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Methods of agar culture of myxomycetes: an overview
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period of stability during which little change occurs, which are available for worldwide distribution, can form a permanent method of storage of type material and a genetic legacy for future work. The use of spore to spore cultures is equally important in the instruction of high school, undergraduate, graduate and post-graduate students, and to provide material for various research projects. In two groups of Eumycetozoa, the Dictyostelids and Protostelids, almost all known species have been cultured [28, 29], but very few of the Myxomycetes have completed their life cycle in laboratory culture.

A comprehensive list of 55 Myxomycete species in the literature that had been cultured from spore to spore on agar, or other media, was given by Gray & Alexopoulus [12], and updated by Clark & Collins [4] and Collins [8] but more species have been cultured by other researchers since then and 98 are listed by Clark [2]. The few additional species that have been cultured since 1995 are *Collaria arcyrionema* [6], *Licea succulenticola* [26], *Didymium megalosporum, Didymium laxifilum* Clark *et al*. [7] and *Didymium wildpretii* Lado *et al*. [21]. In addition, the culture of *Macbrideola cornea* was described in some detail by Wollman [31] in her thesis, but has been omitted from later compilations.

The purpose of this paper is to summarize the culture methods used successfully by the first author over the last 50 years and results obtained, with the aim of encouraging others to attempt these methods and so increase the number of species that can be cultured from spore to spore. These methods were used recently as the basis for three workshops to train researchers on how to get field collections of myxomycetes into agar culture and their subsequent laboratory maintenance. The workshops took place in Fayetteville, Arkansas, USA (October 2004), Madrid, Spain (May 2005), and lastly in Tlaxcala, Mexico in August 2005, as part of the 5th International Congress on the Systematics and Ecology of Myxomycetes.

Material and methods

The following is a synopsis of the most useful methods employed to culture myxomycetes. Specific methods for each species can be found in the original publications.

Source of specimens. Clean sporocarps selected from field and moist chamber culture collections, free from fungal contaminants, were isolated from all other Myxomycete spores in closed wrapped containers, such as small boxes, Petri dishes or Eppendorf tubes. These exsiccata were labelled with the collection number and details. None of the specimens had been frozen, heated to dryness or fumigated.

Cultivation from spores. Germination cultures were set up on sterile 0.75% water agar (WA), weak Malt Yeast (wMY) agar, or *Yucca* bark extract agar (See materials below). A fine marking pen was used to divide the Petri dish bottom into quadrants. An alcohol flamed #5 jewellers forceps was used to pick up spore material and inoculate the surface of the agar in each of the quadrants by using a gentle slashing motion to make sure some of the spores were submerged and others were left on the surface (Figure 1). Individual spores were well-separated on the substratum. The areas of spore deposit were circled on the bottom of the dish, in order to confirm that the spores were from the specimen sampled, and to check the spores at intervals to watch for germination. Parafilm was used to seal the dishes.

Spore germination (Figure 3) occured in hours to days (up to one month). Spore germination was observed by inverting the agar plate, still closed, on the stage of a compound microscope from which the clips or mechanical stage had been removed. A 5X or 10X objective were used with 10X or 15X oculars to view the spores, amoebae (Figure 2), swarm cells (Figure 4) and plasmodial phases at 50X-150X. A

mm agar Petri dish. If soft (0.75%) water agar was used for germination, this may not be necessary.

Sporulation. Some species crawled to the sides or lids of the container to sporulate. Those listed above, sporulated when the fluid was drained from the Petri dish and the dish exposed to light. In other cases, as with *Physarella oblonga* (Berk. & M. A. Curtis) and *Comatricha laxa* Rostaf. it was necessary to cut a block of agar bearing the plasmodia and transfer it to 1.5% water agar and expose it to fluorescent light. In this case sporulation may have been induced by starvation and a light cue. Another method involving making a paper cone for the plasmodium to climb is described in Venkataramani *et al*. [30].

To prepare living cultures for stasis. The International Code of Botanical Nomenclature, recommendation 8 B.1. [13], states that, whenever possible, living cultures be prepared of holotype material of new taxa and deposited in a culture collection such as the American Type Culture Collection (ATCC). The following methods have been used as simple alternative stop-gap stasis approaches in contrast to liquid N_2 stasis employed by the ATCC or other international microbial archives. None of the methods described below guarantee long-term survival of myxomycete cultures. The first author has been able to store and revive cultures after 40 years using a simple lyophilization method [18]. This technique requires the availability of a machine shop to build the lyophilization apparatus.

