1. USEFUL METHODS FOR BRINGING FIELD COLLECTIONS OF *MYXOMYCETES* INTO AGAR CULTURE[1]

Rationale for getting a wide range of Myxomycetes into agar culture.

Although *Physarum* and *Didymium* (Aldrich and Daniel, 1982) have served as excellent model systems for study, they give only a partial view of the biology of the Myxomycetes. There is a pressing need to get a wide range of Myxomycetes into agar culture to be used in the instruction of high school, undergraduate, graduate and post-graduate students as well as to provide material for student research projects. Further, these cultures will serve as primary research organisms in laboratories around the world. As Prof. Spiegel has noted,

> "Chemical sequences derived from a diversity of authentic cultures will make it easier for molecular systematists to build phylogenies. Also, such sequences when derived from cultures representing a wide taxonomic range, will make it easier to recognize myxomycete "chemical signatures" so that we can more confidently use sequences from species that will not go into culture. For those cultures that can be induced to go spore to spore, it will be possible for developmental geneticists to track changes in gene expression and to compare the patterns of gene expression across the Myxomycetes and with other organisms. Also sporulating cultures will allow the design of a range of experiments to determine the stability (phenotypic plasticity) of characters in the sporophore."

Since only morphological features of the sporophores are currently used to identify Myxomycetes, it behooves us to fully understand character variability under different culture conditions.

[1] An overview of these methods is published in Haskins and Wrigley de Basanta (2008).

Lastly, in a topic close to my heart, a taxonomically diverse array of agar cultures will permit a comprehensive evaluation of the reproductive systems in the Myxomycetes.

Suggested Supplies, Tools and Equipment

Watch glasses
Transfer needle with shaft
#5 Jewelers forceps
Arkansas combination (medium/fine) sharpening stone
Flat ended weighing spatula
Alcohol lamp
50 ml glass beaker
GE40W unfrosted appliance bulb
Right angle, glass spreader
Lighter for alcohol lamp
Fine tip marking pen for labeling glass and plastic lab ware
Inoculating loop with metal shaft
Falcon #1029 sterile plastic petri dishes 15 mm X 100 mm
Capped glass tubes
Glass stirring rods
Test tube rack
1 ml and 10 ml graduated pipettes (sterile)
Tubes or flasks of sterile glass distilled water
Compound microscope equipped with 5X or 10X objectives, and 10X or 15X oculars
Dissecting microscope
Glass slides
22 mm^2 cover slips
Millipore filters
Circles of Whatman #1 filter paper
Parafilm
Small spear tip cutter with shaft
Kimwipes, absorbent wipes
Capillary tubes/pipettes
0.15 mm diam Minutien pins
Pin vise
Ballpein hammer
Jewelers bench block
Nichrome, copper or silver wire, 20 or 24 gauge
Wire cutter
Barber shears
Triangular file
Glass tubing cutter (Griffin-type)

The Arkansas sharpening stone is useful in re-sharpening the tips of #5 jewelers forceps. A 40W GE unfrosted appliance bulb is a source of several preformed loops. Such a fine wire loop can be

mounted on the tip of a glass stirring rod using a propane torch. The propane flame has no effect on loop integrity. I use a shop propane torch mounted at a 45º angle in a custom made wooden cradle. The appropriate glass stirring rods can be purchased from Carolina Biological Supply Co. #71-1303. Capillary tubes/pipettes 1 mm o.d. x 0.4 mm i.d. x 75 mm length are available from Friedrich & Dimmock, Inc. Stainless steel Minutien pins 0.15 mm dia are available from Caroline Biological Supply Co. FR-65-4366.

Pin vise, No. 54270 available from Ernest F. Fullam, Inc.

Formulae of media which have proven useful in bringing field collections of Myxomycetes into sustained agar culture.[2]

Agar media and liquid nutrient solutions are standardly autoclaved at 15 lbs. pressure for 15-20 min. Oat flake material is standardly autoclaved at 15 lbs. for 30-45 min. Often it is useful to grind the oat flakes to a flour using a mortar and pestle before autoclaving (see below). Agar media are dispensed to ca one half the depth of sterile petri dishes. For difficult to culture "myxos" agar media can be dispensed as slopes in 20 mm dia capped tubes. Place the tubes in a test tube rack and maintain them in an upright position. Add a water suspension of spores to the base of the slope and developing stages "seek" the appropriate moisture level over time. Liquid media are dispensed to the working volume of their containers.

1. 0.75% Water Agar (.75 WA)

Bacto agar	7.5 g
Glass distilled water	1 L

This agar has been successfully used to germinate a number of Myxomycete taxa. Except for a few echinostelids growing in association with *Cryptococcus laurentii* it doesn't sustain

[2] See Appendix 2 for discussion of rationale of media construction for Myxomycetes in xenic or monoxenic culture.

serial sub-culturing of Myxomycetes. Therefore after germination of spores, transfer the amoebae and swarm cells to other types of agar media (e.g. 1.5% WA, wMY or CM/2 agar) along with appropriate food microorganism(s).

