Chapter 11

Use of Fern Gametophytes to Teach Concepts of Plant Development

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Introduction

Because of their ready availability, ease of culture, rapid growth and ease of observation, fern gametophytes are well suited for laboratory experiments which illustrate key features of plant development. In this article, laboratory exercises which utilize gametophytes of *Pteridium aquilinum, Woodwardia virginica, Lygodium* and *Marsilea* to illustrate sexual propagation, alternation of generations, phototropism, photomorphogenesis, control of cell division by 2-thiouracil, control of growth and sex expression by gibberellic acid, sperm motility, fertilization, embryo development, chemotaxis, regeneration and totipotency are described. These exercises can be used individually or they can be combined to form a module. Although they were designed for particular levels of comprehension, the amount of detail required by the instructor can raise or lower the level of application. The extensive literature concerning the control of gametophyte and young sporophyte growth and development allows these activities to be expanded to include studies on the effects of light and hormones on spore germination (Furuya 1978; Voller 1971), apical dominance (Reynolds and Corson 1979), apogamy (Smith 1979), apospory (White 1979) and the effects of carbohydrates, hormones and light on sporophyte development (White 1971, 1979). An attractive feature of these laboratories is the realization that original discoveries can be made by extending these experiments to previously untested species.

Instructors Materials

Propagation of Ferns from Spores—Peat Pot Method

Each student will need 1 compressed peat pot, 1 clear plastic tumbler and 1 petri plate bottom or top. Either a "Jiffy 7" or "Kys-Kube" is a suitable peat pot. However, fungal contamination has been more of a problem with the latter. If a "Jiffy 7" is used, the nylon netting which surrounds the opening on the top must be slit by radial cuts and peeled back. This provides greater area for gametophyte growth. If the netting is not removed, rhizoids and roots become enmeshed in it and are destroyed when students transfer their gametophytes. Either glass or plastic petri plates can be used. This is a good way to recycle used plastic petri plates.

The peat pot is placed in the petri plate and water is added until the pot is fully moistened (Figure 11.1). With "Jiffy 7's" avoid adding water directly to the top of the pot. To apply spores, remove the plug from the vial of spores, place a piece of aluminum foil over the mouth of the vial and fasten it with a piece of tape placed around the circumference of the vial. Use a sewing needle or a 20-gauge syringe needle to make small holes in the foil. Students use the vial like a pepper shaker (Figure 11.2) to inoculate their pots with

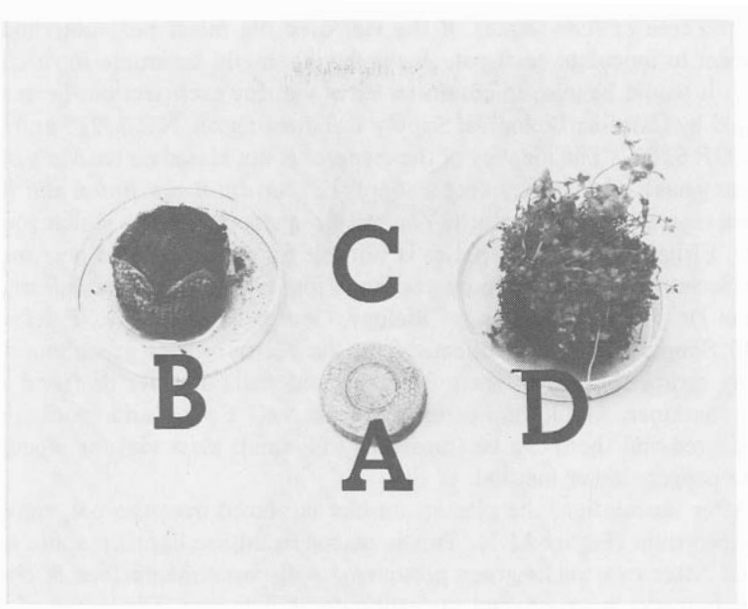


Figure 11.1. Peat pot method of fern spore propagation. A, dry, unexpanded "Jiffy 7" peat pot. Note the imprinted circle on top of the pot where radial slits should be made in the nylon net; B, fully expanded, moistened peat pot in bottom of a petri dish; C, plastic tumbler which is placed over inoculated peat pot to form a mini-terrarium; D, *Pteridium aquilinum* sporophytes after three months of culture on a peat pot.

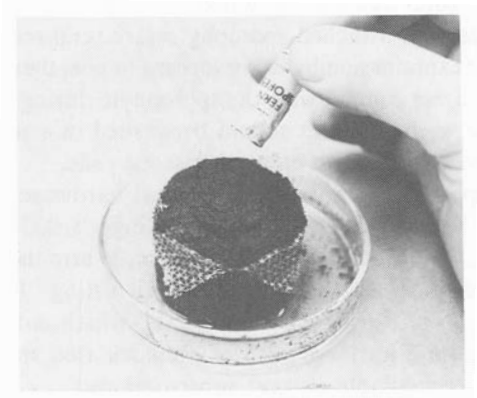


Figure 11.2. Pepper shaker technique for inoculation of a peat pot.

spores. Three or four shakes of the vial over the moist peat pot should be sufficient to inoculate each pot. A single vial should be ample for a class of 20 but it would be wise to obtain an extra vial for each section. Fern spores are sold by Carolina Biological Supply Co., Burlington, NC 27215 and Gladstone OR 92027. The identity of these spores is not stated on the vials but the company has said that they keep a supply of *Pteridium aquilinum* and *Woodwardia virginica* spores in stock. You must request the species which you wish to use. Either of these two species is suitable for this exercise. The American Fern Society maintains a spore exchange for its members. For information, contact Dr. J. E. Skog, Dept. of Biology, George Mason Univ., Fairfax, VA 22030. Spores may also be collected from local ferns or from greenhouse ferns. Simply scrape the brown areas from the underside of a fertile frond into a small container. An old film cannister works well. Spores and sporangia will be removed and these can be transfered to a small glass vial for inoculation by the pepper shaker method.

