

Chapter 10

Laboratory Investigations With *C-Fern*TM (*Ceratopteris richardii*)

Leslie G. Hickok and Thomas R. Warne

Department of Botany
University of Tennessee
Knoxville, TN 37996
(423) 974-2256; cfern@utk.edu

Leslie Hickok received his B.S. in Biology from Murray State University, his M.S. in Botany from Ohio University, Athens, and his Ph.D. in Botany from the University of Massachusetts, Amherst. Dr. Hickok is a Professor at the University of Tennessee, Knoxville. He has studied extensively the biology and use of *Ceratopteris* in basic research ranging from cytotaxonomic to genetic, physiological and molecular genetic studies.

Thomas Warne received his B.S. in Botany from the University of Georgia, Athens and his M.S. and Ph.D. in Botany from Ohio University, Athens. Dr. Warne is a Research Associate at the University of Tennessee, Knoxville and has a research background in the evolutionary and population biology of *Ceratopteris*.

Jointly, Drs. Hickok and Warne have pursued basic research on the physiology and genetics of salt tolerance in plants using *Ceratopteris* and are currently developing educational materials for university-level and grade 7-12 biology courses using this unique organism.

Reprinted From: Hickok, L. G. and T. R. Warne. 1998. Laboratory investigations with *C-Fern*TM (*Ceratopteris richardii*). Pages 143-176, in *Tested studies for laboratory teaching*, Volume 19 (S. J. Karcher, Editor). Proceedings of the 19th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 365 pages.

- Copyright policy: <http://www.zoo.utoronto.ca/able/volumes/copyright.htm>

Although the laboratory exercises in ABLE proceedings volumes have been tested and due consideration has been given to safety, individuals performing these exercises must assume all responsibility for risk. The Association for Biology Laboratory Education (ABLE) disclaims any liability with regards to safety in connection with the use of the exercises in its proceedings volumes.

© 1998 Leslie G. Hickok and Thomas R. Warne

Contents

Introduction	144
Overview of Investigations and Notes for the Instructor	147
Investigation 1. Plant Development and <i>C-Fern</i> Life Cycle.....	150
Investigation 2. Quantitative Measurement -- Serial Dilution And The Mass Of An Individual <i>C-Fern</i> Spore.....	154
Investigation 3. Environmental Controls On Sex Determination - Gametophytic Density And Sex Expression in <i>C-Fern</i>	158
Investigation 4. Ecology in a dish - Environmental Controls On Sporophyte Development - Population Density And Growth of <i>C-Fern</i> Sporophytes	162
Investigation 5. Genetics In Action! Visualizing Mendelian Inheritance Using <i>C-Fern</i>	165
Acknowledgments	171
Literature Cited	171
Appendix A. Surface Sterilization and Sowing of <i>C-Fern</i> Spores	171
Appendix B. <i>C-Fern</i> Nutrient Medium - Composition And Preparation	173
Appendix C. General Culture Instructions.....	175
Appendix D. Sources of Supplies and Materials.....	176

Introduction

Laboratory-based education can serve many functions, such as, the reinforcement and illustration of lecture and text material and the exposure of students to the uses of sophisticated contemporary equipment and methodologies. However, an equally important and often neglected function is the use of the biological laboratory to promote general understanding of the scientific method. Laboratory-based education can teach students how the process of science works, what constitutes scientific information and how scientific information is acquired. In other words, this approach can teach students how to DO science. Comprehension of the scientific method involves understanding and applying a large set of interrelated skills -- problem identification, hypothesis formulation, experimental design, implementation, data collection, analysis and synthesis—that necessitate a different approach to teaching. However, normal constraints on time and equipment can make it difficult to implement such an approach. One option is the introduction of exercises in experimental science using amenable and flexible model organisms relevant to a broad range of disciplines and subject areas. In plants, only a few model systems have attributes that meet the demands of a wide range of situations and levels. Here, we describe a broadly useful model system, *C-Fern*, which is based on the homosporous fern, *Ceratopteris richardii*. *C-Fern* possesses a number of characteristics and developmental features that make it ideal for use in the classroom and in student-initiated research.

Why Use *C-Fern*?

