Modernization of botanical laboratory procedures

Margaret Barton Timnick

University of Iowa

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MODERNIZATION OF BOTANICAL LABORATORY PROCEDURES

by

Margaret Barton Timnick

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science, in the Department of Botany, in the Graduate College of the State University of Iowa

June, 1947
ACKNOWLEDGEMENTS

The author wishes to express sincere appreciation to

Dr. H. L. Dean for suggesting the study and guiding its progress throughout the period of research and to Dr. G. W. Martin for his helpful suggestions relating to the sections on fungi.
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MODERNIZATION OF BOTANICAL LABORATORY PROCEDURES

INTRODUCTION

The following thesis represents a sharp departure from the conventional form usually employed for such writings. Instead of exhaustively developing one subject along traditional lines of development, eleven separate topics are presented, each a more or less complete and workable unit. Each unit contains the results of original and practical experiments by the writer. A review of selected literature is included in each unit. Numerous articles were read which did not apply directly to any of the eleven units. The most useful of these in regard to teaching aids have been brought together and included as a general bibliography.

The primary purpose of these experiments was to develop tested methods for teaching use in an elementary botany laboratory. Prime consideration has been given to experiments which could be performed by students themselves within the limits of an ordinary two hour laboratory period.
CULTURING MYXOMYCETE PLASMODIA FOR CLASSROOM USE*

The Myxomycetes are organisms which exhibit both animal and plant characteristics. This dual nature is of considerable interest and worthy of consideration in planning an elementary botany course. Since the plasmodial and fruiting stages can easily be cultured and recognized, the study of these organisms is a practical project.

In the present study bark was collected from well rotted elm, cottonwood, soft maple, oak stumps and from a decaying elm branch found in cool moist places. Oak, ash, aspen, box elder and elm leaves were collected from pockets in the sod where they had gathered and wintered. These collections were made at Iowa City during the latter part of February.

Collected bark was cut into suitable lengths to fit into standard petri dishes 15 mm. deep. It is essential that the pieces of bark be small to allow some space, preferably 5-10 mm., between the pieces of bark and the top of the petri dish, otherwise the plasmodia may crawl to the top of the petri dish from which place they are less conveniently transferred. For classroom use the standard petri dishes were found to be the most convenient, although larger containers may be used if desired. The pieces of bark were thoroughly moistened with distilled water. If any excess water collected in the bottom

of the dish, it was drained off. The leaves, cut into suitable lengths to fit into the petri dishes, were soaked in distilled water for a few minutes. Several of these pieces were pressed together and placed in dishes.

The material stored at room temperature, but not in direct sunlight, developed plasmodia first. The cultures at room temperature, but in partial darkness, were somewhat slower in producing plasmodia. Some which were placed in an oven at 30° C did not produce any. The development of plasmodia is tabulated in Table I.

The pieces of bark or leaves on which the plasmodia developed were placed on Knop's agar in petri dishes. (Figure 1). The following formula for Knop's solution was used:

\[
\begin{align*}
\text{Ca(NO}_3\text{)}_2 & = 1.0 \text{ gm.} \\
\text{KNO}_3 & = 0.26 \text{ gm.} \\
\text{KH}_2\text{PO}_4 & = 0.25 \text{ gm.} \\
\text{MgSO}_4 & = 0.25 \text{ gm.} \\
\text{FePO}_4 & = \text{trace} \\
\text{Distilled H}_2\text{O} & = 1 \text{ liter}
\end{align*}
\]

Dilute this solution 1:6 with distilled water.

Fifteen grams of agar was dissolved in one liter of diluted Knop's solution and the resulting solution was tubed, autoclaved at 15 pounds for 15 minutes and poured into sterile petri dishes. Plasmodia usually crawled off of the bark or leaves on to Knop's agar in a few hours. It was found that yeast increased the mass of some species (Table I) and these organisms developed along traces of yeast which were streaked on the agar. (Figure 2). The yeast streaks may be made either with yeast from the inner portion
Table I

<table>
<thead>
<tr>
<th>Species</th>
<th>Flasmosia Color</th>
<th>Substrate</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physarum confertum*</td>
<td>white</td>
<td>very rotten inner portion of elm bark</td>
<td>10 days</td>
</tr>
<tr>
<td>Ophiostoma chrysosperma</td>
<td>white, then rosy</td>
<td>outer portion of oak bark</td>
<td>10 days</td>
</tr>
<tr>
<td>Arceya demdata</td>
<td>white</td>
<td>outer portion of elm branch bark</td>
<td>15 days</td>
</tr>
<tr>
<td>Physarum melleum</td>
<td>yellow</td>
<td>elm leaf</td>
<td>19 days</td>
</tr>
<tr>
<td>Unknown</td>
<td>white</td>
<td>elm leaf</td>
<td>19 days</td>
</tr>
<tr>
<td>Unknown*</td>
<td>pink</td>
<td>ash leaf</td>
<td>19 days</td>
</tr>
<tr>
<td>Didymium squamulosum</td>
<td>white</td>
<td>outer portion of cottonwood bark</td>
<td>22 days</td>
</tr>
<tr>
<td>Unknown</td>
<td>white</td>
<td>outer portion of elm bark</td>
<td>22 days</td>
</tr>
<tr>
<td>Physarum diermodies*</td>
<td>white</td>
<td>outer portion of elm bark</td>
<td>26 days</td>
</tr>
<tr>
<td>Physarum sp.*</td>
<td>yellow</td>
<td>outer portion of elm bark</td>
<td>26 days</td>
</tr>
<tr>
<td>Physarum nucleatum</td>
<td>white</td>
<td>outer portion of elm branch bark</td>
<td>29 days</td>
</tr>
<tr>
<td>Unknown</td>
<td>white</td>
<td>inner portion of soft maple bark</td>
<td>28 days</td>
</tr>
<tr>
<td>Didymium nigripes</td>
<td>white</td>
<td>oak leaf</td>
<td>31 days</td>
</tr>
<tr>
<td>Didymium anellum</td>
<td>white</td>
<td>ash leaf</td>
<td>35 days</td>
</tr>
</tbody>
</table>

* Developed along yeast streaks.
of a moist yeast cake or from a yeast culture.

The yeast culture was made by adding a small amount of moist yeast cake to nutrient agar and allowed to grow several days before using. When 2% maltose was added to the agar solution and after hardening was streaked with yeast, the plasmodia increased in mass but the plates were easily contaminated and consequently were not suitable for demonstration purposes.

Plasmodia may be transferred to fresh agar plates by cutting the agar around the plasmodium, slipping a spatula under the piece of agar and placing it on a fresh plate. Plasmodia can be kept active for approximately 50 days, by frequently transferring them and feeding yeast.

These cultures may be made at any time of the year but are less satisfactory during the summer months, unless it is possible to find a cool place to grow them.

For classroom use the petri dishes containing the plasmodia grown on agar may be turned over, placed under the low power of the microscope and the naked protoplasm, gross movement and streaming of protoplasm may be studied. The protoplasmic streaming is observed to be first in one direction, it then slows down, stops and reverses its direction. This process may be timed by students and usually fascinates them.

The swarm cells may be used to illustrate motile gametes, the union of gametes and are usually very interesting. Swarm cells
can be germinated from some species such as Reticularia lycoperdon, and the more common species Euteridium roseum by placing the spores in rain water which had not been collected in a metal container. In this study, the spores of Reticularia lycoperdon were placed in water boiled with a small amount of charcoal to remove metal ions. These were stored at room temperature and germinated in about 48 hours. It is possible for some collections of some species to germinate in 90 minutes, although the time will vary both with the species and with the particular collection. After a collection has once been found to produce swarm cells it may be kept for a number of years and will produce swarm cells when needed.
BIBLIOGRAPHY


Figure 1. Myxomycete plasmodium crawling off bark onto Knop's agar.
Figure 2. Myxomycete plasmodium increasing in mass and developing along traces of yeast streaked on Knop's agar.
Saprolegnia and Aohlyta are saprophytic on plant and animal debris lying in water. Some parasitize fresh-water and marine algae, and a few attack roots of terrestrial flowering plants. Tiffney (21) found these fungi capable of attacking a wide range of hosts, parasitizing frogs and salamanders as well as fish of nine different families. Of all the water animals Tiffney (21) used in his study of Saprolegnia, only the Eel, Anguilla chrysops, could be considered immune to the fungus, since this fish, even when injured, did not contract the disease.