After inoculation, the plastic tumbler is placed over the pot, making a mini-terrarium (Figure 11.1). This is placed in diffuse light for spore germination. After two weeks green protonema will cover the surface of the pot. After six weeks, heart-shaped gametophytes will appear. The leaves of young sporophytes will be observed 10–12 weeks following inoculation. Gametophytes are ready to be transfered at this time.

Fungal growth may be a problem during the initial period of growth. If fungal hyphae appear, remove the plastic tumbler and allow the pot to dry for one hour. Replace the tumbler and repeat this process until fungi are no longer apparent. Pots should be sprayed with water every week or whenever they appear dry. Water can also be added to the petri plate.

Gametophytes with attached sporophytes are removed for transplanting or for microscopic examination by using forceps to peel them from the surface of the pot. Avoid direct contact with the sporophyte during transplanting. For microscopic study, gametophytes should be washed in a petri plate of water before mounting ventral side up on a microscope slide.

Compressed peat pots are available at local hardware stores and garden centers. They can be ordered by mail from Mellinger's, 2310 W. South Range Rd., North Lima, Ohio 44452; Burpee Seed Co., Warminster, PA 18991 and from Carolina Biological Supply Co. under their listing "Instant Pots". Carolina Biological also sells a "Fern Minimarsh" which only requires the addition of water to initiate the growth of preinoculated spores on peat pots. Plastic tumblers are available at most supermarkets.

This laboratory was designed for non-majors but could also be used in general botany. It requires one hour to demonstrate and to inoculate cultures. Periodic observations are necessary to check gametophyte development and for watering. After the appearance of sporophytes, 30–50 minutes are needed to transplant gametophytes with attached sporophytes to their own terraria.

Propagation of Ferns from Spores—Liquid Culture Method

Liquid cultures are initiated by following the procedures described in the section on peat pot culture. To reduce the risk of microbial contamination, the mouth of the vial containing the spores is flamed before the plug is removed. An alcohol lamp or a bunsen burner provide suitable flames. Be careful to avoid igniting the cotton plug during flaming. A piece of aluminum foil, soaked in 80% ethanol and flamed, is placed over the opening of the vial after the cotton plug has been removed. A needle, soaked in 80% ethanol and flamed, is used to make small holes in the foil. The vial is positioned 3–5 cm above the surface of a petri plate which contains 25 ml of sterile fern medium. After the lid has been removed, the vial is shaken twice to inoculate the medium. After the cover is replaced, the dish is sealed with parafilm and placed under the light. Continuous illumination from 40 watt cool white, warm white or grow light fluorescent tubes at a distance of 30–40 cm accelerates gametophyte development.

Further precautions to assure sterility include washing one's hands and forearms with 80% ethanol and performing the inoculation in a fume hood which has been washed with 10% Lysol. The fan in the hood should be off during inoculation. These procedures have been performed in an open laboratory with little resultant contamination.

The culture medium (Johnson and Volpe 1973) is prepared by adding 100 ml of the mineral stock solution (Table 11.1) and 5 ml of the ferric citrate stock solution (Table 11.2) to 895 ml of distilled water. The pH is adjusted to 5.8 prior to autoclaving for 20 minutes at 15 psi.

Table 11.1 Mineral stock solution for fern medium (Johnson and Volpe 1973).

Component	*grams/liter*
NH_4NO_3	5.0
KH_2PO_4	2.0
$MgSO_4 \cdot 7H_2O$	2.0
$CaCl_2 \cdot 2H_2O$	1.0

Table 11.2. Ferric citrate stock solution for fern medium (Johnson and Volpe 1973).

Component	*grams/liter*
Fe citrate	1.0 added to boiling water

Alternation of Generations

For the class a potted fern with sori (Figure 11.3) should be available for study. Suitable plants can be obtained from local garden centers or from Carolina Biological Supply Co. Students observe the organization of the adult sporophyte and obtain a sample of the sporangia by scraping a portion of the sorus onto a drop of water on a microscope slide. By adding a coverslip, they can observe sporangia and spores with a compound microscope.