The advantages of *C-Fern* as a model plant system derive from the unique properties of its development and life cycle (Chasan, 1992; Hickok et al., 1995; Hickok et al., 1997). Both haploid (gametophyte) and diploid (sporophyte) phases exist independently and, thus, experimental studies are possible at the cellular and whole plant level without artificial manipulations of the life cycle. Development from spores to sexually mature gametophytes to young sporophytes can be observed within a 2-3 week period and the complete, spore to spore, life cycle takes less than 12 weeks. The rapid growth and small size of gametophytes permit the ‘miniaturization’ of experiments, such that large numbers of individuals and treatments can be used in a small space and short time. The haploid / diploid genetic system is very simple. *C-Fern* combines features of both 'higher' and

'lower' plant systems. The developmental simplicity and haploid status of the gametophytic phase provides opportunities that are not available in angiosperm models. At the same time, the ability to study any genotype or process within the complex vascular sporophyte phase allows direct comparisons with higher plant systems (e.g., Wisconsin Fast Plants™, *Arabidopsis*) that are not possible with developmentally simpler systems such as mosses (e.g., *Funaria*, *Physcomitrella*) or algae (e.g., *Chlamydomonas*, *Euglena*).

We present here the protocols necessary to culture and use *C-Fern*™ for educational settings. Some investigations that capitalize on the unique attributes of this model plant system are given. Although these investigations are formatted in the 'standard approach', they are easily modified to a 'student-guided' or inquiry-based approach.

What Is *Ceratopteris*?

Ceratopteris is a genus of homosporous ferns found in most tropical and subtropical areas of the world (Lloyd, 1973). Species grow as either aquatics or sub-aquatics and are restricted in habitat to ponds, rivers or other intermittent wet areas such as ditches, rice paddies or taro patches. Although some require an aquatic habitat, most species can be successfully grown in standard greenhouse pot culture under warm, humid conditions. Currently, commercial applications are primarily limited to its use as an aquarium plant, where it is sold under the common name of 'water sprite' and has even been immortalized in plastic replicas.

Although a number of homozygous diploid and tetraploid accessions of *Ceratopteris* are available, the Hn-n strain has been used in most experimental studies (Hickok, 1987; Hickok et al., 1995; Hickok et al., 1997). Hn-n is a diploid ($n = 39$) derived from a collection of *C. richardii* from Cuba (*Killip 44595* GH). During the past several years, an improved diploid strain, RN, has been developed from a series of crosses and backcrosses. All of the exercises presented here are based on the use of the RN strain.

The *C-Fern* Life Cycle (See Figure 10.I.1)

Like all homosporous ferns, *C-Fern* has two independent, autotrophic phases: a developmentally simple haploid gametophyte and a vascular diploid sporophyte. The gametophytic phase, which develops mitotically after germination of the single-celled spore, can be cultured axenically on a simple inorganic nutrient medium. Development of this haploid phase is very rapid. Germination occurs three to four days following inoculation and full sexual maturity is attained within six days after germination. At maturity, the gametophyte consists of a small (2 mm), simple essentially two-dimensional (flat) thallus with rhizoids, vegetative cells and sexual organs (archegonia and antheridia). Archegonia (singular-archegonium) are female organs that contain one egg each that lies at the base of a small neck that sticks out from the surface of the gametophyte. The neck consists of four rows of cells and a few 'central canal cells' in the middle. When mature and in the presence of water, the central canal cells burst open so that a small open canal leading to the egg is formed. The cellular contents from the central canal cells are deposited in the vicinity of the top of the open neck. Meanwhile, the antheridia, or male sex organs, are also active. In the presence of water, they too burst open and discharge swimming sperm (spermatozoids). The sperm swim rapidly in the water and are irresistibly attracted by the discharge from the archegonium. In a few minutes, hundreds of sperm can be seen swarming around the neck of the archegonium. One of them eventually wiggles its way down the neck and fertilizes the egg. After fertilization of the egg, the resulting diploid zygote develops rapidly by mitotic cell division and forms an embryo. Embryos are clearly visible after a few days. In 1-2 weeks small diploid sporophytes can be identified by the presence of roots and leaves. The gametophyte soon dies. The sporophyte grows to maturity to undergo meiosis and produce spores to begin the life cycle again.