In this study, pond water containing small quantities of decaying organic matter was collected in large bottles from ponds near Iowa City. This followed Kauffman's (12) procedure of collecting water from rivers, ponds, lakes, ditches, brooks, and springs which contained algae or other aquatic plants, or merely decaying vegetable matter. Fitzpatrick (8) reported that Saprolegniales occur more or less abundantly in all water, usually preferring those which are clear and relative pure. In stagnant or polluted water their development is retarded by the presence of bacteria and infusoria. An exception is Leptomitus lacteus which may grow in water containing large amounts of organic material. Therefore, stagnant or polluted water should not be collected. About 250 c.c. of the collected pond water was placed in specimen dishes. Flies, clover seeds, cracked hemp seeds, sunflower seeds, corn, buckwheat seeds, bees, and cockroaches were
added to the pond water. Cultures were stored at room temperature away from direct sunlight. Flies were found to be the most successful bait, as bacteria attacked the seeds and cockroaches too readily. Boric or salicylic acid may be used to inhibit bacteria as suggested by Maurizo (16). Various authors have used different kinds of organic matter as bait for Saprolegnia as shown in Table II. Flies may be collected and stored for six months before using. Humphrey (9) pointed out that if house flies are kept dry they retain their nutritive properties for at least six months.

Hyphae ordinarily appeared within two to four days. A good growth develops in about five days and cultures should then be transferred to filtered, sterilized pond water. After pond water is filtered and sterilized it should be allowed to stand over night to absorb oxygen, as suggested by Ward (23) who found the retention of the spores of Achlya polyandra was influenced by poor aeration. These cultures are placed in a refrigerator at 10°C, for about two days and then removed to room temperature. When the surrounding water attains room temperature tips of hyphae are cut off and mounted on a slide. Zoospores usually begin moving about five minutes before escaping from the zoosporangia. (see Figure 3.). Another method of securing zoospore discharge is reported by Coker (3), who found that Saprolegnia on ant larvae in distilled water containing 0.1% Na₂HPO₄ produces many sporangia which discharge zoospores. Kancheese (11) grew cultures on dead fish eggs in a refrigerator at 10°C. The fungus grew "luxuriously" and
produced zoosporangia. Coker (4) found a strong tendency for the spores to be retained in the sporangium as a result of bacterial contamination, foulness from any cause, or when the parts are placed in liquid nutrient media. When discharged under these circumstances the spores germinate at once without developing the second swimming stage.

Various conditions affect the development of oogonia and antheridia. Oogonia develop in about two weeks when the cultures are stored in a refrigerator at 10° C. Peterson (18) suggested that low temperature of the water is probably a factor in the formation of oogonia. Obel (17) reported that mycelium, grown on a pea dextrose or gelatine with yeast extract, when placed in pure water first formed numerous zoosporangia, and later, oogonia. Davis (7) found that Saprolegnia ferax cultivated on boiled whites and yolks of eggs or dried beef, did not produce antheridia but oogonia were formed abundantly. Kolbs (18) placed cultures developing zoosporangia under such conditions that the hyphae were no longer submerged, and oogonia were produced. The material was removed from water and placed in a dish of cold agar, which furnished enough moisture to support the fungus for several weeks. The filaments out of water promptly developed oogonia, even when they had the form characteristic of zoosporangia. Johansen (1) suggested transferring highly nourished mycelium to a 0.1% solution of leucine. The sex organs should appear in about 24 hours. Another method of developing sex
organs suggested by Johanson (10) is to boil corn kernels for 20 minutes then cut into small pieces and place in petri dishes with pond water. The sex organs may appear about four days after inoculation. Coker (3) found sexual reproduction did not occur in ordinary water cultures on flies, ant larvae, gnats, mushroom grubs, etc. Kanouse (11) placed two sterilized hemp seeds in 20 c.c. of sterile distilled water to which zoospores were added. At the end of four weeks many sexual organs in the early stages of development were found.

*Saprolegnia* and *Achlya* grow better in the spring. Coker's (4) studies have shown that, for the great majority of species, spring is the most favorable season for growth. There are eleven species which were found in a greater percentage of the collections in spring, 3 in winter, 3 in summer and 2 in the fall. If comparison is made between the six cold and the six warm months, little or no difference in abundance of these species is found, ten appearing more frequently in the warm and nine in the cold months. Coker (4) placed three young cultures on grubs in an ice box. Growth proceeded similarly to that of cultures at room temperature; few sporangia were formed, with oogonia scattered and very few antheridia present. Trow (22) found that in hot weather bacteria unfavorably affect the healthy development of the culture.

*Saprolegnia* and *Achlya* may also be obtained from the soil. Bose (19) suggested the following procedure for obtaining *Saprolegnia* from the soil. Fifty c.c. of soil, which at times contained the roots
of plants, is shaken with water filtered through charcoal and left standing until the water above the soil had become clear. Several pieces of cracked hemp seeds were added and the bottle was placed in a shaded part of a cold-temperature room. *Saprolegnia* or *Ashlya* usually develop on the cracked hemp seeds in a few days.
<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Substrate</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trow</td>
<td>1895</td>
<td>House flies, hard boiled egg cut up in minute oblong pieces and floated on the surface of the water</td>
<td>Flies were most satisfactory. The house flies were wetted with alcohol and washed with water. Flies were killed with chloroform to prevent injury to their chitinous coat.</td>
</tr>
<tr>
<td>Maurissei</td>
<td>1894</td>
<td>Mealworms, flies, cress-seedlings, peptone, cane sugar, milk sugar, egg albumen, Lycopod's extract, beef broth, and glycerine</td>
<td>The fungi on all of these flourished.</td>
</tr>
<tr>
<td>Davis</td>
<td>1902</td>
<td>Raw beef, fresh insects, boiled white and yolks of eggs, and dried beef.</td>
<td>By cultivating on a rich substratum as raw beef or fresh insects, a much more extensive growth of antheridial filaments was obtained, as in Saprolegnia monoida.</td>
</tr>
<tr>
<td>Hauffman</td>
<td>1908</td>
<td>Flies, small pieces of dry beef</td>
<td>Flies found to be better because bacteria attacked the beef too quickly.</td>
</tr>
<tr>
<td>Peterson</td>
<td>1910</td>
<td>Fish, spawn of toads, frog eggs, toads, frogs, leeches, earthworms, cray fish, snails, insects which fall into the water</td>
<td>These animals were found with Saprolegnia growing on them.</td>
</tr>
<tr>
<td>Lechmere</td>
<td>1910</td>
<td>Small pieces of white of eggs hard boiled, pieces of fish, skins of fish, pieces of beef, dead flies</td>
<td>All of these with the exception of the Abutilon</td>
</tr>
<tr>
<td>Coker</td>
<td>1909</td>
<td>Grats, flies, wasps, mosquitoes and spiders</td>
<td></td>
</tr>
<tr>
<td>Barrett</td>
<td>1912</td>
<td>Sterilized flies, millipedes, Dipterus</td>
<td></td>
</tr>
<tr>
<td>Author</td>
<td>Year</td>
<td>Substrate</td>
<td>Remarks</td>
</tr>
<tr>
<td>-----------------</td>
<td>------</td>
<td>---------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Barrett</td>
<td>1912</td>
<td>Larvae, larvae and pupae of ants, aphids, boiled egg albumen, boiled root of Abutilon</td>
<td>Root and egg albumen proved to be serviceable. The sterilized flies, aphids, and ant pupae were more extensively used.</td>
</tr>
<tr>
<td>Coker</td>
<td>1923</td>
<td>Corn meal agar, boiled butter beans, white of egg, termites, boiled willow twig</td>
<td>Corn meal agar plentiful gemmae, no oogonia, butter beans, white of egg, and termites strong growth, many gemmae, often numerous oogonia</td>
</tr>
<tr>
<td>Couch</td>
<td>1924</td>
<td>Mushroom grubs, bits of boiled corn grain, termites</td>
<td></td>
</tr>
<tr>
<td>Couch</td>
<td>1927</td>
<td>Boiled hemp seeds</td>
<td></td>
</tr>
<tr>
<td>Fitzpatrick</td>
<td>1930</td>
<td>Sterilized flies, bits of egg albumen, ant pupae, aphides, and cooked peas</td>
<td></td>
</tr>
<tr>
<td>Rose</td>
<td>1932</td>
<td>Hemp seeds, oat meal agar, corn meal agar, flies, cockroaches</td>
<td>Cracked hemp seeds were very successful. Saprolegnia was observed on the remains of the woody or herbaceous land plants, dead fish and insects.</td>
</tr>
<tr>
<td>Hanouse</td>
<td>1932</td>
<td>Flies, dead fish eggs, hemp seeds, pea broth, lucine</td>
<td></td>
</tr>
<tr>
<td>Miller and Blaydes</td>
<td>1938</td>
<td>Dried bees, flies, grasshoppers</td>
<td></td>
</tr>
<tr>
<td>Johansen</td>
<td>1940</td>
<td>Dead flies, bees, meal worms, ant eggs, small dead flies</td>
<td></td>
</tr>
<tr>
<td>Sass</td>
<td>1940</td>
<td>Dead fish, water insects, steam sterilized flies</td>
<td></td>
</tr>
</tbody>
</table>
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Botany


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Scci. N.S., 1885, 23:272
Figure 3. *Saprolegnia* zoospores escaping from zoosporangium.
CULTURING RHIZOPUS NIGRICANS

A variety of methods have been reported for culturing *Rhizopus nigricans*. Follette (4) grew mold cultures successfully on a cracker or wafer placed in a petri dish with a small amount of moisture. Walker (7) suggested soaking six lima beans about 48 hours in water and then placing them in a 125 ml Erlenmeyer flask stored in a moist chamber. The mycelium developed along the side of the flask. Rhizoids, stolons, and sporangiophores are easily observed and distinguished under the low power of the microscope. Portions of this culture may be transferred by means of a scalpel to a drop of 10% glycerine on a microscope slide and covered with a cover glass. In this condition the streaming of protoplasm in the sporangiophores may be observed. Miller and Blaydes (6) and Briscoe (2) have suggested other more complicated methods for culturing *Rhizopus nigricans*.