Gametophytes of different developmental stages which have been grown in liquid culture can be observed by transfering small samples to a microscope slide and adding a coverslip before examination with a compound microscope. Young gametophytes can be removed with an eyedropper. Because the spores aggregate on the surface of the culture medium, students must take their samples from these areas. Students tend to insert their eyedroppers at random and expect to obtain specimens. Older gametophytes which are easier to see, can be removed with a bacterial loop or a dissecting needle. One-dimensional filamentous gametophytes will be present in 1–2 week cultures. Heart-shaped

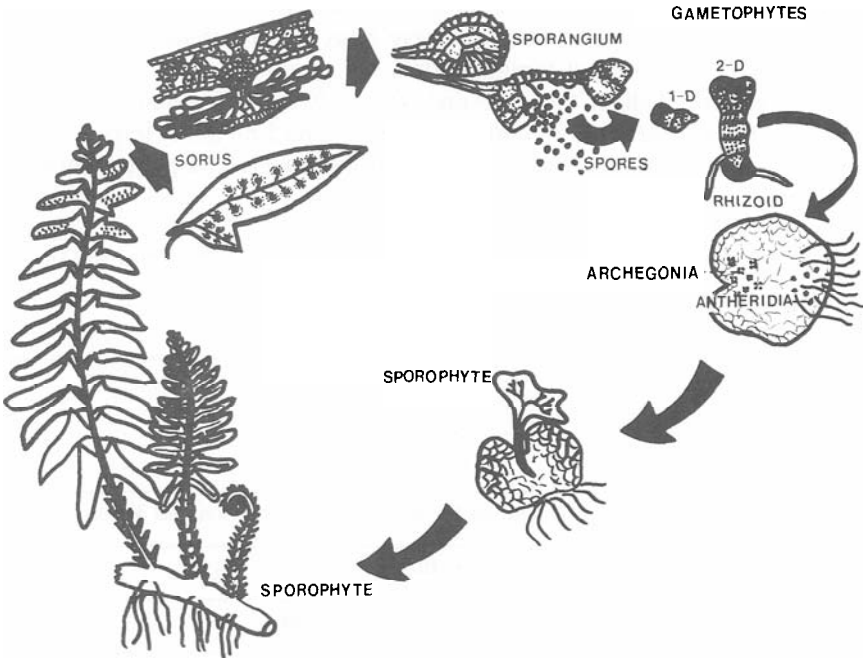


Figure 11.3. Typical fern life cycle. 1-D, one-dimensional gametophyte; 2-D, two-dimensional gametophyte. (Adapted from illustration by Carolina Biological Supply Company, copyright © 1977.)

gametophytes will be present in 4–6 week cultures. Gametophytes with sex organs require 8–10 weeks to develop. Gametophytes with attached sporophytes can be observed in 12–14 week cultures. It is best to inoculate cultures at two-week intervals starting 12–14 weeks prior to the date of use. Two cultures at each developmental stage are needed for a class of 20.

Spore walls remain attached to the gametophytes for long periods in liquid culture. This observation clearly illustrates the relationship between the spore and the gametophyte (Figure 11.4). Archegonia are produced below the apical notch on the ventral surface of the gametophytes and antheridia are produced near the base of the gametophyte (Figure 11.3). Young sporophytes

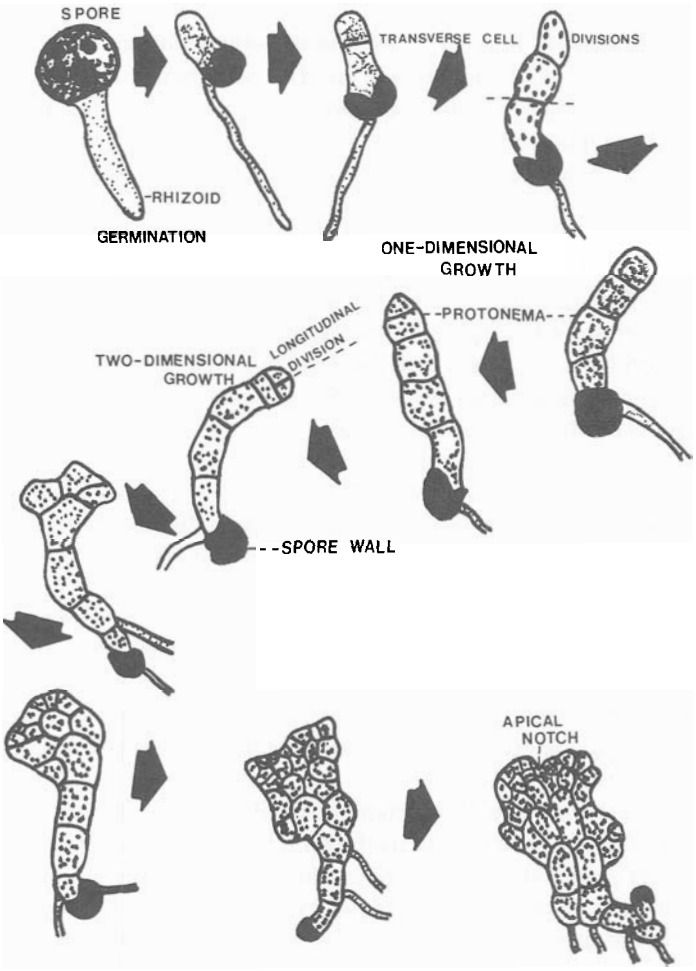


Figure 11.4. Early gametophyte development of *Asplenium nidus*. (Redrawn with permission of Raghavan, 1974.)

permission of Raghavan, 1974.)

permission of Raghavan, 1974.)