2. 1.5% Water Agar (1.5 WA)

Bacto agar	15 g
Glass distilled water	1 L

3. Half Strength Bacto Corn Meal Agar (CM/2)

Bacto corn meal agar	8.5 g
Bacto agar	12.5 g
Glass distilled water	1 L

4. Scratch Corn Meal Agar (SCM)

Albers yellow corn meal	20 g
Difco agar	17 g
Glass distilled water	1 L

Recipe courtesy of Prof. H.C. Whisler

5. Corn Meal + Agar (CM+)

Difco corn meal agar	17 g
Yeast extract	2 g
Glucose	1 g
Glass distilled water	1 L

Recipe courtesy of Prof. F.W. Spiegel

6. Bark Extract Corn Meal (BECM) Agar

If feasible, use oak bark (*Quercus fusiformis* or *Q. alba*). Collect ca 50 g of outer bark pieces and soak in 1 L glass distilled water ca 24 h. Filter the supernatant through cheese cloth and bring final volume to 1 L; if necessary, add additional water. To this liquid add:

Bacto agar	17.5 g
Bacto corn meal agar	8.5 g

7. Weak MY (wMY) Agar

Malt extract	0.002 g
Yeast extract	0.002 g
Dibasic potassium phosphate	0.75 g
Bacto agar	15 g
Glass distilled water	1 L

Recipe courtesy of Prof. F.W. Spiegel

8. Soft Weak MY (swMY) Agar

Malt extract	0.002 g
Yeast extract	0.002 g
Dibasic potassium Phosphate	0.75 g
Bacto agar	7.5 g
Glass distilled water	1 L

9. Penicillin-Streptomycin Agar (PS)

Bacto agar	20 g
Potassium penicillin G	2 million units
Streptomycin sulfate	1 g
Glass distilled water	1 L

Melt the agar by steaming. Autoclave at 15 lbs for 15 min. and add sterile solutions of antibiotics to cooling agar. Modified from Gray and Alexopoulos, 1968.

10. Yucca Bark Extract Corn Meal (YBECM) Agar

Use outer bark pieces. Collect ca 25 g and soak in 1L glass distilled water ca 24 h. Filter the supernatant through cheese cloth and then filter again through filter paper. Bring the final volume to 1L; if necessary, add additional water. To this liquid add:

Bacto agar	17.5 g
Bacto corn meal agar	8.5 g

Recipe courtesy of Diana Wrigley de Basanta

11. Green Jungle™ (GJ) Agar[3]

Green Jungle orchid food – fertilizer	40 ml
Difco agar	7.5 g
Glass distilled water	1 L

Green Jungle™ agar is an efficacious medium for the isolation of echinostelial taxa.

12. Trypticase Soy (TS) Agar

BBL Trypticase soy agar	40 g
Glass distilled water	1 L

Useful in growing some food organisms, often at 30°C

13. NTGY Medium (NY)

Tryptone	6 g
Yeast extract	6 g
Dextrose	1 g
KH_2PO_4	1g
Glass distilled water	1 L

Used in growing some difficult to cultivate organisms. Especially useful in growing *E. col*. at 30-37°C. To solidify add 15 g Bacto agar. Modified after Raper, 1984 p. 53.

14. Difco Nutrient Broth

Difco nutrient broth	8 g
Glass distilled water	1 L

Dispense in capped tubes or 125 ml Erlenmyer flasks.

15. GPY Broth

Dextrose	10 g
Difco Bacto Peptone	10 g

[3] Green Jungle ™ Fertilizer which is available from Orchids Limited, Plymouth, MN 55446 U.S.A. is purported to contain all the essential plant macronutrient and micronutrient elements needed for the growth of chlorophyllous land plants and its inclusion produces a mineral medium. For additional ideas for constructing mineral media for eumycetozoans consult Schuster, 1990, Stein, 1973, Andersen, 2005 and Morholt and Brandwein, 1986.

Difco yeast extract	1 g
Mg SO_4 • 7 H_2O	1 g
Glass distilled water	1 L

Useful in growing some food organisms, also used in dilutions of 1/5 or 1/10 and grown at 20-30°C.

16. Mueller Hinton Broth

Difco Mueller Hinton Broth	21g
Glass distilled water	1 L

Useful in growing some difficult to cultivate organisms, often at 30°C.

17. BSS (Bonner's Salt Solution)

K Cl	0.75 g
Na Cl	0.6 g
Ca Cl_2	0.3 g
Glass distilled water	1 L

Useful in harvesting amoebae and small plasmodia from agar (chill to 5°C). Bonner, J.T. 1947. J. Exp. Zool. 106:1-26.

18. Buffers

KH_2 PO_4	1.36 g
Na_2 H PO_4	0.38 g
Glass distilled water	1 L

Final pH value 6.6-6.8

Consult Barker, 1998 for additional information concerning useful biological buffers.

19. Ground Old Fashion Quaker Oat Flakes

Use a mortar and pestle to prepare oat flake flour from Old Fashion Quaker Oat Flakes. Dispense flour to a depth of ca 1.5 cm in capped or cotton plugged tubes or 125 ml Erlenmyer flasks and autoclave at 15 lbs. for 30-45 min. Sprinkle sterilized oat flour sparingly on agar

cultures of Myxomycetes. This additive isn't necessary or appropriate for all taxa. One must use a "trial and error" approach to determine if it is beneficial to a Myxomycete in agar culture. NB- IMPROPERLY STERILIZED OATS CAN BE THE SOURCE OF FUNGAL CONTAMINATION!

Microorganisms Useful in Agar Culture of Myxomycetes

Often the microbial "melange" that develops from the spore inoculum proves useful in feeding the amoebal and subsequent plasmodial phase. However, for biosafety and/or establishing more vigorous serial subcultures, the following microorganisms, available from the ATCC, have proven effective in agar culture of Myxomycetes.

Escherichia coli	ATCC # 23437
Escherichia coli B	ATCC # 11303
Cryptococcus laurentii	ATCC # MYA-2946
Flavobacterium sp.	

These microbes grow well with Bacto nutrient broth and/or Corn meal + agar.

Quality control during the culturing process.