Chapman (3) inoculated small pieces of moist bread placed on black and white squares of cardboard. After they had been in a moist chamber for a few days the black squares contrasted with the white mycelium and the white cards with the black, mature sporangia of the fungus.

Following this principle the writer used two white one inch squares of filter paper and two black one inch squares of paper, all well soaked but not dripping wet with orange juice and placed in a checkerboard pattern in a petri dish. These squares were inoculated
with spores of *Rhizopus nigricans* and stored at room temperature in subdued light. The mycelium should develop enough in two days so that it may be observed on the black squares. Black sporangia may also be observed on the white squares. The petri dish may be turned over and placed under the low power of the microscope for detailed study of the fungus. By this method it is possible for students to observe the natural growth of *Rhizopus*. It is possible to see protoplasmic streaming by removing the cover of the petri dish and focusing on a hypha at the edge of the paper.

**Germination of Spores**

Aquin (1) demonstrated a simple and easy method of obtaining early stages in the germination of *Rhizopus nigricans* spores. Spores were sowed on water in which a few pieces of bread had been soaked for a few minutes. Good germination of the spores occurred in about six hours. A longer period is required if more advanced stages are desired. A drop of the liquid may be placed on a slide and gently covered to avoid damaging the germinating spores. Robbins and Rickett (8) reported that spores germinated in 2-3 hours in dilute prune juice. Fleming and Smith (6) germinated mold spores on cellophane, on nutrient material.

The writer found that excellent germination occurs when *Rhizopus nigricans* spores are added to a 50% solution of filtered orange juice and stored in an oven at 30°C. (Figure 4). Under these conditions spores germinate within four hours. Spores will also germinate in about five hours at room temperature in a 10% molasses solution. Spores placed in distilled or tap water do not germinate.
Rhizopus spores were experimentally placed on various substrates stored at room temperature in subdued light. Results of these experiments are shown in Table III.

From these experiments it is evident that cooked mashed white potato, orange juice, grapefruit, cooked buckwheat flour, moistened buckwheat flour, bread and mash potatoes are very good substrates for the growth of Rhizopus. Filter paper soaked with orange juice, placed in a petri dish and inoculated with Rhizopus spores provides a very good substrate and the cultures are easily prepared and handled.
### Table III

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>lemon juice</td>
<td>did not grow</td>
</tr>
<tr>
<td>raw white potato</td>
<td>did not grow, dried out too quickly</td>
</tr>
<tr>
<td>cooked mashed white potato</td>
<td>in two days a very good growth</td>
</tr>
<tr>
<td>hard boiled egg white</td>
<td>did not grow, bacteria attacked it</td>
</tr>
<tr>
<td>hard boiled egg yolk</td>
<td>after four days started to grow, bacteria</td>
</tr>
<tr>
<td>white karo syrup</td>
<td>attacked it</td>
</tr>
<tr>
<td>brown karo syrup</td>
<td>did not grow</td>
</tr>
<tr>
<td>molasses</td>
<td>grew slightly</td>
</tr>
<tr>
<td>orange juice</td>
<td>did not grow</td>
</tr>
<tr>
<td>grapefruit juice</td>
<td>a good growth in 2 days</td>
</tr>
<tr>
<td>cooked buckwheat flour</td>
<td>a good growth in 4 days</td>
</tr>
<tr>
<td>buckwheat flour moistened bread</td>
<td>a good growth in 2 days</td>
</tr>
<tr>
<td>sweet potatoes</td>
<td>a good growth in 4 days</td>
</tr>
<tr>
<td></td>
<td>a good growth in 2 days</td>
</tr>
</tbody>
</table>
BIBLIOGRAPHY


9. Turtox Service Leaflet: Culturing the common molds. No. 32
Figure 4. *Rhizopus* spores germinating.
YEAST

The usual directions given for the demonstration of alcoholic fermentation by yeast, call for the inoculation of a "sugar solution" by a "yeast culture." This solution is usually allowed to stand several hours to a day or two until fermentation has occurred. These typical directions fail to indicate what percentage of sugar solution is optimum or what sugars are best. They do not indicate the most favorable concentration of the yeast suspension, and do not recommend temperatures best suited for rapid fermentation. A major objection to carrying this experiment over until the next laboratory period, is that students themselves are not able to follow the progress of fermentation and thus have a legitimate right to question the results. It is desirable that fermentation of yeast be adequately demonstrated within an ordinary two hour laboratory period.

Typical directions for yeast fermentation usually omit methods of testing for carbon dioxide and alcohol. It is obviously advantageous for students to be able to make these tests to identify the products of fermentation.

The writer has met the objections named above by determining experimentally the ideal concentration of sugar solution, the most favorable yeast suspension and the optimum temperature necessary to secure rapid and positive fermentation well within a two hour laboratory period. Tests for carbon dioxide and alcohol have been developed that may be performed by the student in the laboratory.
Various methods for inducing sporulation of yeast cells have been reported but the asci have been small and the methods not always dependable. A further purpose of this study is to report a reliable method for inducing sporulation of yeast cells.

Fermentation of Various Carbohydrates

Prepare a yeast solution by thoroughly mixing one cake of moist Fleischmann's yeast with 100 c.c. of distilled water. Add 5 c.c. of this solution to calibrated fermentation tubes containing 10 c.c. of various carbohydrate solutions. For quick results these tubes should be placed in an oven at 60° C. The time ordinarily required for the formation of 5 c.c. of carbon dioxide gas due to the fermentation of various sugars under these conditions is listed in Table IV.

<table>
<thead>
<tr>
<th>Sugar solution</th>
<th>Time for formation of 5 c.c. of CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% dextrose</td>
<td>60 minutes</td>
</tr>
<tr>
<td>10% dextrose</td>
<td>40 minutes</td>
</tr>
<tr>
<td>20% dextrose</td>
<td>50 minutes</td>
</tr>
<tr>
<td>5% maltose</td>
<td>95 minutes</td>
</tr>
<tr>
<td>10% maltose</td>
<td>40 minutes</td>
</tr>
<tr>
<td>5% maltose / 5% dextrose</td>
<td>35 minutes</td>
</tr>
<tr>
<td>10% sucrose</td>
<td>40 minutes</td>
</tr>
<tr>
<td>50% brown karo syrup</td>
<td>40 minutes</td>
</tr>
<tr>
<td>50% amalzo corn syrup</td>
<td>40 minutes</td>
</tr>
<tr>
<td>50% white karo syrup</td>
<td>50 minutes</td>
</tr>
<tr>
<td>25% molasses</td>
<td>40 minutes</td>
</tr>
<tr>
<td>50% molasses</td>
<td>45 minutes</td>
</tr>
</tbody>
</table>

From the above results a shortened experiment for the
alcoholic fermentation of various carbohydrates by yeast to be carried out in general botany laboratories could be as follows. Prepare the yeast solution as above (one cake of yeast per 100 c.c. of distilled water), add 5 c.c. of this solution to separate calibrated fermentation tubes containing 10 c.c. of 10% dextrose, 10% maltose, 10% sucrose, 60% brown karo syrup, 50% molasses and 10% lactose (a nonfermentable sugar). Starch or dextrin may be included as other nonfermentable carbohydrates. Place these cultures in an oven at 60° C. A chart should be made by the student to indicate the rate of carbon dioxide formation by the various solutions. Under teaching laboratory conditions it may be expected that fermentation will take from 5 to 10 minutes longer than under the experimental conditions listed above.