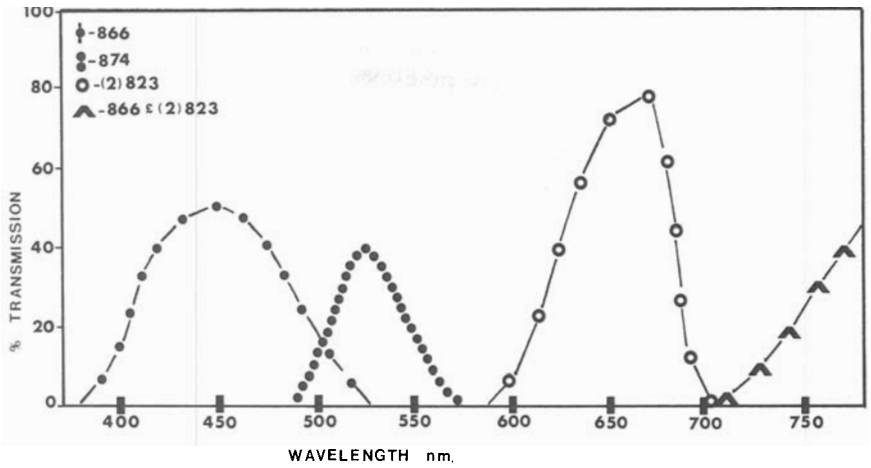
are easily distinguished from the gametophytes. The presence of spirally thickened tracheary elements in the sporophyte leaf blade and petiole as well as the presence of stomates in the leaf blade and the presence of true roots are readily seen with the microscope. The origin of the sporophyte can be traced back to the archegonial region. This observation clearly illustrates the relationship of the sporophyte to the gametophyte (Figure 11.3).

This laboratory was designed for general botany but it can be used in advanced morphology courses by increasing the amount of detail required. This exercise requires a full laboratory to complete. Preparation of the stock solutions and culture medium requires 2–4 hours. Each inoculation can be accomplished in 5–10 minutes.

Photomorphogenesis of *Pteridium aquilinum* Gametophytes

Each team of four students will need 8 petri plates of fern medium. Students prepare their own plates by pipetting 20 ml of sterile medium into each plate. The liquid culture medium is the same as that described previously in this chapter. Students inoculate their plates by the previously described pepper shaker technique. Plates are sealed with parafilm and placed into different light boxes. Each team places 2 plates into either a white, blue, green or red light box. These boxes are constructed from shoe boxes or typewriter paper boxes. A rectangular opening is cut into the top of the box and this is covered with either a blue, green or red light filter or with a piece of frosted acetate. Colored acetate filters with discrete transmissions in the visible spectrum can be purchased from Edmund Scientific, 101 E. Glouchester Pike, Barrington, NJ 08007. Catalog # 82,039 (Dark Urban Blue 866) is a good blue filter with fluorescent light (Figure 11.5). Number 82,041 (Medium Green 874) is a good green filter with fluorescent light and two layers of # 82, 051 (Medium Red 823) make a good red filter with fluorescent light (Figure 11.5). If desired, a far-red filter can be made by combining one layer of the blue filter with two layers of the red filter and illuminating with incandescent light (Figure 11.5). Frosted acetate is available at most art supply and stationery stores. The filters are sealed over the openings with black plastic tape which is available at most hardware and variety stores. Black tape is used to seal the edges of the boxes after the inoculated plates have been placed inside. The light boxes are placed under fluorescent lights which are 20 cm above the tops of the boxes.

Students examine one plate from each light treatment after one week. The surface of the culture is examined with a dissecting microscope to locate the spores and germinated gametophytes. In this way the percentage of spore germination can be estimated. Gametophyte samples are withdrawn with eyedroppers and examined with a compound microscope. Students estimate the percentage of one-dimensional and two-dimensional gametophytes (Figure 11.4).



Dark Urban Blue (#866) filter in fluorescent light

Medium Green (#874) filter in fluorescent light

Two medium Red (#823) filters in fluorescent light

Dark Urban Blue (#866) filter plus two Medium Red (#823) filters in incandescent light

Figure 11.5. Transmission spectra of acetate filters.

With constant illumination with 40 watt fluorescent tubes, *P. aquilinum* gametophytes grown in white light should be two-dimensional after one week. Those grown in blue light will also become two-dimensional but it may take two weeks for this to occur. Gametophytes grown in green and red light will remain one-dimensional and their component cells will be greatly elongated compared to those of gametophytes grown in white or blue light. Red light may also induce vertical growth of the protonema. Students can count the number of cells present in gametophytes grown under different light qualities. If ocular micrometers are available average cell length can be determined. The second plate in each treatment is examined after two weeks and the same observations are made. Students enter their data in tables on the blackboard and representative microscopic preparations are placed on demonstration for class observation. If a microprojector is available, these slides can be shown to the entire class.

Gametophytes of *Woodwardia virginica* become two-dimensional in both red and blue light as well as in white light. Gametophytes grown in red light are more elongated and have larger cells than those grown in either blue or white light. *W. virginica* spores do not germinate in green light. Spores of this

species can be used to illustrate the variation in photomorphogenetic responses which occur in different ferns. They can also be used for group or individual research projects after the class has performed the experiment with *P. aquilinum*.