1. Source of specimens. If you are going to culture Myxomyctes it is useful to have a list of target taxa in mind. This does not preclude an opportunistic approach to collecting field or moist chamber specimens. When you find a candidate collection, make sure it is clean of contaminating fungi and ideally not mixed with other Myxomycetes. The specimen should be glued immediately into a clean, fresh herbarium box. Record collector, site of collection, habitat, provisional identification on each collection. Record, preferably photographically the habitus of the sporophore, peridial, stalk, columnar characters and spore ornamentation (observe with oil immersion). All of this should be part of the record associated with each culture. That way, if another worker disagrees with your identification, there will be voucher material to use to help

determine the accuracy of the identification. And once cultures sporulate, place an accession into the herbarium as an additional voucher.

One must use extreme caution at all steps of specimen processing to avoid cross species contamination. I and other ardent culturists have repeatedly seen more than one species of Myxomycetes develop from an inoculation. It is “a dirty world” out there! To minimize this problem, place the freshly glued specimen box in a clean paper bag and roll it up to prevent contaminating spores from getting into the air spora. Air dry but do not freeze or fumigate. Store the specimen separately from other collections of Myxomycetes.

Even though freshly collected spores, i.e. collections less than one year old are thought to be most appropriate for agar culture, herbarium material collected some time previously may be used. It should be noted that Prof. Jim Clark has found that some specimens of tropical Myxomycetes lose spore viability within weeks after sporulation. It is standard that material is first frozen before inclusion in an herbarium collection (Bridson and Forman, 1998). Further, the anecdotal view that slime mold collections are non-viable when they have been fumigated, heated or frozen to minimize animal or fungal development needs to be challenged by systematic study. One must be wary of cross species contamination that may have occurred during herbarium processing and take all the precautions elaborated in this presentation.

2. Cultivation from spores. If sufficient spore material is available, set up germination cultures on 0.75% WA, 1.5% WA, wMY, and CM/2 agar (see Formulae of Media). If there is a paucity of material, try 0.75% WA first; then if no germination occurs, 1.5% WA, wMY or CM/2 agar. Use a fine marking pen to divide the bottom of the petri dish into quadrants. Draw small circles in each quadrant which will serve as areas for inoculation of spores. These circles will allow you to quickly locate the deposited spores as you check at intervals for germination.

I prefer to use an alcohol flamed #5 jewelers forceps to pick up spore material and inoculate the surface of the agar in each of the quadrants using a gentle slashing motion to make sure some of the spores are submerged and others are left on the surface. Other workers use a sterile transfer needle to pick up a small sample of spores to spread on a germination medium. Some workers heat the needle, plunge it into an agar plate, thus coating the tip with agar which makes the end of the needle conducive for picking up spores. In both methods, individual spores should be well separated on the substratum. Germination usually takes 12-36 hours but may in some species occur within a few hours.

Spore germination can be observed by inverting the agar plate on the stage of a compound microscope from which the clips or mechanical stage have been removed. Use a 5X or 10X objective with 10X or 15X oculars to view the spore, amoebal and plasmodial phases at 50X-150X. Typically a dissecting microscope provides less resolution at a comparable magnification.

Frequently one gets some spore germination in each inoculated quadrant but the amoebae fail to actively multiply during the first few days. To get a successful agar culture it is necessary to find an appropriate food organism before the amoebae encyst and growth ceases. Leave one quadrant with any mélange that may be developing and inoculate the remaining quadrants with e.g. *E. coli*, *Flavobacterium* or *Cryptococcus* etc. Pick up these microbes with a transfer needle and place them near the amoebae. Hopefully the amoebae will crawl over to the provided food organisms and begin to feed and multiply. If encystment of amoebae has occurred, sometimes it is possible to induce excystment by subculturing the encysted amoebae to a medium of low nutrient content e.g. 0.75% WA or 1.5% WA. However, if an appropriate food organism isn't provided, reencystment will ensue.

Although standardly I only use germination media in petri dishes, other methods may be useful. Some workers inoculate and germinate spores on agar slices (see Formulae of Media) 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.

Prof. Kalyanasundaram (2005) suggests that spore germination is facilitated by several hours of immersion in 0.5 ml sterile drop of 0.001% (v/v) tween 80 (Polyoxyethylene sorbitan monooleate). Subsequently, ca. 2 ml (w/v) of carrot extract is added to the spore suspension. This nutrient rich solution favors the modest growth of microbes originally associated with the spores. The resulting amoebal cells and microbes can then be transferred to appropriate media.

Gray and Alexopoulos (1968) summarize some of the work of earlier investigators who reported that the wetting and drying of spores increases the percentage of spore germination.

Transferring of amoebae or plasmodia can be done in various ways. If these cells are growing in a microbial film transfer blocks can be cut out of the culture agar. The blocks can be placed either cell side down, cell side up or even perpendicular to the agar surface. It is ony necessary that the transfer doesn't cause lysis of the cells or prevent them from swarming onto the new plate. A sterile loop can also be useful to transfer myxomycete cells. If the culture is grown under a water layer, a pipette is the transfer tool of choice.

Keep careful records of all collections that germinate so that future attempts at agar culture are possible. After germination occurs, amoebae and plasmodia often flourish on the microbial melange (bacteria and/or yeasts) that develops from the original spore inoculum. All too often there is also a subsequent development of filamentous fungi and/or mycelial Actinomycetes. For successful Myxomycete subculture, these "contaminants" must be got rid of. A useful method to clean up your cultures is Outrun The Fungi (OTF) described below (see "Trouble shooting"

culture problems). The OTF method separates small populations of amoebae or plasmodia from mycelial taxa and also the mélange. OTF may have to be repeated to eliminate yeasts. If bacteria persist, transfer to Penicillin-Streptomycin agar (see Formulae of Media) for a period of time to be determined by trial and error. After the original mélange has been removed in the name of biosafety, the bacterium *Escherichia coli*, the bacterium *Flavobacterium* sp, or the yeast *Cryptococcus laurentii* can be inoculated as food organism replacements. Sometimes eliminating the original mélange leads to stoppage of growth and development of the Myxomycete of interest. Prof. Kalyanasundaram (pers. comm.. 2007) reports that she typically grows Myxomycete in agar culture using the bacterial/yeast mélange derived from the initial specimen.