**Rapid Fermentation of a Single Sugar**

The above experiments demonstrate fermentability of various carbohydrates. However, it is often desirable to demonstrate fermentation, using a single sugar, when the speed of the reaction is of primary importance. Experiments were conducted to determine the strength of sugar and yeast combinations that would give the most rapid fermentation possible. The inoculum is a very active yeast suspension prepared by dissolving one moist Fleischmann's yeast cake in 100 c.c. of 5% dextrose solution. This should be freshiy prepared and placed in an oven at 50° C. 20 to 30 minutes before using. Five cubic centimeters of this suspension was added to calibrated fermentation
tubes containing 10 c.c. of different concentrations of sugar solutions. The time required for the formation of 5 c.c. of carbon dioxide under these conditions is listed in Table V.

Table V

<table>
<thead>
<tr>
<th>Sugar solution</th>
<th>Time for formation of 5 c.c. of CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% dextrose</td>
<td>40 minutes</td>
</tr>
<tr>
<td>10% dextrose</td>
<td>45 minutes</td>
</tr>
<tr>
<td>20% dextrose</td>
<td>45 minutes</td>
</tr>
<tr>
<td>50% amaiso corn syrup</td>
<td>40 minutes</td>
</tr>
</tbody>
</table>

The above procedure was repeated, except that a moist Fleischmann's yeast cake was dissolved in 100 c.c. of 10% dextrose to make the inoculum. The time required for 5 c.c. of carbon dioxide gas to form due to fermentation of different concentrations of different sugar solutions under these conditions is listed in Table VI.

Table VI

<table>
<thead>
<tr>
<th>Sugar solution</th>
<th>Time for formation of 5 c.c. of CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% dextrose</td>
<td>40 minutes</td>
</tr>
<tr>
<td>10% dextrose</td>
<td>40 minutes</td>
</tr>
<tr>
<td>20% dextrose</td>
<td>35 minutes</td>
</tr>
<tr>
<td>10% sucrose</td>
<td>45 minutes</td>
</tr>
<tr>
<td>50% amaiso corn syrup</td>
<td>40 minutes</td>
</tr>
</tbody>
</table>

In another group of experiments, a moist Fleischmann's yeast cake was dissolved in a 50% amaiso corn syrup solution to form the inoculum. When added to a 50% white karo syrup solution 5 c.c. of carbon dioxide was produced in 50 minutes; a 50% amaiso corn syrup
solution with the same inoculum produced 5 c.c. of carbon dioxide in 55 minutes. A different inoculum was prepared by suspending one Fleischmann's yeast cake in a 50% molasses solution. When added to a 50% amaloso corn syrup solution it produced 5 c.c. of carbon dioxide gas in 46 minutes, 5 c.c. of carbon dioxide in 50 minutes with a 50% white karo syrup solution and 5 c.c. of carbon dioxide in 55 minutes with a 50% molasses solution.

The above procedure may be modified by using 10 c.c. of yeast solution and 10 c.c. of sugar solution and placing in an oven at 50°C. Prepare the inoculum by adding one moist Fleischmann's yeast cake to 10 c.c. of 5% dextrose solution. When added to a 50% amaloso corn syrup solution it produced 5 c.c. of carbon dioxide in 35 minutes. When the inoculum is prepared by dissolving a yeast cake in 100 c.c. of 10% dextrose solution and added to a 50% amaloso corn syrup solution it produced 5 c.c. of carbon dioxide in 50 minutes.

In another group of experiments the above procedure was modified by using 10 c.c. of yeast solution and 5 c.c. of sugar solution placed in an oven at 50°C. The inoculum was prepared by adding one cake of yeast to a 100 c.c. of 5% dextrose solution. When added to a 50% amaloso corn syrup solution 5 c.c. of carbon dioxide gas was formed in 20 minutes. When a yeast cake was dissolved in 100 c.c. of 10% dextrose solution and 10 c.c. of this inoculum added to 5 c.c. of a 50% amaloso corn syrup solution, 5 c.c. of carbon dioxide gas formed in 25 minutes.
From the above experiments it appears that the most rapid fermentation is secured when 5 c.c. of 50% amaze corn syrup solution is inoculated with 10 c.c. of yeast solution prepared by dissolving one moist Fleischman's yeast cake in 100 c.c. of 5% dextrose solution and placed in an oven at 50° C. Five cubic centimeters of carbon dioxide are formed in only 20 minutes. When 10 c.c. of a 50% amaze corn syrup solution is inoculated with 10 c.c. of yeast solution prepared by dissolving one cake of yeast in 100 c.c. of 10% dextrose solution and placed in an oven at 50° C. it required 50 minutes for 5 c.c. of carbon dioxide gas to form.

**Test for Carbon Dioxide**

After fermentation has proceeded until the side arm of the tube is completely filled with carbon dioxide, drop 20 pellets of potassium hydroxide into the solution so that they come in contact with the gas. As the carbon dioxide is absorbed by the potassium hydroxide, the liquid gradually rises in the side arm. This should require approximately 4 minutes. A control may be set up by pouring the unfermented sugar-yeast solution into a fermentation tube and adjusting so that air remains in the side arm. Twenty pellets of potassium hydroxide added as above should cause no appreciable rise of the liquid in the side arm of the tube.

**Fermentation at Room Temperature and Tests for Carbon Dioxide**

Fermentation may be carried out and carbon dioxide tested for
at room temperature by the following method. Two cakes of moist Fleischmann's yeast are pulverized and suspended in 200 c.c. of 50% brown karo syrup solution in an Erlenmeyer flask. Gas formed in the resulting fermentation is conducted by a bent glass delivery tube to another Erlenmeyer flask containing 200 c.c. of saturated barium hydroxide (baryta water). The set-up is shown in Figure 5. After preparing barium hydroxide solution for this experiment care must be taken to protect it from the atmosphere by keeping it in a closed container.

Figure 5.
If a precipitate forms it should be filtered before use. After fermentation has proceeded for approximately 5 minutes, bubbles of gas can be observed rising in the barium hydroxide solution. When fermentation has proceeded approximately 16 minutes a white precipitate forms in the barium hydroxide solution. This is due to the reaction between the carbon dioxide produced by fermentation and the barium hydroxide, resulting in the formation of insoluble barium carbonate.

Another method of testing for carbon dioxide formed by yeast fermentation is by the use of indicators. The above procedure and apparatus may be used, except an indicator is substituted for the barium hydroxide. The carbon dioxide bubbling through the indicator solution will cause it to change from its basic color to the acidic color, due to the formation of carbonic acid. The time required for various indicators to change color under these conditions is listed in Table VII.

Table VII

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Concentration</th>
<th>Basic Color</th>
<th>Acidic Color</th>
<th>Time to change color</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.015% phenol</td>
<td>3 drops per 50</td>
<td>red</td>
<td>yellow</td>
<td>1 minute</td>
</tr>
<tr>
<td>red</td>
<td>c.c. of water</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.04% prom-thymol blue</td>
<td>5 drops per 50</td>
<td>blue</td>
<td>yellow</td>
<td>2 minutes</td>
</tr>
<tr>
<td>phenol red</td>
<td>c.c. of water</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.015% neutral red</td>
<td>1 drop per 50</td>
<td>yellow</td>
<td>red</td>
<td>4 hours</td>
</tr>
<tr>
<td></td>
<td>c.c. of water</td>
<td>orange</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Other indicators probably could be used to test for carbon dioxide but time limitations prevented further experiments in the present study.
Test for Alcohol as a Reducing Substance

The usual methods of testing for ethyl alcohol are time consuming and require equipment not found in the usual elementary botany laboratory. For example, colorimetric methods of testing for alcohol obviously require a colorimeter, a rare instrument in elementary botany laboratories. There is also the iodoform test for alcohol. This test depends upon the characteristic odor and crystal formation. These characteristics are not easily observed by beginning botany students.

A simple test for alcohol as a volatile reducing substance may be performed as follows. To prepare the testing reagent dissolve .15 gm. of potassium dichromate in 15 c.c. of distilled water, add 28 c.c. of concentrated sulfuric acid and dilute the solution to 50 c.c. with distilled water. Place 1 c.c. of this solution in a 50 ml. Erlenmeyer flask. A piece of filter paper wrapped around a cork is moistened with a fermented sugar-yeast solution, exercising extreme care to blot away excess liquid in order to prevent any trace of this liquid from dripping into the reagent (Figure 6). The dextrose in this liquid, like alcohol, is a reducing agent, and therefore, would quickly cause a color change if accidentally mixed with the testing reagent. The filter paper and cork are quickly placed in the flask. The alcohol evaporates, the fumes are oxidized by the potassium dichromate and sulfuric acid with the coincident reduction of
the dichromate to chromium sulfate. This reduction occurs in
approximately 15 minutes and is accompanied by a change in color from
orange to green. If the reaction is allowed to continue 1-2 hours a
blue color develops. Controls may be set up employing the above pro-
cedure, except that the filter paper in one flask is moistened with a
plain sugar solution and that in another flask with an unfermented yeast
solution. No change in the color of the reagent occurs in these controls.