The total amount of light energy incident on the gametophyte in the 24hr period also influences the rate of transition from one-dimensional to two-dimensional morphology. Experiments with different photoperiods or different light intensities at a constant photoperiod could also be designed as research projects. An additional light treatment with far-red light would be valuable if *W. virginica* is studied. Raghavan (1973) demonstrated that gametophytes of *Lygodium japonicum* became two-dimensional in both red and blue light but remained one-dimensional in far-red light. It is possible that *W. virginica* gametophytes would respond similarly to far-red light. It would also be desirable to quantify with a quantum radiometer the amount of light transmitted by each filter so that the results of each treatment could be more strictly compared.

Control of Cell Division by 2-thiouracil (Johnson and Volpe 1973)

Students work in groups of six. Each student is responsible for one of the following treatments:

a control (plain fern medium)
b fern medium with 4 mg/1 2-thiouracil
c fern medium with 8 mg/1 2-thiouracil
d fern medium with 12 mg/1 2-thiouracil
e fern medium with 8 mg/1 2-thiouracil plus 30 mg/l uracil
f fern medium with 30 mg/1 uracil

Each student prepares two plates of the appropriate medium and inoculates them by the pepper shaker technique. Each plate is sealed with parafilm, labeled and placed under 40 watt fluorescent lights with a 24 hr photoperiod. During the next two succeeding laboratories students examine the growth form of the gametophytes by removing samples with an eyedropper and observing them with a compound microscope. As in the previous exercise, students estimate the percentage of one-dimensional and two-dimensional growth occurring in each treatment. Measurements of cell number and length can also be made.

Two-dimensional growth should occur after one week in plain medium and in medium containing uracil. Some two-dimensional growth may also occur in 4 mg/1 2-thiouracil. Gametophytes in 8 mg/1 and 12 mg/1 2-thiouracil will remain one-dimensional. Germination may also be retarded in these two latter treatments and few gametophytes may be present after one

week. Total gametophyte growth may also be inhibited by 2-thiouracil. Uracil by itself, stimulates gametophyte growth and overcomes the inhibitory effects of 2-thiouracil. Gametophytes of *P. aquilinum* and *W. virginica* have the same response to uracil and 2-thiouracil.

Uracil and 2-thiouracil are available from SIGMA Chemical Co., P.O. Box 14508, Saint Louis, MO 63178. Stock solutions of 1 mg/ml are prepared. At this concentration each ml contains 1 mg and the culture solutions are easily prepared by substituting the appropriate volume of uracil and/or 2-thiouracil for an equal volume of water. Medium containing 2-thiouracil should be prepared promptly since the stock solution may precipitate. The pH of the media is adjusted to 5.8 prior to autoclaving. More alkaline solutions may form a precipitate after autoclaving. Preparation of the media requires 2–3 hours. Inoculation requires 1–2 hours. Subsequent observations and discussion take 1.5–2 hours at each laboratory meeting. The two preceding laboratories were designed for courses in developmental biology or plant morphogenesis.

Phototropism of *Pteridium aquilinum* or *Woodwardia virginica* Gametophytes

Students work in groups of three. Each student obtains two petri plates of fern medium which has been solidified with 2% agar. Students inoculate their plates using the pepper shaker technique, observing all of the precautions to avoid microbial contamination. Fungal contamination has been a problem with this laboratory. Fungal hyphae can overgrow the gametophytes and obscure them from observation. The "washing machine" technique of Basile (1973) eliminates most of the contamination problems associated with this experiment. This procedure has not been used by students in my courses but I have found it to work well. Spores are placed on filter paper (Whatman #2) or glass microfibre paper (Whatman GF/C) which has been placed under the o-ring of a Delrin (25 mm) syringe filter holder (See Figure 11.6). The filter unit is closed by adding the upper half of the unit which is attached to a 5 ml or 10 ml syringe. A sterile hypodermic needle (3", 20 gauge) is placed on the open end of the filter holder. This assembly is the "washing machine." A solution of 7% (v/v) bleach and 0.5% Alconox is drawn into the syringe from a culture tube stoppered with a serum cap containing a syringe needle plugged with cotton. The bleach solution is pumped back and forth for one minute. Pressure must be applied sparingly during the pumping since severe pressure may rupture the filter.