It is critical to have simpler methods available for longer term storage of cultures. Some cultures preserved 10 years earlier by the following method have been revived successfully, but be advised that the viability of the stasis stocks must be checked from time to time. A number of No. 1 or No. 1-1/2, 22 mm^2 glass cover slips were placed in a glass Petri dish and sterilized in a drying oven for 2 hr or more at ca 160°C. After sterilization and cooling, several sterile cover slips were placed into a 100 X 15 mm sterile plastic dish. Using a flame sterilized and cooled flat-ended spatula, 1 cm^2 agar blocks of actively growing Myxomycete cultures were cut out. These were placed one block on each cover slip, culture side up. The Petri dish was labelled with the name, and other data of the culture, and closed. It took up to 1 month for the agar block to dry down to a thin film. When dehydration had occurred, the dish was sealed with Parafilm to avoid fungal, etc. contamination and stored at 18-25°C in the dark. To revive a culture, a drop of sterile distilled water was added to the top of the agar fragment, and after 10-20 minutes the film was lifted with a sterilized spatula. The agar fragment was place cell-side down on the surface of an appropriate agar plate and resumed growth was looked for in 1-14 days. When growth occurred, food micro-organisms were added. A second air-drying method was used for four years and is still being assessed for its effectiveness. All details of handling the culture blocks, dried agar fragments and revival methods are the same. However, in the second method about 12, 1 cm^2 agar blocks of culture material were cut out aseptically and placed cell side up on the bottom of the 100 X 15 mm sterile plastic Petri dish. The dish was labelled, dehydrated for up to a month, sealed with Parafilm, and stored as described. Some cultures stored this way were revived after four years.

Agar cultures of Myxomycetes wrapped in parafilm strips and stored at 5°C in a refrigerator can survive for 1-2 months as amoebae or microcysts and occasionally up to a year.

Media. These are the most useful media mentioned above and what they were used for. Details of other media and media preparation can be found on www.slimemold.uark.edu

1). 0.75% Water Agar (0.75% WA)

Bacto agar 7.5 g, Glass distilled water 1 L [excellent for spore germination]

2). Weak Malt Yeast (wMY) Agar **(**courtesy of Prof. F.W. Spiegel)

Table 1. Myxomycete species cultured on agar for the first time in the laboratory of E.F. Haskins

Taxon	Reference
Comatricha lurida	[24]
Echinostelium arboretum	[16]
Echinostelium coelocephalum.	[15]
Echinostelium corynophorum	[15]
Semimorula liquescens	[14]

more readily on agar than others.

Germination has been accomplished in many more taxa than those that have completed the full life cycle (see above, and (12, pp. 26-27), also *Cribraria zonatispora* [20]). Similarly some species such as *Cribraria violacea* [25], *Kelleromyxa fimicola,* [9], have been grown on agar from sclerotia and plasmodia, but without germination from spores. *Cribraria fragilis* [10] has been grown on columnar cactus remains from plasmodia, and *Hemitrichia imperialis* completed its cycle on rotten wood from spores [23].

Although only germination on media in Petri dishes has been described here, other methods may be useful. Some workers inoculate and germinate spores on agar slices on sterile microscope slides or cover slips, agar drops on cover slips set as hanging drops, or hanging drops of sterile liquid medium on sterile cover slips. These can then be transferred to agar plates to complete the cycle.

Contaminants such as *Acanthamoeba* [27], filamentous fungi, Ascomycetes and even food organisms can cause serious difficulty in culturing, and increase the cost in both time and money by making frequent sub-cultures necessary. The strict use of aseptic techniques, and close and frequent observation of the cultures can limit this. It is important to start with only one species, and to keep the number of cultures down in order to facilitate close control. However, it is also important to set up multiple cultures once germination has occurred, in order to keep a clean "stock" culture of amoebae, but also have others to try out different media and conditions until the optima for completing the cycle for that species have been found.

Clark [2] commented that most major genera have at least one species cultured and "thus a reasonable and fairly representative accumulation of culture studies for the myxomycetes" have been done. However there are some obvious genera where spore to spore culture work is almost totally lacking, such as *Cribraria, Licea, Lycogala. Hemitrichia, Trichia, Lamproderma, Colloderma, Listerella, Barbeyella, Tubifera* and *Brefeldia*.

Certain species of these genera, with importance for taxonomic and genomic studies, should be considered to be target species, and attempted first. These should include the type species of the different genera such as *Licea pusilla* Schrad., although the age of spores in the actual type specimens may preclude their germination, and in these cases other collections would have to be used. All future species descriptions should be accompanied wherever possible by spore to spore culture [17]. It is also important to have descriptions of all life cycle stages, including microcyst development and details of plasmodial and sporocarp development as Collins [8] suggested.

Taxonomic or phylogenetic studies, whether traditionally done or with molecular techniques, that are based on morphospecies, which may include clonal populations or ecotypes, or may be species complexes, are susceptible to error. These and other lines of investigation need the complete base research of each species to underpin the accuracy of their systems. For this, spore to spore culture, clarification of biological species by investigation of mating systems, and the deposit of authentic, uncontaminated living material for research by future generations is therefore urgently needed.

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