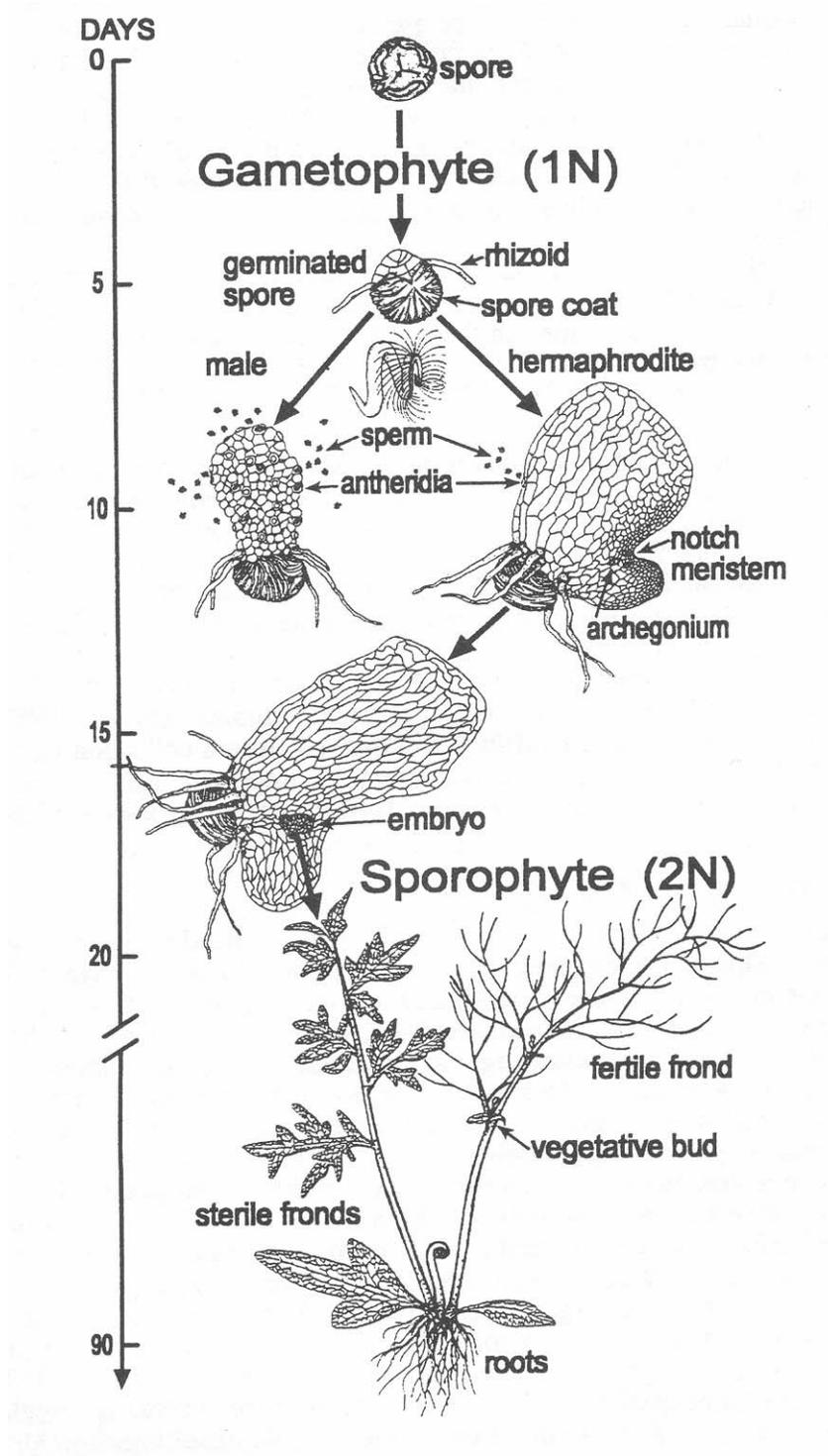


Figure 10.I.1. C-Fern Life Cycle.