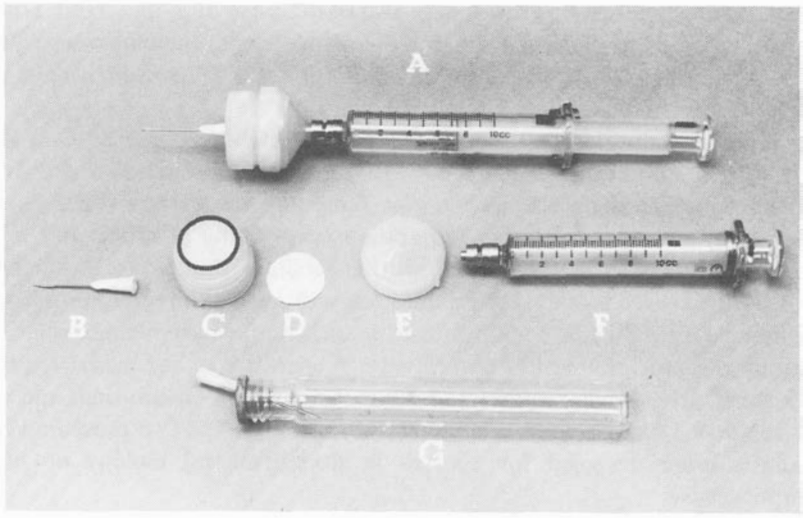


Figure 11.6. Components of the "washing machine" technique (Baslie, 1973) for sterilization of fern spores. A, assembled "washing machine" composed of B, sterile three inch, 20 gauge, syringe needle; C, lower half of "Delrin" filter holder with 25mm Whatman GF/C glass microfibre paper (D) and o-ring; E, top half of "Delrin" filter holder; F, glass syringe. G, culture tube capped with serum cap containing syringe needle plugged with cotton. Decontaminating solution or sterile water are placed in G and are drawn into the assembled washing machine (A). See text for complete description of this technique.

The decontaminated spores are rinsed by repeating the above procedure with three tubes of sterile distilled water which have been autoclaved for 20 minutes at 15 psi. Before inserting the syringe of the "washing machine" into the tubes of sterile water, the syringe needle must be flamed and the serum cap must be swabbed with 80% ethanol. Following one minute rinses in the tubes of sterile water, sterile culture medium from a fifth tube is drawn into the syringe. The syringe is removed from the filter holder and the Luer-lock is flamed. The needle is removed from the filter holder, flamed and attached to the Luer-lock. Petri plates are inoculated by carefully lifting the cover, inserting the needle and delivering 0.3–0.4 ml of spore suspension. The edge of the plate is sealed with parafilm and the plate is rotated at a 45 degree angle to distribute spores over the agar surface.

Excess surface moisture can obliterate the orientation of the gametophytes. To avoid this problem, excess medium can be removed with a sterile pipet before the parafilm is added. The spores tend to stick to the agar so that many will remain in the plate after the excess medium has been removed. Spores can be seen with high power under a dissecting microscope and the agar surface can be checked to assure the presence of spores. Students should

be cautioned about this problem and they should attempt to keep their plates in a horizontal orientation at all times following inoculation. As an added precaution, the plates can be incubated in an inverted position so that the agar is on top and excess fluid will accumulate in the lid which is on the bottom. The spores can be studied through the agar which is firm enough to remain intact under these circumstances.

An arrow is drawn on the top and bottom of each sealed plate. Plates are positioned in shoe boxes so that the arrows point directly away from a lateral slit which has been cut into one end of the box. The arrows indicate the direction of the light gradient. Cellophane tape is used to secure the plates in the boxes. The box lids are added and sealed with black plastic tape. The lateral slits are covered with either one layer of blue or green acetate light filters or two layers of red acetate. One layer of frosted acetate is applied to boxes serving as white light controls. A dark control may be added.

After three weeks the petri plates are removed from their light boxes, the parafilm is removed and the agar surface is examined with a dissecting microscope. Protonema of *P. aquilinum* display positive phototropism to blue, green and white light. The rhizoids are negatively phototropic to these light qualities. The orientation of the protonema to directional white light is complex. In addition to being positively phototropic, the protonema grow at a right angle to the direction of illumination (Furuya 1978). In red light the protonema grow at a right angle to the direction of illumination. The response of protonema to white light appears to be a combination of its responses to blue and red light. Gametophytes of *W. virginica* do not germinate in green light but otherwise they show the same responses to directional illumination as gametophytes of *P. aquilinum*.

This laboratory was designed for a course in developmental biology but it would also be appropriate for plant morphogenesis. Preparation of the culture medium takes 1–2 hours. Preparation of materials for spore sterilization takes 1 hour. Spore sterilization requires 15 minutes. Inoculation of cultures takes 1 hour. Preparation of the directional light boxes takes 20 minutes for each box. The boxes can be reused many times. Examination of the cultures and discussion requires 1.5–2 hours.

Totopotency of Isolated Gametophyte Cells

Each student will need four 35-x-10-mm petri plates or two 60-x-15-mm petri plates containing fern medium solidified with 2% agar. Using dissecting needles or bacterial loops, students remove heart-shaped gametophytes from liquid cultures and place them dorsal side up on the surface of the agar fern medium. One or two gametophytes fit into the small dish while three or four can be placed in the larger dish. These are placed on the stage of a dissecting microscope which has been wiped with 80% ethanol. A fine dissecting needle is made by heating a pasteur pipet tip and drawing it into a fine thread which

is broken at its narrowest point. Viewed at 25–30X, the glass dissecting needle is used to press the edge of the gametophyte flat against the agar surface. The sharp point is used to cut away a small piece from the margin of the gametophyte. The students should focus on a small group of cells and try to puncture all but one with the needle. As a consequence of this action a small marginal piece will become separated from the gametophyte. This piece should ideally contain a single living cell. Usually, several living cells will be present in these pieces. The detached pieces are gently pushed away from the parent gametophyte. This isolation process is repeated at different locations around the circumference of the gametophyte. At the termination of this dissection the parent gametophyte is surrounded by small satellites, each of which contains one to several living cells.