Not infrequently amoebal growth is poor on 0.75% WA or even 1.5% WA. It is then I resort to the SIN (Sequential increase in nutrients) approach. I first subculture the isolate to slightly more nutrient rich agar, e.g. from 0.75% WA to 1.5% WA or 1.5% WA to CM/2 agar or CM/2 agar to oak bark CM/2 agar. I stop this procedure as soon a good growth of amoebae and plasmodial and sporophore development is achieved.

Further, if growth in xenic culture is sparse, try adding sterile “Old Fashion” oat flakes or oat flake flour. Some Myxomycetes require the presence of a thin water film for amoebal growth and subsequent plasmodial development, e.g. Stemonitales. The water film should be maintained at a depth of ca. 1-2 mm. This may require the addition of sterile water from time to time. Some species require water for only the early stages of plasmodial growth. For difficult to grow taxa try subculture of amoebal cells to CM/2 or bark extract CM/2 agar with or without a water film.

3. Cultivation from plasmodia or sclerotia. Since you cannot *a priori* know the identity of a culture derived from a plasmodium or sclerotium, one must induce sporulation and confirm the

identification using sporophores. With viable spores available, subsequent agar cultures can be established.

In summary, although agar culture can be established using plasmodia or sclerotia of some Myxomycetes (Gray and Alexopoulos, 1968), it is more commonly obtained from spore germination. When appropriate food microorganisms have been selected, subsequent plasmodial development may be encouraged by adding ca 5 ml of sterile, glass distilled water to the surface of a 15 mm X 100 mm agar petri dish. If this favors plasmodial development, be sure to add additional liquid as required to maintain this shallow film. One must use trial and error to induce sporulation. Some species crawl to the sides or lids of the container to sporulate. Or it may be necessary to drain the fluid from the petri dish and expose the plasmodia to light. In other cases it is necessary to cut a block of agar bearing the plasmodia and transfer to 1.5% water agar and expose to light (e.g. fluorescent light). In this case sporulation may be induced by starvation and a light cue. It should be noted that it was long the contention of the laboratories of Professors Martin and Alexopoulos that it was necessary to “run” each new agar culture of a Myxomycete “spore-to-spore” at least once to be confident of its taxonomic identity. Be aware that all too often aberrant fruiting bodies form in culture. Accurate taxonomic identification of an isolate depends on the availability of normal fruits.

Venkataramani et al., 1977 have provided a simple technique for inducing sporulation of myxomycetes. When plasmodial growth has completely covered the agar surface the petri dish lid is removed and a filter paper cone ca. 15-20 cm (sterile) is placed on the agar surface. The cone should be moistened with sterile distilled water to encourage plasmodia to move onto the cone. This preparation is then covered with a large beaker or bell jar thus establishing a humid chamber. When the plasmodia moves onto the filter paper the cone can be transferred to a dish

containing water to a depth of 1-2 mm and covered once more. As the cone evaporates, hopefully the plasmodia will ascend the water gradient produced by evaporation and sporulate somewhere near the apex of the cone. For some species a light/dark regime will have to be maintained in order to induce sporulation. Also carrying out this procedure 2-3°C above the initial culture temperature has often proved useful in inducing sporulation.

Zitierform:

HASKINS, E.F., N.S. KERR und INST. WISS. FILM: Vergleich der Plasmodien-Typen und der Sporulation bei Myxomyceten. Film C 1220 des IWF, Göttingen 1976. Publikation von E.F. HASKINS, Publ. Wiss. Film., Sekt. Biol., Ser. 11, Nr. 27/C 1220 (1978), 34 S.