A pheromone-like substance, the antheridiogen A_{Ce} , secreted by developing gametophytes controls differentiation of two distinct sexual forms of gametophytes. A_{Ce} is likely biosynthetically related to gibberellins and is effective at exceedingly low concentrations (Warne and Hickok, 1989). In the absence of A_{Ce} , gametophytes develop initially as heart-shaped (cordate) females with archegonia and subsequently as hermaphrodites with both archegonia and a few antheridia. In hermaphrodites, a defined meristematic region (notch meristem) is present and growth is indeterminate until fertilization of an egg occurs via swimming sperm. Meristematic activity ceases shortly after the fertilization event. In contrast to hermaphrodites, gametophytes that mature in the presence of A_{Ce} develop into tongue-shaped males, which are small, determinate, lack a meristem and produce large numbers of antheridia. The vascular sporophyte stage consists of 5-20 cm plants composed of a short upright stem (rhizome) with roots and leaves (fronds). In contrast to many ferns, the *Ceratopteris* sporophyte is not woody and grows rapidly as an annual. Spore production via meiosis occurs within sporangia that are located on the margins of fertile leaves. Upon maturity, spores are produced continually and are unlimited in number. Compared to many ferns, spores are quite large (ca. 120 μm) and relatively easy to handle. Because individual haploid gametophytes can be self-fertilized, sporophytes completely homozygous across all genetic loci can be produced in one generation of selfing. Such sporophytes produce an unlimited number of genetically identical spores. If kept dry, spores remain viable for many years.

Notes for the Instructor

Overview of Investigations:

Using 'DarkStart'

In some Investigations, sterilized spores are presoaked in complete darkness for 3-7 days (DarkStart, Refer to Appendix C). Following this presoaking step, initial germination will occur about 36-48 hours after exposure to light. This procedure also helps to synchronize early gametophytic development.

Culture Conditions and Timing of Investigations

Times given for monitoring the various developmental stages of gametophytes are based upon ideal growth conditions. Because growth rate and development are dependent upon the environmental conditions (principally, temperature and light), it is recommended that teachers set up a trial run to determine when stages will be present under specific conditions. Specific temperature and light requirements are given in Appendix C.

Medium Preparation and Spore Sterilization

The use of prepared medium and presterilized spores greatly simplifies set-up. However, all the investigations can be combined with training in basic tissue culture techniques by having students formulate and pour the medium (Appendix B) and surface sterilize spores (Appendix A). (Prepared *C-Fern* medium and spores, both presterilized and bulk unsterilized, are available through Carolina Biological Supply Company.)

Aseptic Technique - Handling Petri dishes

All of the Investigations can be performed without the use of a laminar flow hood or sterile bench. Simple precautions and careful attention to technique can minimize or eliminate nearly all contamination. When pouring medium and inoculating or sampling Petri dishes the lid should be lifted up at an angle only as far as necessary. Should some contamination appear on cultures, it will not usually significantly alter the outcome of the experiment. The same precautions should be taken when sampling cultures for observations and when watering cultures to facilitate fertilization.

Investigation 1. Plant Development and the C-Fern Life Cycle

This exercise serves as an introduction to the plant life cycle in general and that of *C-Fern* in particular. This exercise provides experience in manipulating and making observations of the organism. Students can develop familiarity with the organism so that its use in subsequent investigations or student-initiated research is facilitated. This investigation focuses on the development of sexually mature gametophytes from single-celled spores in less than 2 weeks, but this can be extended to over 4 weeks to include observations of sporophyte development following fertilization. Since the establishment of cultures and subsequent periodic observations are not time-consuming, additional subject matter can be easily combined with this exercise. For instance, this vivid demonstration of plant development can be used as a focal point to introduce or discuss subjects such as genetics, mating and breeding systems and evolution. In addition, observation of the effects of environmental variables on development could be incorporated by use of simple experimental treatments, e.g., manipulation of temperature or light conditions.

Investigation 2. Quantitative Measurement -- Serial Dilution And The Mass Of An Individual C-Fern Spore

This investigation uses a suspension of a known weight of *C-Fern* spores and some simple tools and procedures to estimate the mass of a single spore. It introduces the handling of *C-Fern* spores as a prelude to further investigations. This investigation provides intensive practice with measurement and observations using a microscope. A complete analysis of data involves extensive arithmetical manipulations. A fundamental concept introduced by this investigation is *dilution*, an extremely simple and routine technique used widely in medical, scientific, research and commercial laboratories. *Serial dilution*, an interconnected succession of dilutions, yields an ultimate effective dilution that otherwise would require an exceedingly large volume of *diluent*, the inert substance used to dilute. In microbiology, serial dilution is fundamental to the determination of the original concentration of bacteria in a suspension culture. In molecular biology, cell biology and physiology, the techniques and approaches of serial dilution can be used to formulate complex reagent mixtures from more concentrated stock solutions. The mathematical (arithmetic) manipulations necessary to make complete calculations underscore the necessity of handling units appropriately and understanding the relationships among units.