After four weeks, regeneration should easily be observed with a dissecting microscope. Individual regenerated gametophytes recapitulate the stages of normal gametophyte development from spores. These regenerated gametophytes can be removed with a dissecting needle and mounted in a drop of water on a microscope slide. After adding a coverslip, the origin of the regenerated protonema from individual cells can be observed with a compound microscope. Fungal contamination may occur but, except in very severe cases, it will not interfere with regeneration.

This laboratory was also intended for use in developmental biology and plant morphogenesis courses. Preparation of the culture medium takes 1.5 hours. Microdissection of the gametophytes requires 1.5–2 hours. Analysis of regeneration and discussion takes an additional 1.5–2 hours.

Control of Antheridium Development in *Lygodium* by Gibberellin

Students work in pairs. One student inoculates a petri plate of plain liquid fern medium with *Lygodium* spores. The second student inoculates a plate of liquid medium containing 10 mg/1 gibberellic acid (GA_3) with *Lygodium* spores. The plates are marked and sealed with parafilm and placed under 40 watt fluorescent tubes which are 20–30 cm above the level of the plates. Germination and development of the gametophytes can be observed periodically with a dissecting microscope and samples can be withdrawn with eyedroppers for study with a compound microscope. Gibberellic acid delays germination and retards initial gametophyte growth. After two weeks, GA_3-treated gametophytes should be covered with clear bumps. These are the antheridia. With a compound microscope, the outline of the jacket cells (Foster and Gifford 1974) and the clear contents of the antheridia can be seen. The lack of well-developed chloroplasts in the antheridial cells contrasts sharply with the large, densely packed chloroplasts present in the vegetative

cells of the gametophyte. Gibberellic acid promotes fungal growth. The sterilization of *Lygodium* spores by the previously described "washing machine" technique is recommended but not required for good results.

A stock solution of GA_3 (1 mg/l) is prepared by dissolving 0.1 g of GA_3 in 1–2 ml of 95% ethanol. The final volume of the solution is brought to 100 ml by adding distilled water. Gibberellic acid is available from SIGMA Chemical Co. To make 1000 ml of fern medium containing 10 mg of GA_3, measure 885 ml of distilled water, 100 ml of mineral stock solution, 5 ml of ferric citrate stock solution and 10 ml of GA_3 stock solution. Add these together in the order mentioned above with constant stirring. Follow this same procedure but substitute distilled water for the GA_3 stock solution to make the control medium. Adjust the pH to 5.8 prior to autoclaving for 20 minutes at 15 psi. The students can dispense the medium with pipets during the laboratory.

Several species of *Lygodium* spores are available from the spore exchange of the American Fern Society. This service is only available to members. Write to the previously mentioned address of Dr. J. E. Skog for membership information. *L. circinatum* has been successfully used in this experiment but other *Lygodium* and *Anemia* species should respond similarly to GA_3 (Naf 1979).

This laboratory was designed for plant morphogenesis but it could also be used in an advanced course in plant morphology or in general botany. It could also be used to illustrate hormone action in developmental biology. Preparation of culture media requires 2 hours. Inoculation with spores takes 30 minutes. Final observations and discussion takes 1.5–2 hours.

Sperm Release, Fertilization and Embryo Development of *Marsilea*

Marsilea is a semi-aquatic fern. The entire plant (Figure 11.7) is composed of a horizontal rhizome which produces leaves and roots. Near the junction of the leaf pediole and the rhizome, a short stalk diverges bearing several seed-like sporocarps. These sporocarps contain two types of sporangia. Microsporangia produce short-lived microgametophytes which release multiflagellated sperm. Each megasporangium produces a single megagametophye which consists of an archegonium and a large basal cell. The archegonium produces a single egg. These sporangia are attached in units called sori. The individual sori are all attached to a gelatinous sorophore. Gametophyte development begins when water enters the sporocarp. At 25 C, sperm release and fertilization occur 7–10 hours following the beginning of hydration. Further details regarding the timing of sperm release and fertilization in *Marsilea* can be found in the chapter by M. Albert in this volume. The sperm remain motile for approximately one hour following their release from the microgametophyte. They are chemotactically attracted to the archegonial region of the megagametophyte where they become embedded in the gelatinous sheath