.Formation of Ascospores

Various methods have been reported for inducing sporulation
of yeast cells but most of the asci produced have been so small that
it is difficult for students to study them. Lindegren and Lindegren (5)
tried various media and finally developed an optimum medium containing
beet leaf extract, beet root extract, apricot juice, grape juice, yeast
(dried), glycerin, agar and calcium carbonate. This medium produced
large, four spored asci. Wickerham, Flickinger and Burton (15) devised
a modification of Henrici's vegetable-juice sporulation medium for
yeast by using mixed juices from eight vegetables (commercially obtain-
able as V-8) and obtained large, four-spored asci. This medium is pre-
pared by adjusting the pH of the contents of one can of V-8 juice (1 pt.,
2 fluid ounces) to 6.8. Disperse one-half a cake of moist Fleischmann's
yeast in this solution, and steam the mixture 10 minutes to kill the
yeast cells and liberate the acid they contain. Again adjust the pH
to 6.8 and then add an equal volume of hot distilled water containing
4% melted agar. Mix the two solutions, tube, and autoclave 15 minutes
at 15 pounds pressure. Avoid unnecessary heating.
Freshly slant the medium not more than 5 hours before use. The medium may also be poured into petri dishes but cultures are more easily contaminated than when in test tubes. Inoculate the medium with a 24 hours yeast culture grown in a 10% dextrose solution (one cake of moist Fleischmann's yeast per 100 c.c. of 10% dextrose solution) or as Wickerham, Flickinger, and Burton (15) have suggested, the medium may be inoculated with 24 hour yeast culture grown on a yeast-extract malt-extract slant incubated at approximately 28° C. After inoculation of the medium the cultures are incubated at room temperature. Good sporulation should occur in about 20 days. The asci will be large and mostly four-spored (Figure 7). The average measurement of the four-spored asci was 10 microns. The spores are easily observed by placing them in a drop of Delafield's hematoxylin.

Good sporulation may be induced by inoculating a 2% plain agar medium with yeast from a 24 hour yeast culture grown in a 10% dextrose solution (one cake of moist Fleischmann's yeast per 100 c.c. of 10% dextrose solution). Ascospores develop in about 4 to 5 days. The asci are usually two-spored and smaller than when grown on the V-8 juice agar. Asci are usually much more numerous on the 2% plain agar than on the vegetable agar. The two-spored asci grown on 2% plain agar averaged 7 microns in diameter.

In order to obtain fairly large asci in a shorter time, the yeast cells may be started as above on the V-8 juice agar medium, allowed to grow for about 4 days, and then transferred to the plain 2% agar medium. In about 5 days asci may be found but there are not as many
four-spored asci nor are they as large as the ones grown on the V-O juice agar. These asci, however, are larger than the ones grown on the plain 2% agar but are not as numerous.

Smith (11) suggested that ascospores could be stained with malachite green and the vegetative cells counterstained with safranine. A drop of distilled water is placed on a clean glass slide and mixed with a sample taken from the yeast culture. After the film has thoroughly dried, flood with a 5% aqueous solution of malachite green and warm gently (for 1 to 3 minutes). Rinse the slides in distilled water and counterstain with 0.5% aqueous safranine-iod for a few seconds. Wash in distilled water to remove excess safranine, dry and mount in clarite. The ascospores are easily distinguished from the other yeast cells by this method of staining but the asci and yeast cells appear to shrink in the process.

Fermentation of Wild Yeasts.

Fermentation by naturally occurring yeasts may be obtained by allowing grapes, dried figs, apples and bananas to ferment. Cultures are prepared by crushing these fruits separately, covering each with a 10% sucrose solution and allowing to remain at room temperature 3–4 days. The naturally occurring, external, wild yeasts on the skins of the fruit cause this fermentation.


Figure 7. Ascospores of yeast cells.
CULTURING STROMATA IN CLAVICEPS

Various methods have been developed in attempts to produce stromata in Claviceps sclerotia. Whetzel and Reddick (4) produced stromata by leaving sclerotia outdoors until spring, then bringing them into the laboratory and placing on moist sand in a covered chamber. They were kept at room temperature. Twelve days later evidence of germination was observed. Kirchoff (2) experimented with sclerotia exposed to varying periods of cold. He found that after 3-6 weeks exposure at 2-3° C., followed by a period of 4-8 weeks at 15° C., good head formation occurred. Moderately moist sand was the substrate. Hensen and Valleau (1) planted Claviceps purpurea sclerotia on water agar in September. He placed some in a cold room at 3° C. and others outdoors. About the middle of the following April, a few of both those in the cold room and outdoors had produced stipes. Some of the sclerotia were overgrown by other fungi and failed to produce stromata. Tiffany (3) surface sterilized Claviceps sclerotia, collected from brome grass, for five minutes in a 10% solution of hypochlorite. These were placed in test tubes on sterile 1% water agar slants and tightly plugged with cotton. The tubes were kept in three chambers at temperatures of 5° C., 10° C., and 15° C., respectively. All sclerotia were tube August 25, 1945, and on February 8, 1946, heads bearing mature perithecia were beginning to form on a sclerotium in one of the tubes held at 5° C., but none were apparent on sclerotia in the 15° C. chamber.
In this study Claviceps purpurea sclerotia, collected from Elymus virginicus, were sterilized for five minutes in a 10% solution of hypochlorite (Clorox). A number of sclerotia were placed in test tubes on sterile 1% water agar slants and others on sterile sand in test tubes. All tubes were plugged tightly with sterile cotton and stored in a refrigerator at 10°C. Other unsterilized sclerotia were placed on sand in a dish, covered with a glass plate and stored in the greenhouse. These cultures were started May 26, 1946, and on August 1, 1946, heads began to appear (Figure 8) in the sterilized cultures on 1% water agar slants. The unsterilized cultures placed in the greenhouse, were badly contaminated with other fungi and did not develop stromata. Similarly, sclerotia placed on sterilized sand and stored at 10°C did not develop stromata and the majority of them were contaminated with other fungi.
BIBLIOGRAPHY


Figure 8. Stromata of Claviceps developing from a sclerotium.
GERMINATION OF MOSS AND FERN SPORES

Moss protonema and early stages of fern prothallia are often needed for laboratory work when it is not possible to collect them outdoors. Therefore, it is advantageous to culture them in the laboratory, making them available at any season.

Robbins and Rickett (5) stated that spores of species of *Fumaria* and *Polytrichium* germinate easily in a suitable nutrient solution. Stages of development ranging from 5–20 days are most useful for laboratory demonstrations. Buds formed in about 3 months. Dark-walled spores retain their vitality longer than those with more transparent walls.

Miller and Blaydes (5) suggested crushing a dry sporangium on the surface of diluted Knop's solution. Spores may germinate in 2 weeks and branched protonema appear in about 4 weeks. Spores germinate in about 10 days when scattered over a medium of 2% agar containing diluted Knop's solution. Buds and young gametophytes may appear abundantly within 2 or 3 months.

In the present study the opercula of the capsules were cut off from various moss species and the spores dusted on sterile plates of Knop's agar. Care was taken to keep the cultures sterile. The following formula for Knop's solution was used:

\[
\begin{align*}
\text{Ca(NO}_3\text{)}_2 & \quad \text{1.0 gm.} \\
\text{KNO}_3 & \quad \text{0.25 gm.} \\
\text{KH}_2\text{PO}_4 & \quad \text{0.25 gm.} \\
\text{MgSO}_4 & \quad \text{0.25 gm.} \\
\text{FePO}_4 & \quad \text{trace} \\
\text{Distilled H}_2\text{O} & \quad \text{1 liter} \\
\text{Dilute this solution 1:4 with distilled water}
\end{align*}
\]
Dissolve fifteen grams of agar in one liter of diluted knop's solution, tube, autoclave at 15 pounds for 15 minutes and pour into sterile petri dishes.

Incubate cultures at room temperatures in a window but do not expose to direct sunlight. The time required for various species of moss spores to germinate under these conditions is shown in Table VIII. A moss protonema growing on knop's agar is shown in Figure 9.