In addition, this investigation introduces students to basic sampling concepts. One of the key elements of the scientific method is the use of approaches that summarize the characteristic elements of a *population*. In order to obtain information that represents the entire population accurately, *sampling* procedures are required.

It is especially important in this investigation to suspend spores thoroughly *immediately* prior to each and every sowing and to sow spores quickly onto their respective dishes. Since *C-Fern* spores are exceptionally large they rapidly sink in the tube. If spores are not handled promptly high variability in sowing results.

Investigation 3. Environmental Controls Of Sex Determination - Gametophytic Density And Sex Expression in the C-Fern

Fern spores are haploid, single celled resting structures that germinate under appropriate environmental conditions to develop into the small sexual phase of the life cycle, i.e., the gametophyte or prothallium (prothallia). Fern gametophytes are analogous to the minute gametophytic parts of flowering plants that develop from pollen grains (male) or are found deep within the ovary (female) of the flower. Unlike flowering plants, the gametophytes of ferns contain chlorophyll, are free living and physiologically and developmentally independent of the diploid sporophyte portion of the life cycle.

Investigation 3 may be used as an extension of Investigation 2, in which students collect data and determine the percentage of sexual types of gametophytes (male and hermaphroditic) after about 10-12 days of culture. However, if cultures for this investigation are established *de novo*, it is not necessary to take the careful precautions to minimize and account for spore loss indicated in Investigation 2. It is sufficient to establish cultures with a range of spore densities from 1 per dish up to about 1000 per dish. This exercise provides students with experience in the acquisition of and graphic display of data.

Investigation 4. Ecology in a dish - Environmental Controls Of Sporophyte Development - Population Density And Growth of C-Fern Sporophytes

Investigation 4 may be used with no additional setup as an extension of Investigation 3. It provides a dramatic demonstration of the effect of population density on plant growth. Following data acquisition in Investigation 3, dishes can be watered to effect fertilization and returned to culture for an additional 14-21 days. Once the first leaves and roots of the resultant population of sporophytes have reached a measurable length, i.e. a few to several mm, they can be measured. The plotted data compares the effect of population density on growth.

One possible complication may occur if fertilizations are differentially successful at the different densities. Typically, in the higher density cultures, a large number of successful fertilization will occur. The competitive response will be clearly demonstrated both qualitatively and quantitatively. In some cases, in lower density cultures, a single watering may not yield successful fertilization at the same time as the higher density cultures. If this occurs, sporophyte production may be substantially delayed and result in smaller sporophytes than those from higher density cultures. However, given sufficient time, these delayed sporophytes will eventually grow quite large.

Investigation 5. Genetics In Action! Visualizing Mendelian Inheritance Using C-Fern

Mendelian genetics typically presents a challenge to many students at many academic levels. A full understanding of Mendelian genetics depends on the integration of basic principles of cell biology (mitosis and meiosis) with arithmetic rules of probability. Although reproduction and inheritance are certainly dynamic processes in most organisms, constraints on time and facilities limit the ways in which these basic principles can be demonstrated. In *C-Fern*, because all stages of sexual reproduction following meiosis take place within a two week period, basic Mendelian principles can be demonstrated in a powerful and visual manner not possible with most other organisms. This exercise is constructed so that students are active in data acquisition and manipulation of the organism. The ability to follow a trait over two distinct generations (gametophyte to sporophyte) permits meaningful hypothesis formulation and testing. The culture of small populations provides experience in population sampling. Adequate numbers can be generated from a single Petri dish. Class data can also be combined to demonstrate the effect of sample size on ‘goodness of fit’.