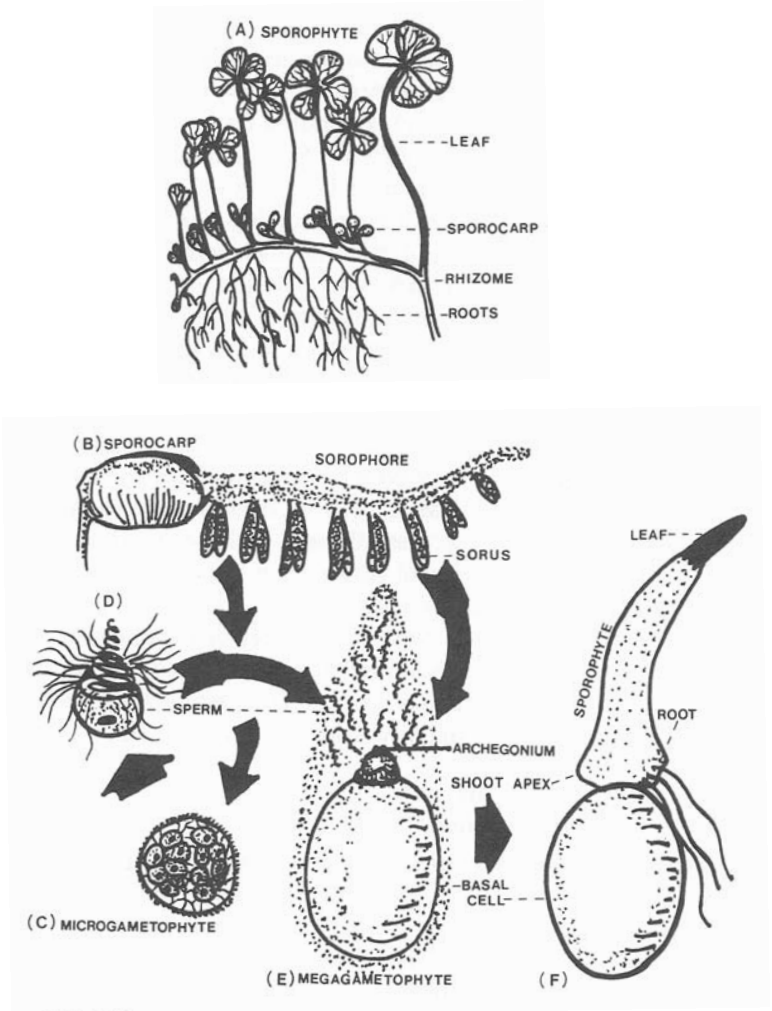


Figure 11.7. Life cycle of *Marsilea*. A, sporophyte; B, hydrated sporocarp with expanded, gelatinous sorophore; C, microgametophyte containing spermatocytes; D, individual flagellated sperm; E, megagametophyte with expired sperm trapped in gelatinous sheath; F, young sporophyte growing from archegonium of the megagametophyte. A-D adapted from *Morphology of Vascular Plants* by A. J. Eames, copyright 1936. Used with permission of McGraw-Hill Book Company. E and F from *Comparative Morphology of Vascular Plants*, Second Edition, by Adriance S. Foster and Ernest M. Gifford, Jr., W. H. Freeman and Company, copyright 1974.

which helps the megagametophyte to stay afloat. Trapped sperm remain active and can be seen slowly rotating in their frustrated effort to reach the egg. The flagellar apparatuses of expired sperm become uncoiled and appear as spirals in the gelatinous sheath of the megagametophyte.

Marsilea is heterosporous since it produces two distinct types of spores. This type of life cycle should be contrasted with the homosporous life cycle of *Pteridium* or *Woodwardia.*

Embryo development is rapid and virtually superficial. After two days a globular green embryo can be seen in the archegonial region. After 7 days the first leaf and root can be seen (Figure 11.7)

Students work in pairs. Each pair obtains one *Marsilea* sporocarp, one plastic petri dish, one pasteur pipet, one razor blade or scalpel, and two microscope slides with coverslips. At the start of the exercise, students hold the sporocarp against a hard surface with one of their fingers. Using the razor blade, they make shallow cuts at the two narrow ends of the sporocarp. After making these cuts, they should be able to see the yellow color of the sporocarp's contents. The nicked sporocarp is placed in a petri plate of water. The sorophore should emerge within 30 minutes after the sporocarp is exposed to water. Students should observe and sketch the initial events of germination and they should identify the sori, sorophore, microsporangia and megasporangia. The sporangia become detached from the sorophore after one hour.

To observe sperm motility, chemotaxis and fertilization, students use pasteur pipets to remove megagametophytes from a culture which has been incubated at 25 C for 8 hours. Fast-moving sperm can be seen swimming in the water. Many trapped sperm can be viewed near the archegonium of the megagametophyte. By reducing the aperture of the condenser iris diaphram, students can see the outline of the gelatinous sheath surrounding the megagametophyte. The archegonium is yellow and the basal cell is white. To see microscopic details of the megagametophyte it is helpful to illuminate from above with a dissecting microscope lamp as well as from below with the microscope lamp. Careful examination of the archegonium will reveal the outermost cells of the neck which protrude slightly beyond the outline of the archegonial papilla.

Following these observations, the petri plates are sealed with parafilm and placed in the light for further development. Students repeat their observations at 48, 96 and 168 hours, and record the initial events of embryo and early sporophyte development (Figure 11.7). The sporophytes will continue to grow slowly in plain tap water. Substitution of plain liquid fern medium should stimulate growth. The addition of carbohydrates to the medium has been shown to influence heteroblastic leaf development (White 1971) as well as regulate the general morphological form of the sporophyte. Sporocarps are easily sterilized by soaking in 10% bleach with 0.5% Alconox for 20 minutes

followed by three rinses in sterile water (Laetsch 1967). Using sterile instruments to nick the sporocarp, and allowing fertilization to occur in sterile medium, experiments on the effects of carbohydrates on sporophyte development can be performed.

Marsilea plants and sporocarps can be purchased from Carolina Biological Supply Co.

This laboratory was designed for developmental biology but it could easily be adapted for many botany courses. Demonstrating sporocarp nicking and having the students nick their own sporocarps requires 30 minutes. Observations during the first period take 1.5 hours. Brief 30 minute observations are necessary at prescribed intervals.

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