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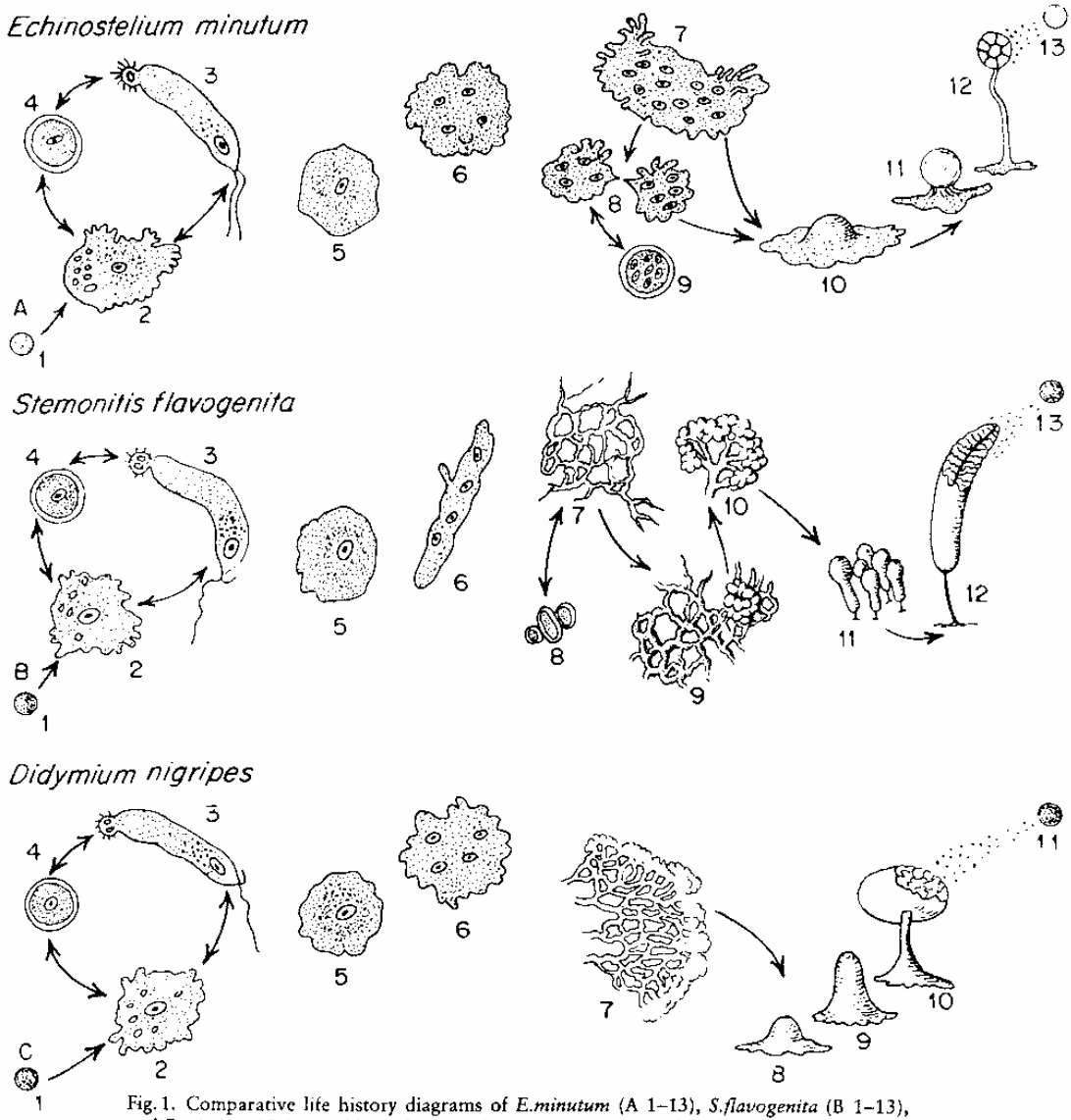


Fig. 1. Comparative life history diagrams of *E.minutum* (A 1–13), *S.flavogenita* (B 1–13), and *D.nigripes* (C 1–11). Natural proportions are not represented in all instances, and nuclei are not drawn in all cells

E.minutum. A-1: spore; A-2: myxamoeba; A-3: swarm cell; A-4: myxamoebal cyst; A-5: uninucleate plasmodium; A-6: 4-nucleate plasmodium; A-7: mature multinucleate protoplasmodium; A-8: daughter protoplasmodia formed by fission (plasmotomy); A-9: protoplasmodial cyst; A-10: protoplasmodium with central sporangial initial; A-11: later stage of sporulation; A-12: mature sporophore with apical cluster of spores; A-13: cloud of air-borne spores with one spore enlarged

S.flavogenita. B-1: spore; B-2: myxamoeba; B-3: swarm cell; B-4: myxamoebal cyst; B-5: uninucleate plasmodium; B-6: linear, 4-nucleate plasmodium; B-7: multinucleate, hyaline aphanoplasmodium; B-8: aphanoplasmodial cysts; B-9: opaque aphanoplasmodial phase forming the coralloid stage; B-10: coralloid stage; B-11: cluster of immature sporophores formed from the coralloid plasmodium; B-12: solitary sporophore showing details of the capillitial network; B-13: cloud of air-borne spores with one spore enlarged

D.nigripes. C-1: spore; C-2: myxamoeba; C-3: swarm cell; C-4: myxamoebal cyst; C-5: uninucleate plasmodium; C-6: 4-nucleate plasmodium; C-7: multinucleate phaneroplasmodium; C-8: young sporangia: primordium; this represents a single intial from the cluster of initials which differentiate from a plasmodium; C-9: a later stage of differentiation; C-10: mature sporophore showing details of the capillitial network; C-11: cloud of air-borne spores with one spore enlarged

4. **"Trouble shooting" culture problems.** As Prof. Spiegel has said,

> "Do not rush; do one culture at a time. If you try more than one specimen at a time make sure to use specimens whose spores are morphologically distinct from one another. Do not be discouraged that it may take weeks to establish some cultures. The most important goal is getting an agar culture with a single myxomycete present. Therefore, do not discard a culture that may have a fungal contaminant or growth of a mycelial Actinomycete. A simple technique to free a culture from a fungal contaminant, etc. that works well with protostelids and dictyostelids was dubbed Outrun The Fungus (OTF) during a recent training session at the ATCC. In a fungus contaminated culture, find an area of the plate with numerous amoebae and as little mycelium as possible. Cut out a small block from this area and place the block amoebae side-down on a plate of 1.5% WA. The amoebae will usually migrate out more rapidly than the hyphae will grow, and individual amoebae can be picked clean and transferred to a fresh culture plate. This also helps to get the amoebae to crawl free of some unwanted bacteria or yeasts."

I have used an alternative method to separate myxamoebae from hyphal contaminants. An area with amoebae and few contaminants is selected under 100 X and a drop of sterile distilled water is added. A sterilized capillary tube is then touched to the drop. The suspended amoebae are then dispensed to the surface of a fresh agar plate by repeatedly touching the capillary pipette to the surface of the agar. When an area is found on this transfer plate containing one or more amoebae but lacking unwanted contaminants it is cut out and subcultured to another plate along with a drop of food microorganisms placed near the transfer block.

Another problem is failure of spores to germinate. The reasons for this vary: sporulation in the presence of excess microbial waste (Kerr and Sussman, 1958); a problem in properly wetting the spores (this can be overcome by brief treatment with 0.001% tween 80, Indira, 1969) or 0.5-1% bile salts (Elliott, 1948); problems with a blocked meiosis; or unidentified additional factors.