Table VIII

<table>
<thead>
<tr>
<th>Name</th>
<th>Time for Germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dicranum caparum</td>
<td>3 days</td>
</tr>
<tr>
<td>Rhodobryum roseum</td>
<td>4 days</td>
</tr>
<tr>
<td>Bryum caespiticum</td>
<td>4 days</td>
</tr>
<tr>
<td>Leptodiorym riparium</td>
<td>15 days</td>
</tr>
<tr>
<td>Bertramia pomiformis</td>
<td>did not germinate</td>
</tr>
<tr>
<td>Polytrichum sp.</td>
<td>did not germinate</td>
</tr>
<tr>
<td>Atrichum angustatum</td>
<td>4 days</td>
</tr>
<tr>
<td>Atrichum undulatum</td>
<td>6 days</td>
</tr>
<tr>
<td>Brachythecium sp.</td>
<td>6 days</td>
</tr>
<tr>
<td>Brachythecium sp.</td>
<td>7 days</td>
</tr>
<tr>
<td>Brachythecium sp.</td>
<td>8 days</td>
</tr>
<tr>
<td>Heterophyllum haldianianum</td>
<td>7 days</td>
</tr>
<tr>
<td>Plagiothecium sp.</td>
<td>10 days</td>
</tr>
</tbody>
</table>

Moss spores were also scattered on various other media, including soil extract agar, 2% agar and 10% gelatin, with light and temperature conditions the same as above. The soil extract solution was prepared by mixing one liter of distilled water with 1000 grams of garden soil. The mixture was shaken every day, and stored in a refrigerator at 10°C, after which it was filtered and 2% agar added. It was sterilized at 15 pounds pressure for 15 minutes and poured into sterile petri dishes. The results of these experiments are shown in Table IX.
Table IX

<table>
<thead>
<tr>
<th>Name</th>
<th>Medium</th>
<th>Time of germination</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brachytheicum sp.</td>
<td>soil sol, agar*</td>
<td>10 days</td>
<td>prolific growth</td>
</tr>
<tr>
<td>Brachytheicum sp.</td>
<td>soil sol, agar*</td>
<td>8 days</td>
<td>prolific growth</td>
</tr>
<tr>
<td>Brachytheicum sp.</td>
<td>soil sol, agar*</td>
<td>4 days</td>
<td>prolific growth</td>
</tr>
<tr>
<td>Atrichum sp.</td>
<td>soil sol, agar*</td>
<td>9 days</td>
<td>germinated but died</td>
</tr>
<tr>
<td>Brachytheicum sp.</td>
<td>Dil. soil sol, agar**</td>
<td>8 days</td>
<td>prolific growth</td>
</tr>
<tr>
<td>Brachytheicum sp.</td>
<td>2% agar</td>
<td>9 days</td>
<td>cells distorted</td>
</tr>
<tr>
<td>Brachytheicum sp.</td>
<td>10% gelatin</td>
<td>10 days</td>
<td>contaminated and</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>gelatin liquified</td>
</tr>
<tr>
<td>Brachytheicum sp.</td>
<td>dil. Knop's sol.***</td>
<td>8 days</td>
<td>prolific growth</td>
</tr>
</tbody>
</table>

* A soil extract solution was prepared by mixing one liter of distilled water with 1000 grams of garden soil. The mixture was shaken every day, and stored in a refrigerator at 10°C after which it was filtered and 2% agar added. It was then sterilised at 15 pounds pressure for fifteen minutes and poured into sterile petri dishes.

** Soil extract solution was diluted 1:8 with distilled water, 2% agar was added, sterilised and poured into sterile petri dishes.

*** Knop's solution was diluted 1:8 with distilled water, 2% agar was added and the resulting solution was sterilised.
The effect of different temperatures was determined by placing one petri dish with Brachytesciom spores on Knop's agar in an oven at 87°C. four days, after which it was placed in subdued light at room temperature. Another culture was stored in a refrigerator at 10°C. four days and then placed in subdued light at room temperature. Controls started at the same time from the same collection of Brachytesciom were left at room temperature in subdued light. The controls germinated 20 days later, and the culture which had been held 4 days in the oven did not germinate up to the time it was finally discarded at the end of two months.

Various methods of culturing fern prothallia have been reported. Robbins and Biskett (5) recommended using spores of Pteris longifolia which germinate on a suitable nutrient solution in 3 to 4 days and are ready for use within 10 days. Fern gametophytes may be obtained by sowing spores lightly on Sphagnum moss which has been boiled, pressed dry, soaked with nutrient solution and then placed in covered sterile jars. Another method is to sow spores on inverted flower pots placed under bell jars. Prothallia ordinarily mature in 3 months and young sporophytes will appear soon after. The jars should be kept in diffuse light.

A Turtox Service Leaflet (1) contains a simple method of growing fern prothallia. A large flower pot is partially filled with broken pieces of pots or shells. Over this placed a layer of rich loam, bringing the surface up to within an inch or so of the top of the
A layer of fine, clean sand is sprinkled over the top of the layer. Spores are sown over the surface of the sand, the pot covered with a glass plate and placed in a saucer for watering.

Miller and Blaydes (3) recommend drying the fern spores 2 or 3 days before sowing them on a moist pot inverted in a flower pot saucer filled with water. Germination occurs within a few days and prothallia mature in 6-10 weeks. Knop's solution diluted 1:2 with water is used for watering. These authors also suggested using a sterilized Knop's solution containing 2% agar.

Heris (2) made a study of the effect of light intensity on fern development in sterile cultures. She found that 10-50 foot-candles of light were needed for germination and 50-75 foot-candles for development after germination. The light intensity was then gradually increased to about 150 foot-candles until reproductive organs formed.

The writer sowed Polystichium spores on Knop's agar plates prepared as previously described and placed in shaded light. These spores germinated in seven to thirteen days. Spores sown on soil solution agar prepared as previously described germinated in 13 days and grew well. Aspidium spores sown on Knop's agar plates germinated in 25 days and also grew well. A developing fern prothallium grown on Knop's agar is shown in Figure 10.
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1. Anna: Growing fern prothallia in the laboratory. Turtox Service Leaflet No. 44


4. Patterson, Sister May Thomasine: Growing fern prothallia in the laboratory, Turtox News, 23:24-25


Figure 9. Moss protonema growing on Knop's agar.
Figure 10. Fern prothallium growing on Knop's agar.
GERMINATION OF POLLEN

Philpott (9) dusted a small quantity of pollen in a small drop of culture solution previously placed on clean slides. The slides were inverted and placed over troughs of a galvanized-iron moist chamber, designed after that described by Bigsti (6). Water heated to 50° C. was poured in the troughs to increase the humidity and retard drying out of the hanging drop. The moist chamber remained at room temperature 4-6 hours after inoculation. Very good germination of the pollen secured.

Lilling (7) germinated pollen in a drop of solution consisting of 1 gm. of gelatine, 4-5 gm. of cane sugar, and 50 c.c. of distilled water. Pollen was added to the drop, covered with a cover glass and kept in a moist chamber at about 18° C. Good pollen tubes developed in 8-10 hours.

Brink (2) found a semi-solid medium of agar, containing 10% sucrose and a trace of yeast extract to be the most favorable for optimum growth of pollen tubes.

Martin (8) tried using small squares of parchment paper soaked in distilled water and in 0.6646, 0.7314, and 0.662 volume normal sucrose solution. Surface moisture was removed, the pollen added, and the culture placed under a bell jar. Parchment paper proved unsatisfactory because its opacity and fibrous character made microscopic observations difficult. Hog bladder was substituted but germination appeared closely correlated with the amount of water
in the membrane, and it was difficult to dry the membrane sufficiently to secure consistent germination.

La Cour and Faberge (6) suggested germinating pollen on small squares of permeable cellophane floated on a nutrient solution.

For the present study, small squares of permeable duPont cellophane 0.04 mm. thick was soaked in the nutrient solution. When the cellophane was first dropped into the solution it curled up. After becoming thoroughly soaked it uncurled and was then removed from the solution and well blotted. These squares of cellophane were floated on a small quantity of nutrient solution in a watch glass, taking care to avoid wetting the upper surface of the cellophane. Tobacco, Nicotiana affinis (variety crimson better), pollen was sown on the cellophane and the watch glass covered with another watch glass to prevent drying out. Various percentages of sucrose and dextrose were tried as a nutrient medium. In addition experiments were conducted using a soil extract solution to which various percentages of sucrose had been added. The soil extract solution was prepared by mixing 1000 gm. of garden soil in one liter of distilled water. The mixture was stored in a refrigerator at 10° C, and shaken every day for a week. Shortly before using this soil extract the various percentages of sucrose were added. The optimum medium for germination was found to be 6% sucrose in a soil extract solution. Figures 11 and 12 illustrate the difference in pollen tube growth when using 6% sucrose solution and when using 6% sucrose plus the soil extract solution. Cultures were allowed to
remain at room temperature as no significant difference in growth was observed when they were placed in an oven at 30° C. Pollen usually began to germinate in 2-3 hours. These cultures may be suitable for demonstration purposes for a day or two but after that time they become badly contaminated with bacteria and fungi.
BIBLIOGRAPHY


Figure 11. Tobacco pollen tubes cultured on cellophane and 6% sucrose solution.
Figure 12. Tobacco pollen tubes cultured on cellophane and 6% sucrose containing soil extract.
OSMOSIS

Because of confusion in terminology relating to osmosis

Eyster (5) suggested that: "Since it is difficult to change a
definition of a term, it is recommended that we use the term "osmotic
diffusion pressure", and define it as the diffusion pressure of a solvent
diffusing through a membrane. —— The term "osmotic pressure" could
still be used to designate a potential maximum hydrostatic pressure.
—— The term "activity" can be used in place of osmotic diffusion
pressure. In this case, the major trend of osmotic water diffusion
would be from the side of the membrane where the water molecules are
less active."