The use of *C-Fern polka-dot* mutation as a segregating nuclear gene provides visual interest for students. *Polka-dot* affects chloroplast distribution within cells and is expressed in both gametophytes and in sporophytes, where it is recessive. The mutation results in tissue that appears to have green ‘polka-dots’ in contrast to the wild type condition that shows uniformly green tissue. Although it is dramatically different from the wild type, the polka-dot mutation has minimal effect on growth and development so that expected gametophytic ratios of 1:1 resulting from meiotic segregation of alleles in the F1 hybrid and F2 sporophytic ratios of 3:1 resulting from random fertilizations and recessive expression of *polka-dot* are highly reproducible. Although *polka-dot* males can be distinguished from wild type, scoring of phenotypes only in cordate hermaphrodites simplifies data acquisition. Since the differences between male and hermaphrodite gametophytes are developmental and not genetic, segregation data are not affected when only one sexual type is scored.

In animal models (e.g., *Drosophila*, humans) meiosis results directly in gametes and phenotypes cannot be readily observed until after fertilization and development of the embryo into the adult. In

150 C-Fern™

flowering plants (e.g., peas, *Arabidopsis*, Wisconsin Fast Plants™), although male and female gametophyte generations do exist (e.g., the embryo sac within the ovary and the bi-nucleate pollen grain), they are small, not readily observed and do not typically exhibit a clearly visible phenotype. As in animals, scoring of phenotypes can only be readily accomplished in the diploid phase. In contrast, the products of meiosis in *C-Fern* develop into independent small plants (gametophytes) that, as in this example, can show a clear difference between mutant and wild type phenotypes. As a result, 'gametic' segregation ratios resulting from meiosis in an F1 hybrid are visible and directly demonstrate the Mendelian principle of 'segregation of alleles'. Data acquired from cultures at this stage can be used to construct hypotheses about phenotypic segregation ratios in the diploid stage that result from random combination of gametes during fertilization. Using *C-Fern*, students actually initiate and visualize fertilization by adding water to the cultures and viewing the hundreds of actively swimming sperm seeking out mature archegonia containing eggs. Subsequent examination of the resulting sporophytes allows students to determine the F2 phenotypic ratio and whether the trait is dominant or recessive.

The total time required is three partial class periods with manipulations and observations once each week. Although under ideal conditions inoculation and scoring of cultures can be done on a weekly basis, for most situations it is suggested that cultures be inoculated three to four days prior to the first class period. Subsequent observations can then be made on a weekly basis. A suggested time table is: Set-up - basic medium preparation (1.5 hr) and inoculation of cultures 3-4 days prior to the first class meeting (0.5 hr). First Week - Observation of cultures (15 minute). Second Week - observations and determination of gametophyte segregation ratio, formation of hypotheses for mode of inheritance and visualization of swimming sperm and fertilization (1 hr). Third or Fourth Week - sampling of cultures to estimate sporophyte phenotypic ratio, Chi-Square analysis to test hypotheses (1.5 hr).

Directions are given to establish cultures (2 dishes / student) at 2/3 standard sowing density using spores of the F1 hybrid: polka-dot x wild type. Pre-sterilized spores (10 mg) may be sown at 2/3 the standard density by adding 6 ml sterile water to the vial and sowing up to sixty 60 mm (diameter) Petri dishes. If using bulk unsterilized spores, sterilize using standard procedures and sow at 2/3 standard density.

Depending on your culture conditions, sporophyte phenotypes can be scored on either the third or fourth week to allow clear determination of sporophyte phenotypes

Options: This exercise could be combined with Investigation 2 to allow determination of the effect of population density on expectations from random fertilization. Very small populations (lowest density) can show significant variation from expectations, although the hypothesis concerning inheritance of the trait may be valid.

As an option for interested students, this investigation can be extended by culturing sporophytes to maturity and then separately collecting spores from different individuals from the F2 generation. Gametophyte cultures produced from polka dot sporophytes (homozygotes) would uniformly show the trait while the two types of green sporophytes (homozygous wild type and heterozygotes) would show all green and 1:1 segregations, respectively.

After individual data have been tested with Chi-square analysis, class data can be pooled to demonstrate the effect of sample size.

Investigation 1. Plant Development and the *C-Fern* Life Cycle

Background

How do plants develop and reproduce? Many plants can reproduce *sexually* - but what does this mean? Land plants are typified by a special life style, commonly referred to as the *alternation of generations*, in which two distinct phases occur defined by the events of *meiosis* and *syngamy* (fusion of gametes). In land plants, meiosis results in the production of haploid *spores*. Spores are single cells that germinate and develop under the appropriate conditions into multicellular plants

that produce the sex cells or *gametes*. These *gametophytes* not only bear the male and female gametes (spermatozoids and egg cells, respectively) but also serve as the site for fertilization. The completion of fertilization by the formation of the *zygote* initiates the diploid phase of the life cycle, the *sporophyte*. In both ferns and seed plants, the sporophyte is the visibly dominant generation. The gametophyte of seed plants is very small and develops within special structures of the sporophyte, whereas in ferns the gametophyte, though tiny, develops and matures independent from the sporophyte.