After prolonged laboratory maintenance some cultures of Myxomycetes lose vigor, cease growing and die (Aldrich and Daniel, 1982). The work of Prof. Clark and others suggest that this display of senescence is due to genetic and nongenetic factors. This points out the need for having stasis stocks available to start fresh laboratory cultures.

It is perplexing that some amoebal cultures can't be induced to go spore-to-spore. Imagine in the first case a culture started from spores that produces only amoebae. One must attempt to eliminate the possibility this is an isolate of a soil or freshwater amoeba (Page, 1988), protostelid, or cellular slime mold. Soil amoebae often have a typical, distinctive shape when translocating over the substrate (Smirnov and Brown, 2007; Page, 1988). Some soil amoebae have a unique cyst morphology, e.g. *Acanthamoeba* (Page, 1988). Rarely do soil amoebae become flagellate. And when they do, as in the case, for example, of *Naegleria* or *Tetramitus* their swimming motions are unlike myxomonads which have a typical corkscrew swimming motion. In addition to amoebo-flagellates the flagellate *Cercomonas* has been reported co-occurring with eumycetozoans (Gilbert, 1927).

As for possible confusion with cellular slime molds or protostelids, one hopes for the development of characteristic stalked fruiting bodies. Be aware that development of plasmodia and fruiting bodies from Myxomycete amoebal populations may be delayed or prevented because one is using an inappropriate growth medium and/or an unsatisfactory food microorganism.

If one has eliminated the possibility of non-myxomycetous contamination then what could explain the failure of plasmodial formation? Various abiotic factors may influence the amoeba-to-plasmodium transition. Among these are the source of water used to compound the agar medium e.g. tap water, reverse osmosis, or glass distilled. There are anecdotal stories of cultures of microorganisms ceasing growth when the water source was changed. The inclusion of an infusion made from the original substrate may be needed (Gray and Alexopoulos, 1968). Selecting an appropriate pH, achieved by using an effective buffer system, may be essential for plasmodial formation (Collins and Tang, 1973). Temperature may be a cue for development as well as the light regime (Reinhardt, 1968). I always grow eumycetozoan cultures in unsealed Petri dshes to minimize gas exchange problems. Gases of importance include, O_2, CO_2, ammonia (Lonski, 1976) and possibly ethylene (Amagai and Maeda, 1992). Raper (1984) discusses the use of activated charcoal to ameliorate the effect of gases on cellular slime mold development. Possibly this material would aid plasmodial development and differentiation.

A more remote possibility is that mutations may have blocked the normal amoebal to plasmodial transition. Theoretically, but of low probability, a heterothallic isolate (see below Reproductive Systems) e.g., *Comatricha lurida* (McGuinness and Haskins, 1985) displaying an extreme skewing of mating type could produce a population of amoebae possessing a single mating type and hence no plasmodia would form. Can you think of ways to test this contention?

Imagine, in the second case, an amoebal population that produces plasmodia but for unknown reasons sporulation is blocked. Currently there is no accepted way to identify a Myxomycete in the amoebal or plasmodial phase; one must have sporophores available for positive taxonomic identification. Is there a way that molecular systematists can utilize these agar cultures? This might be feasible if the cultures had been established using the extremely

stringent collection and culture methods described previously and if the taxonomic identities of the non-sporulating cultures were confirmed by the detection of appropriate molecular signatures.

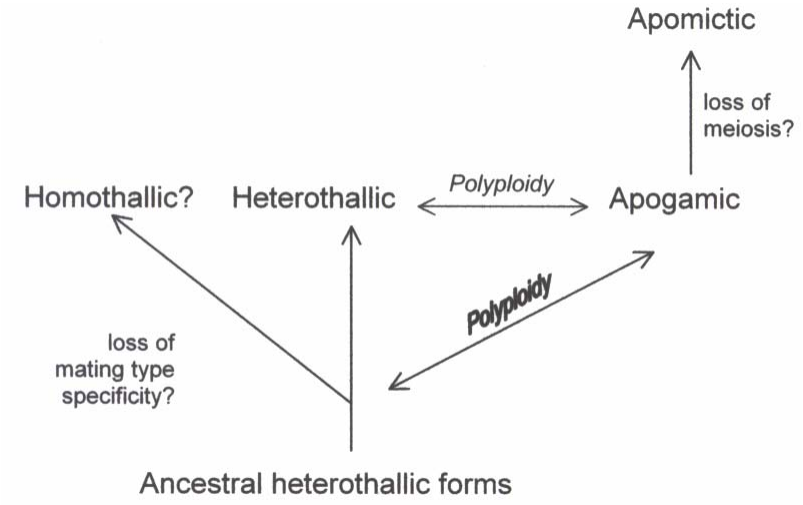
Matrix Modeling as the Basis for Experimental Design and Comparative Analysis

To sort out culture problems one must employ a logical research approach. I often use 3M (Matrix Model Method). The classic matrix model is the Punnett Square of genetics fame. If your goal is to culture and sporulate your newly isolated myxomycete using a matrix allows you to systematize possible combinations of media components, their concentrations, growth temperatures, light quantity/quality regimes, etc. 3M is also useful in identifying similarities and differences in Mycetozoan life histories or developmental cycles (see Fig. 1).