So many methods of demonstrating osmosis have been reported
that only a few can be discussed in this study. Court (3) dissolved
10 gm. of parlodion in 45 c.c. of ether and 45 c.c. of absolute
alcohol. He poured 15-30 c.c. of this solution into a 400 c.c.
Kjeldahl flask with the neck cut down to a length of 1 inch. The
flask was whirled so that the drying film inside the flask would be
of uniform thickness. The edges of the film were loosened with a razor
blade and tap water run into the flask. After a few minutes the water
was decanted and the film pulled out of the flask. The membrane is
filled with \( \frac{M}{2} \) potassium ferrocyanide and soaked in a beaker of \( \frac{M}{2} \)
copper sulphate. A brownish film of copper ferrocyanide forms inside
or around the pores of the parlodion membrane. The membrane is now
readily permeable to water molecules but almost impermeable to sugar molecules. The potassium ferrocyanide is poured out after several hours and the membrane bag rinsed well with distilled water. The bag is filled with a one molar solution of sucrose and stoppered with a one holed rubber stopper connected to a long "capillary" glass tube. The connection is sealed with rubber cement. This apparatus is immersed in a larger beaker of distilled water and osmosis begins almost immediately.

Chadwick (8) and Miller and Blaydes (7) used a collodion bag to demonstrate osmosis. Broadhurst (1) fastened half a gelatine capsule in an upright position to a little disk or metal cap by touching its base to the surface of a drop of glue or melted gelatine. Molasses is poured into the half capsule and water added to the dish. The capsule swells materially. In 2-3 hours sufficient water passes into the capsule to make the inward passage of water clearly evident. Broadhurst (1) also suggested that a good imitation of a cell may be constructed by filling a whole capsule with molasses and placing it in water. Lacroix (6) reported that a large carrot or medium sized turnip could be used to demonstrate osmosis.

The writer found the following methods of demonstrating osmosis to be satisfactory for a general botany laboratory.

Bore a hole with an auger bit in the center of a large Irish potato. Peel the potato and remove all excess tissue until a
wall approximately ¼ inch thick remains. Pour molasses into the hole, stopper with a one-holed rubber stopper connected to a length of 1 mm. bore capillary glass tubing. Clamp the tubing to a ring stand. In the present experiments the liquid has been observed to rise in the tube 15 inches in 1½ hours. If the potato is not peeled the liquid in the tube rises only about 1 inch in 2 hours.

A carrot or a beet may be used in the same procedure as above but the liquid in the glass tubing has not been observed to rise as rapidly as when using the potato.

The Lyons osmometer has been used quite satisfactorily in the general botany laboratory at the State University of Iowa. Parchment paper or permeable duPont cellophane 0.4 mm. thick are satisfactory membranes for this type of osmometer. Fill the osmometer with molasses, tighten the metal ring and place in a beaker of water. A length of 1 mm. bore glass tubing attached to the osmometer is clamped to a ring stand.

The writer has developed original, and visual, methods for demonstrating osmosis. Pour approximately 1.5 c.c. of 2% plain agar into a 15 c.c. U-tube. After the agar has hardened into a plug, pour a 10% sucrose solution into one arm and water into the other arm of the U-tube. Add an indicator to each side and 2 drops of concentrated hydrochloric acid to the water. As the water and acid diffuse through the agar into the sugar solution, the pH of the sugar solution is lowered and the indicator changes color. In Table X is listed the time
ordinarily required for the indicator to change color, thus demonstrating
that osmosis has occurred. Any of the indicators listed below could
be used. If more agar is placed in the U-tube than is necessary it will
naturally require a longer time for the indicator to change color.

Table X

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Concentration</th>
<th>Basic Color</th>
<th>Acidic Color</th>
<th>Time to change color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl orange</td>
<td>1 drop per 5 c.c. of liquid</td>
<td>green</td>
<td>purple</td>
<td>25 minutes</td>
</tr>
<tr>
<td>Xylene cyanol</td>
<td>2 drops per 5 c.c. of liquid</td>
<td>red</td>
<td>yellow</td>
<td>25 minutes</td>
</tr>
<tr>
<td>0.04% Chlorophenol red</td>
<td>1 drop per 5 c.c. of liquid</td>
<td>yellow</td>
<td>red</td>
<td>20 minutes</td>
</tr>
</tbody>
</table>

Another method of using indicators to demonstrate osmosis
is to place in a thistle tube 30 c.c. of a 10% sucrose solution to
which one drop of 25% sodium hydroxide and 5 drops of a given indicator
have been added. Moisten a suitable square of duPont permeable cellophane
0.04 mm. thick and with a rubber band tightly fasten it over the opening
of the tube. Clamp thistle tube to a ring stand with the membrane
immersed in 50 c.c. of distilled water to which 10 drops of the same
indicator, as used in the sugar solution and 10 drops of concentrated
hydrochloric acid has been added. As the acid water diffuses into the
sugar solution a change of color from the basic to the acidic color
occurs.
In Table XI is listed the time ordinarily required for the indicator to change color in the 10% sucrose solution.

Table XI

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Basic Color</th>
<th>Acidic Color</th>
<th>Time to change color</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.04% bromthymol blue</td>
<td>blue</td>
<td>yellow</td>
<td>25 minutes</td>
</tr>
<tr>
<td>0.04% chlorophenol red</td>
<td>red</td>
<td>yellow</td>
<td>6 minutes</td>
</tr>
<tr>
<td>methyl orange</td>
<td>green</td>
<td>purple</td>
<td>15 minutes</td>
</tr>
<tr>
<td>xylene cyanole</td>
<td>red</td>
<td>yellow</td>
<td>10 minutes</td>
</tr>
<tr>
<td>0.015% phenol red</td>
<td>yellow</td>
<td>red</td>
<td>20 minutes</td>
</tr>
<tr>
<td>0.015% neutral red</td>
<td>orange</td>
<td>red</td>
<td>20 minutes</td>
</tr>
<tr>
<td>0.04% methyl red</td>
<td>yellow</td>
<td>red</td>
<td>20 minutes</td>
</tr>
</tbody>
</table>

The 50 c.c. of water may be made alkaline by adding 10 drops of 25% sodium hydroxide and 10 drops of an indicator. The 30 c.c. of 10% sucrose in the thistle tube containing 5 drops of a indicator may be made acid by adding 1 drop of 25% hydrochloric acid. Neutral red will change from red to yellow-orange in about 25 minutes and methyl red will change from red to yellow in 5 minutes.
BIBLIOGRAPHY


INDICATORS

Various authors have suggested the use of indicators to help illustrate plant functions. Abbott (1) reported the following methods to demonstrate respiration of seeds. A filter paper is folded to form a pointed spill. The pointed half is then dipped in melted paraffin and cooled, after which the larger, absorptive end is wetted with a brom-thymol blue solution. The paraffined end is then thrust into a mass of germinating seeds and the container tightly closed. In a short time the carbon dioxide released due to respiration of the seeds will cause the brom-thymol blue to turn yellow.

Datar and Kulkani (4) reported that an aqueous extract of hollyhock flowers (presumably colored) evaporated to a total solid content of 2%, could be used for titrations of 0.1 N solutions of acids and bases. It is green in basic and red in acidic solutions. The author does not state that this is due to anthocyanin but it probably is. Therefore, it would appear that any colored flower containing anthocyanin could be used for this purpose.

Miller and Blydes (5) suggested the use of phenolphthalein as an indicator in testing for carbon dioxide.

Brandwein (3) reported that brom-thymol blue and phenol red may be used to indicate that carbon dioxide is used in photosynthesis.

The writer has developed the following methods to demonstrate various plant functions by the use of indicators. To demonstrate that carbon dioxide is given off during respiration of germinating seeds,
Wrap germinating wheat seeds in cheesecloth and suspend them above 80 c.c. of a basic aqueous indicator solution in a wide mouthed 250 c.c. Erlenmeyer flask or a bottle of approximately the same size. Stopper the flask. The carbon dioxide given off by the seeds unites with the water forming carbonic acid. This will lower the pH of the aqueous solution causing the indicator to change color. Phenol red solutions caused the most rapid color change of all the indicators employed in this experiment. The times ordinarily required for various indicators to change color are listed in Table XII.

