinate: either spores lost viability because of long dry storage, or the collected fruiting bodies were not sufficiently mature.

It is known that spore longevity in myxomycetes varies greatly from species to species (from several months to more than half a century, as shown by de Bary, 1864; Macbride and Martin, 1934; Elliott, 1949; Erbisch, 1964), and also depends on the conditions of specimen development and storage (Gilbert, 1929; Gilbert, 1935). However,

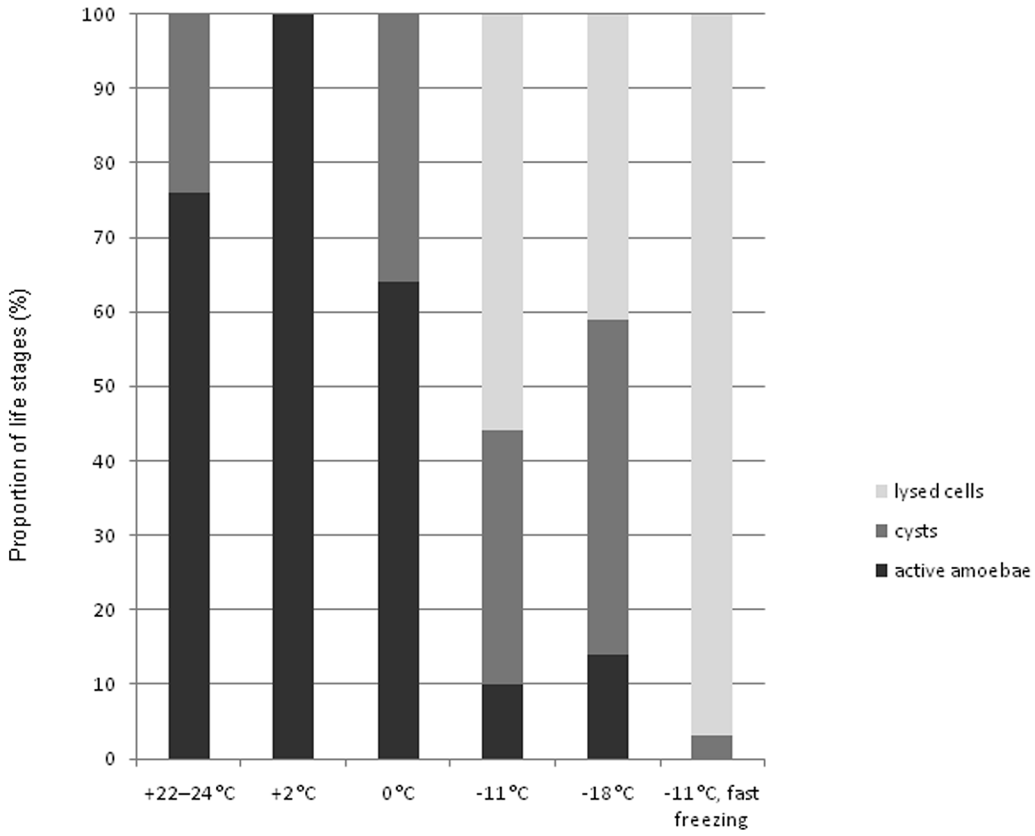

Fig. 2. Proportions of active, encysted and lysed amoebae after treatment with different temperature regimes, followed by a growth period at +2 °C for four days (+22–24 °C – without growth period). Shown are the mean of per cent proportions of life stages for cultures from three specimens of *Lepidoderma chailletii*.

data analysis showed no correlation between age of specimens and their germination. Analysis was performed for Physarales and Stemonitales separately. Additional attempts should be made with more fresh and ample material to determine the ability of these species to germinate in laboratory conditions.

Amoebae of *L. chailletii* and *P. nivale* readily proliferated at room temperature and even microplasmodia started to appear in some isolates, but further development of plasmodia was observed only at +2 °C, otherwise amoebae and microplasmodia started to encyst. Similarly, sudden cooling induced excystment of amoebae. It was also shown that germination in *L. chailletii* can occur at +2°C. These observations allow assuming that amoebae of nivicolous myxomycetes grow best in cryophilous conditions as occurring under a snow cover where temperatures typically vary from –5 to +3 °C (Freppaz et al., 2008; King et al., 2010). However, germination and sometimes microplasmodia occur as well at room temperatures; possibly, these stages will survive warm spells in winter. It seems as well possible that microplasmodia form already in autumn or even earlier and live actively or wait as solitary macrocysts until the winter snow cover creates conditions allowing them to develop into plasmodia. The observed, readily occurring encystment of amoebae can be seen as an adaptation to resist early frosts that can happen before a thick snow cover accumulates and buffers temperature drops. Experiments showed that amoebae of *L. chailletii* can actively live at temperatures from 0 to +2 °C. Some amoebae can survive temperatures as low as –18 °C, but only if they are refrigerated slowly enough to allow encystment. Another limiting factor, not yet investigated, may be the viability of macrocysts after freezing.

An interesting observation was that plasmodia in *L. chailletii* and *P. nivale* appeared in cultures only at 2 °C and required the constant presence of a thin layer of water. These conditions are consistent with the microclimate in water-saturated soil under the snow. At low humidity and at room temperature plasmodia either died or sclerotized. Apparently, these changes are not sufficient to trigger formation

of fruiting bodies. Tests including different culture media and combinations of light, temperature and humidity are necessary to find the proper sporulation conditions for nivicolous myxomycetes.

In addition, the appropriate food sources for trophic stages of nivicolous myxomycetes are not yet known. Most myxomycete species successfully cultured so far were grown on bacteria serving as food (Henney and Henney, 1968). For this reason, we did not sterilize spore mass and some amount of bacteria and yeasts associated with this material was transferred in culture. What is important, in our experiments only highly nutritive SM agar supported growth rate of these food organisms at +2 °C that was sufficient to provide development of dense amoebal populations and large plasmodia in *L. chailletii*. Whether these microorganisms associated with spores represent members of under-snow microbial community or they are an accidental combination of microorganisms remains unknown. Non-living, organic food sources have as well been used to hasten growth of plasmodia in culture, like oat flakes (Madelin, 1984; Haskins and Wrigley de Basanta, 2008). Surprisingly, in our experiments we did not observe positive taxis of *L. chailletii* plasmodia towards oat flakes; in contrast, sometimes plasmodia avoided this food source. However, oat flakes were attractive for plasmodia of *P. nivale*. In cultures of *L. chailletii* we observed deep hollows on agar surface as traces of plasmodium activity. Recently we recorded this phenomenon also for cultures of some species of *Diderma*. This can be a result of production of extracellular agarolytic enzymes that was previously described in myxomycetes only for *Fuligo septica* (Murugan et al., 1996). The ability of plasmodia of some myxomycete species to use agar as food source was also confirmed by Knowles and Carlile (1978) who showed that plasmodia of *Physarum polycephalum* can utilize agarose.

Production of agarolytic enzymes is a quite rare property among microorganisms; it has been reported only from several genera of marine bacteria and a few soil bacteria (Van der Meulen et al., 1974). Current research on under-snow microbial communities may provide useful hints towards natural food sources for nivicolous myxomycetes (Schmidt and Lipson, 2004; Segawa, 2005; Lipson, 2009; King et al., 2010), and co-culturing of cryophilous bacteria found in such communities may be a promising way to understand the food networks where nivicolous myxomycetes are involved.

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