Study of Reproductive Systems of Myxomycetes in Agar Culture

Clonal cultures of amoebae can be obtained by plucking single spores (use dissecting microscope at ca. 60X) or by serially diluting a spore suspension such that ca 50 spores are plated per petri dish. I have used a slender wedge of Millipore Filter held by a forcep to pick up an *Echinostelium* spore ball and spread it on an agar surface. In these cases clonal populations of amoebae are observed as plaques in a confluent film of the microbial food organism. See Ashworth and Dee (1975) p. 24 and Kerr and Sussman (1958) for photomicrographs of amoebal plaques. These plaques of amoebae should be cut out and subcultured to fresh plates of the selected agar inoculated with the appropriate food microorganism. If a clonal population develops plasmodia and fruits, this indicates the isolate is non-heterothallic (either apogamic or homothallic). As Clark et al., 1995 point out, homothallism has not been convincingly demonstrated in the Myxomycetes. Even Gray and Alexopoulos, 1968 stated “the existence of homothallism has not been proved and so-called homothallic species may prove to be

apogamic". If, on the other hand, the clonal cultures don't form plasmodia they should be crossed in appropriate pairwise combinations (Clark, 2000; Clark et al., 2003). If plasmodia and fruits develop, a heterothallic system is indicated. If more than one collection of this taxon is available, further isolations can be done. And, if a heterothallic mating system is present in the second, third, etc. isolate, studies can be made to determine if a multiple-allelic heterothallic system is present in that particular taxon (Clark et al., 2004). Further, one can test if a morphospecies consists of sibling species. Cross two sets of heterothallic mating clones which have been derived, for example, from two separate locations. If these two sets of clones fail to produce plasmodia when crossed in all possible pairwise combinations, each set of clones represents a sibling and/or cryptic species. For an interesting discussion of species concepts see Mayr (2001).



Different reproductive systems in *Didymium iridis*. From: Mulleavy, W.P. 1979. Genetic and cytological studies in heterothallic and non-heterothallic isolates of the myxomycete, *Didymium iridis*. Ph.D. Dissertation. Univ. of California, Berkeley. 129 p.

Method for Obtaining Myxomycetes via Humid Chamber Culture.

Place equidistant pieces of bark, wood or leached, dried small fruits on a 9 cm diameter Whatman #1 filter paper in a 50 X 90 mm crystallizing dish. Add ca 50 ml or more glass distilled water (enough to submerge material) and soak 12-24 h. Subsequently drain off the water and incubate the material in a light/dark environment at 18-25ºC. Alternatively, make the same sort of preparation in a 15 X 100 mm Petri dish. I often also place samples of the bark, wood, fruits, etc. on plates of 0.75% WA or 1.5% WA. Any richer agars lead to the overgrowth of fungi. If the dish contents remain wet, aphanoplasmodial species may develop. Examine the preparation daily with a stereomicroscope. Hopefully plasmodia and/or fruiting bodies will develop within several days to 1 mo.

Another approach: use banana peels in sterile humid chambers, e.g. 15 X 100 mm Petri dishes lined with filter paper dampened with sterile distilled water. Davis (1967) added 1 X 2 inch pieces of banana peels to such chambers and obtained plasmodia and fruiting bodies within 1 mo. Most isolates were species of *Didymium* followed by *Physarum*, *Stemonitis*, *Comatricha* and *Arcyria*. Ling (1999, and personal communication) used 23 X 33 cm metal cake pans lined with two layers of paper towels. The towels should be saturated with chlorine free water. If sterile lab distilled water is unavailable, bottled drinking water can be used. After draining the excess water add four entire banana peels and add an additional 1/2 cup of water and cover tightly with plastic wrap.Too much free-standing water is to be avoided as it inhibits plasmodial development. Keep away from bright light and excessive heat. When plasmodia develop, add sterile Quaker Old Fashion Oat Flakes. Ling (1999) reports he obtained one plasmodium from 12-20 Panamanian banana peels and one plasmodium per 15-30 Costa Rican and Honduran banana peels. He, like myself, got no Myxomycetes from Ecuadoran banana peels.

Simple Methods for Preparing Stasis Cultures of Myxomycetes

The following methods are simple alternative stopgap 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 methods are presented in order of decreasing long time survival effectiveness.

I have been able to store and revive cultures after 40 years using a simple lyophilization method (Kerr, 1965). However, if one doesn't hear a pop when the tube is opened, there is little chance the cells will revive. This technique requires the availability of a machine shop to build the lyophilization apparatus.

Agar cultures of Myxomycetes wrapped in parafilm strips and stored at 5°C in a refrigerator can survive for 1-2 mo and occasionally up to 1 yr.

I formerly advocated using air-drying of cultures for short term storage of cultures, either on sterile 22 mm^2 coverslips or in sterile plastic Petri dishes. I no longer endorse this approach.

I am currently experimenting with the storage of Myxomycete cultures on silica gel (Raper, 1984 p. 83). My results will be presented in due time.

Challenges for the Future

Close to 100 % of the species of cellular slime molds and protostelids have been grown in agar culture (Haskins and Wrigley de Basanta, 2008). Gray and Alexopoulos (1968) pointed out that only 15% of Myxomycetes had been cycled spore-to-spore on agar. Due to the subsequent increase in described species this number is currently closer to 10% (Haskins and Wrigley de Basanta, 2008). Why is agar culture of Myxomycetes so difficult? There is probably no single answer. Was there insufficient sampling of multiple collections of a taxon? Were improper culture approaches used and/or was there poor choice of media? Did blocked meiosis prevent germination? Determined future researchers will lead us out of this impasse.

I challenge you to get in spore-to-spore agar culture species of the following genera: *Lycogala, Dictydium, Hemitrichia, Trichia, Lamproderma, Colloderma, Listerella, Barbeyella, Tubifera, Cribraria,* and *Brefeldia*. This list is only a fraction of what remains to be done but serves as a challenge for us all. More practical is getting agar cultures of the following and subsequently depositing them in an archival repository such as the ATCC. These taxa have previously been reported in agar culture: *Badhamia utricularis, Physarella oblonga, Diachea splendens, Lamproderma splendens, Calomyxa metallica, Leocarpus fragilis, Amaurochaete comata, Perichaena vermicularis, Metatrichia vesparium,* and *Mucilago crustacea*. This abbreviated list only hints at the need to properly comb the literature to make a proper "wanted" list of the Myxomycetes that can be "tamed" for laboratory use.