Table XII

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Concentration</th>
<th>Basic Color</th>
<th>Acidic Color</th>
<th>Time to change color</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.015% phenol red</td>
<td>2 drops per 50 c.c. of water</td>
<td>red</td>
<td>yellow</td>
<td>35 minutes</td>
</tr>
<tr>
<td>0.04% bromothymol blue</td>
<td>2 drops per 50 c.c. of water</td>
<td>blue</td>
<td>yellow</td>
<td>1 hour</td>
</tr>
<tr>
<td>0.015% neutral red</td>
<td>1 drop per 50 c.c. of water</td>
<td>yellow-yellow</td>
<td>red</td>
<td>1 ¼ hours</td>
</tr>
<tr>
<td>0.04% chlorophenol red</td>
<td>1 drop per 50 c.c. of water</td>
<td>red</td>
<td>yellow</td>
<td>1 ½ hours</td>
</tr>
</tbody>
</table>

It is usually difficult to demonstrate that carbon dioxide is used by plants in photosynthesis, but it is easily shown by the use of proper indicators. However, it is necessary to have sunlight or a good source of light in order to carry out this experiment, otherwise it may require an unreasonably long time for the indicator to change color.

Wash algae (Cedogonium) in distilled water, and place in a...
test tube containing distilled water to which an indicator has been added. Adjust the pH by blowing gently through a glass tube into the indicator solution until it just changes to the acidic color. Place the culture in the sunlight or under a very bright light. As the algae absorbs carbon dioxide for photosynthesis the indicator will change back to the basic color. Any of the indicators listed in Table XIII may be used in this demonstration, but phenol red and chloro-phenol red give quicker results. The time ordinarily required for various indicators to change color in this manner is listed in Table XIII.

Table XIII

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Concentration</th>
<th>Acidic color</th>
<th>Basic Color</th>
<th>Time to change color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl orange cyanine</td>
<td>1 drop per 40</td>
<td>purple</td>
<td>green</td>
<td>40 minutes</td>
</tr>
<tr>
<td>Xylene cyanine</td>
<td>0.05% of water</td>
<td>yellow</td>
<td>red</td>
<td>10 minutes</td>
</tr>
<tr>
<td>0.016% phenol red</td>
<td>2 drops per 20</td>
<td>yellow</td>
<td>red</td>
<td>25 minutes</td>
</tr>
<tr>
<td>0.04% chloro-phenol red</td>
<td>4 drops per 20</td>
<td>blue</td>
<td>yellow</td>
<td>40 minutes</td>
</tr>
<tr>
<td>0.04% brom-thymol blue</td>
<td>0.05% of water</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Place healthy Elodea tips in a large test tube to which has been added 50 c.c. of an acidic aqueous indicator solution. Adjust the pH by gently blowing through a glass tube into the indicator solution. Place the cultures in the sunlight or a bright source of light. As the carbon dioxide is used in photosynthesis the color of the indicator will change. The time ordinarily required for the indicators to change color is listed in Table XIV.
Table XIV

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Concentration</th>
<th>Acidic Color</th>
<th>Basic Color</th>
<th>Time to Change Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.06% brom-thymol blue of water</td>
<td>4 drops per 50 c.c.</td>
<td>yellow</td>
<td>blue</td>
<td>1 hour</td>
</tr>
<tr>
<td>methyl orange</td>
<td>1 drop per 50 c.c.</td>
<td>purple</td>
<td>green</td>
<td>20 minutes</td>
</tr>
<tr>
<td>xylene cyanol of water</td>
<td>8 drops per 50 c.c.</td>
<td>yellow</td>
<td>red</td>
<td>25 minutes</td>
</tr>
<tr>
<td>0.06% chloro phenol red of water</td>
<td>1 drop per 50 c.c.</td>
<td>yellow</td>
<td>red</td>
<td>40 minutes</td>
</tr>
</tbody>
</table>

Indicators may be used in testing of carbon dioxide in yeast fermentation, as suggested in the section on yeast. Another use of indicators is in the demonstration of osmosis as discussed under the section on osmosis.
BIBLIOGRAPHY


LEAF SKELETONS

Leaf skeletons are desirable for use in a general botany laboratory when students study vein patterns.

Plymale (4) obtained leaf skeletons by placing leaves in vessels filled with river water, allowing bacteria to decompose their softer tissue. This requires from 1-4 weeks, depending upon the type of blade.

Turtox News (2) suggested placing poplar or willow leaves in an aquarium and allowing the pond snails to eat everything but the skeletal parts.

Loomis and Shull (5) recommended making leaf skeletons by boiling rose leaves one half an hour to an hour in a solution of soda-lime (1 teaspoon per 100 c.c. of water).

Another issue of Turtox News (1) suggested that leaf skeletons be prepared by placing fresh leaves in a 10% solution of sodium hydroxide. The time required for the caustic to eat away the softer parts of the leaves varied according to the kind of leaf used. Ordinarily the process should be completed in two weeks.

Haupt (3) used a mixture of sodium carbonate and calcium hydrate to rid fresh leaves of all soft green tissues. A modification of his reagent was prepared by dissolving 115 gm. of sodium carbonate in 1000 c.c. of boiling water. To this was added 60 gm. of calcium hydrate and the solution boiled for fifteen minutes. The solution was filtered when it had cooled. Leaves were added to the filtrate and boiled.
The length of boiling varies with type of leaf. Softer leaves such as orange, tulip tree, or Eucalyptus are boiled approximately one-half hour while others, such as elm or oak leaves, must be boiled approximately one hour. The leaves are then placed in a large pan of cool water and carefully rubbed between the fingers under the water to remove the softer tissue.

When all of the soft tissue have been removed the leaves are floated onto lantern slide cover glasses and allowed to dry. Another lantern slide cover glass is placed over the dried leaf skeleton and a binding tape applied exactly as for a lantern slide. (Figure 13).
BIBLIOGRAPHY


Figure 13. Leaf skeleton prepared by using calcium hydrate and sodium carbonate.
PROTOPLASMIC STREAMING

In his extensive work on protoplasmic streaming Selfrig (4) reported that streaming is not frequently found in young cells, though in some, such as the cambium, it is quite active. Streaming is usually most active in highly vacuolated cells. Protoplasmic streaming is accelerated by higher temperatures within a definite range. With tissues commonly exposed to light there is no stimulating effect by light on streaming, whereas tissues not naturally exposed to light, such as the inner cells of stems, are sensitive to light and respond by and increased rate of streaming. Elodea leaves when soaked in 1%-2% barium or strontium chloride for several hours, or longer, depending on concentration, are aroused to pronounced and often abnormal activity.

Sweeny (6) found that indole-3-acetic acid accelerated protoplasmic streaming in root hairs of Avena. The optimum concentration was $10^{-4}$-$10^{-5}$ mg. per liter. Concentration of 10 mg. per liter or above was inhibitory. Diurnal periodicity was observed in the degree of acceleration brought about by indole-3-acetic acid, the maximum occurring in the morning.

Harvey (2) reported the optimum temperature for maximum cyclosis in Nitella flexilis is 35-36°C. He also suggested that a sudden increase or decrease in temperature may cause shock stoppage of cyclosis.

Wylie (7) found Vallisneria leaves better than Elodea to demonstrate cyclosis, but these thicker leaves should be sectioned in
a flat plane for a good view of the cells.

Serena (5) suggested chopping Vallisneria leaves and letting them stand in a glass on a window sill for a few days before using to demonstrate protoplasmic streaming.

Gordon (1) found that thiamin chloride stimulated protoplasmic streaming Elodea leaves, but did not state the minimal or optimum effective concentration.

In attempting to determine whether thiamin chloride stimulated protoplasmic streaming Elodea leaves it was found that if an untreated leaf was left on the microscope stand for some time it would show an increase in protoplasmic streaming. Therefore it is difficult to determine exactly whether thiamin chloride stimulated protoplasmic streaming. After preliminary experiments on the problem, a standard procedure was devised. Leaves were picked quickly from near the tip of a healthy Elodea plant and submersed in various concentrations of thiamin chloride as follows: 6 mg., 3 mg., 1.5 mg., .75 mg., and .575 mg. per 200 c.c. of distilled water. Leaves were also placed in a control solution of distilled water at the same time. Leaves were left in the test solutions for 5 minutes, then quickly removed and placed on slides in a drop of the respective solution. A quick survey was made of all the leaves. It was found that the most rapid streaming occurred in the solution containing .75 mg. of thiamin chloride per 200 c.c. of distilled water. There was some streaming in the solution containing .575 mg. and 1.5 mg. per 200 c.c. of distilled water, but 3 mg. and 6 mg. of thiamin chloride per 200 c.c. of distilled water seemed to inhibit protoplasmic streaming. In the
first quick survey of the leaf the one in the water usually showed very little streaming. However, as mentioned above, after the leaf had been mounted on the slide and studied for some time on the microscope stand the streaming seemed to increase.

It would appear that a solution containing .75 mg. of thiamin chloride per 200 c.c. of distilled water stimulated protoplasmic streaming but further experiments are necessary to verify this work.
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