If one is determined to start agar cultures from spores, it would be useful to develop techniques to assess spore viability. Cell culturists use a 0.4% (w/v) Trypan blue solution to determine cell viability. If spore viability is indicated but germination doesn't occur, try various methods to induce hatching. Agar cultures can also be started with plasmodial and sclerotial material. Of course one must induce sporulation to verify the taxonomic identity of the culture using characteristics of the sporophores.

Even with the paucity of agar cultures (see list of Myxomycetes available from the ATCC), those available, along with the considerable fresh field and herbarium specimens of sporophores, have allowed us to begin the first steps toward building phylogenetic trees based upon nucleotide chemistry (Fiore-Donno, 2005). And the genome analysis of *Physarum* polycephalum is all but complete (Physarum genome project). Forward march! and as Max cried: "Let the wild rumpus start!"

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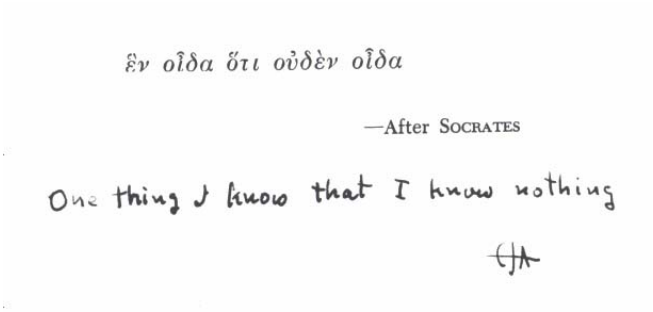
Socrates quote & CJA translation



Or as two of my research colleagues have said of my scientific ideas and contributions: "Very interesting, if proven correct"[4] —Prof C.J. Alexopoulos and "Ich bin skeptisch."—Dr. Hans-Henning Heunert.

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I take no credit for any of these ideas but admit to any errors as mine alone.

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[4] See Sir Karl Poppers' views on the concept of scientific proof.
[5] "He led where any dared to follow."

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Appendix 1

Sample checklist for culture methods

1) Source of culture material

Note: spores, plasmodia, amoebae, swarm cells, amoebal cysts, plasmodial cysts, sclerotia.

2) Analysis of growth phase

Note: lag, log, stationary and decline/death phases. **NB** It can even be difficult for experienced culturists to identify these stages.

3) Media

Note: brand of agar, agar concentration (w/v), type of water used in preparation, buffered or unbuffered, if buffer system used, its molarity, and pH range during culture, additives to agar e.g. cornmeal extract, bark extract, etc. List all media attempted and their effectiveness.

4) Petri dish used

Note: glass or plastic, 60 mm or 100 mm dia cultures grown unsealed or sealed.

5) Food organisms used to feed culture

Note: if original microbial mélange is used or specific taxon of bacterium or yeast.

Note: if grown in GPY, nutrient broth, trypticase soy broth, Mueller Hinton broth, etc. or on the surface of agar versions of these media and subsequently harvested and suspended in e.g. sterile distilled water, etc. and spread as a lawn on an appropriate agar medium.

6) Additives to surface of agar plates

Note: Sterile oat material, sterile rice material or other grains or e.g. if a film of sterile distilled water used.

7) Temperature

Note: temperature range during culture.

8) Light/Dark regime

Note: length of light and dark periods and length of time in light when cultures are examined, is light natural or fluorescent.

9) Special conditions

Note: e.g. activated charcoal, etc.

10) Additional resources for culture ideas

Note: dictyBase, etc.

Appendix 2

Rationale for media construction.

Axenic or pure culture, although usually very difficult, is the ultimate goal (Gray and Alexopoulos, 1968; Balaji et al., 1999). For the difficult to cultivate Myxomycetes, it might initially be necessary to employ a xenic culture consisting of various bacteria, yeasts, fungal conidia, protozoa, algae or even micro-animals as food organisms. But every effort should b emade to get the culture monoxenic. In this case most investigators will be growing the eumycetozoan of interest in association with a food organism such as a bacterium or yeast. Therefore, in addition to the nutritional needs of the eumycetozoan one must consider the growth needs of the food organism. More problematical, a medium must be chosen that supports growth of both.

Concerning the rationale used in the construction of microbial media, Prof. James T. Staley, Department of Microbiology, University of Washington, Seattle, WA states:

> "Media composition is a topic in and of itself. Clearly all of the elements found in organisms must be provided to them in some form. These are CHONPS plus elements such as Ca, Fe, Mg and trace elements too.
>
> The form in which these are supplied to the microorganism depends on what its genetic machinery is capable of accomplishing. For example, a nitrogen-fixing bacterium would not require ammonia or nitrate, but can use N_2 from the air. Likewise, a photosynthetic organism (aside from the non-sulfur purples) do not need organic carbon but can use CO_2. Some organisms such as the lactic acid bacteria require some amino acids but do not need Fe and other bacteria may require vitamins, etc.

As regards sources of energy for growth, microbes fall into one of two categories. Photosynthetic organisms require light as an energy source whereas chemotrophic organisms require chemical compounds. The chemotrophic group can be further subdivided into two groups (a) those that can use inorganic chemical compounds as energy sources; these are termed chemolithotrophs and (b) those that require organic compounds for their energy source which are called chemoorganotrophs or chemoheterotrophs or simply heterotrophs."

Students of the eumycetozoa can take this as wise advice from a skilled practitioner of microbiology as we select the most effective media for our needs.