Objective

To observe basic events of the plant life cycle, focusing on the sexual phase of a fern. This will include observations of development from spore germination to sexual maturation and the formation of the young sporophyte.

Materials

- surface sterilized wild type *C-Fern* spores (unsterilized bulk or presterilized, preweighed in screw cap vial), 10 mg is sufficient to inoculate about 30+ dishes.
- Petri dishes (60 mm diameter) containing agar-solidified *C-Fern* nutrient medium, 2 per student or group
- One 5 ml volumetric pipette, sterile
- about 40 ml distilled water, sterile (SD-water)
- If spores are unsterilized, 1 sterile conical bottom centrifuge tube and spore sterilization supplies (see Appendix B)
- Pasteur pipette, sterile, with bulb,
- small spatula, probe, or forceps, sterile
- spore spreader, sterile
- alcohol (70%)
- stereomicroscope
- Culture Dome

Procedures and Observations

First Period (Day 0)

Today you will manipulate *C-Fern* spores – sterilize them (if necessary), sow them onto nutrient medium, and carefully observe them. If you are using presterilized spores add the appropriate amount of water (4 ml SD-water for 10 mg spores), then proceed to sow spores. If you are using unsterilized spores, first weigh out 10 mg spores and transfer them to a sterile conical centrifuge tube. Surface disinfect the spores according to the method in Appendix A and suspend them in 4 ml SD-water.

Sow spores by thoroughly suspending them in solution and then putting 3 drops of spore suspension onto the agar surface. Sterilize the spore spreader by dipping it into alcohol and allowing it to air dry. Distribute spores across the agar surface using a sterile spore spreader. Remember to work quickly and carefully. After spores are dispersed and they germinate, what do they grow into?

Let us contemplate the spore! The spores you are using come from a special strain of the tropical fern, *Ceratopteris*. You may have seen a different species of this fern (called water sprite) sold as a plant for tropical fish aquaria. Observe the spores under the highest power of your dissecting microscope. If you have a compound microscope, prepare a *wet mount* by placing one

152 C-Fern™

drop of the spore suspension onto a microscope slide – be sure to put on a cover slip. Believe it or not, *C-Fern* spores are very large compared with typical fern spores! Why are spores so small? How many cells are in a spore? How does this compare with the seed from higher plants? Before wetting and sowing did the spores appear to be dry? When dry, how can they survive? What factors could be responsible for the germination of spores? Can you see any decorations on the spore walls? Is the spore wall ornamentation and conformation uniform on all surfaces? Draw one or more spores to show the wall ornamentation and conformation.

Over the next several laboratory sessions, you should carefully watch what happens to these spores. During the period from 4-7 days following the sowing of your spores, you will want to make several observations. During each laboratory period you should check your cultures and record your qualitative and quantitative observations by taking good notes and making drawings of the different things that you see. Each time you make observations, you should try to handle your cultures carefully to avoid contamination by keeping the lid in place as much as possible. Remember to return your cultures to the Culture Dome promptly when you are finished.

Second Period (Day 4-7)

Using the dissecting microscope, look through the lid of the dish at the spores you sowed last time. Observe the overall appearance of the spores -- their size, shape and color. How have the spores changed since last time? Many spores should have germinated. What is happening when spores germinate? You can identify the germinating or developing ferns because they will always be growing out of spores. Have all of the spores germinated? How would you quantitatively describe the relative number of germinated spores? What could be the reasons for germination of some spores and not others? Are there fungi, bacteria or algae growing on the dish? Where could these contaminants come from? When doing experiments, why is it important to try to eliminate these types of contaminants completely?

Make the following observations under high magnification with a dissecting scope or prepare a wet mount of the germinated spores and observe them under a compound microscope. When you remove a few germinated spores using a small spatula or forceps, try to choose a variety of different looking plants. For each developing gametophyte, locate the original spore wall. What has happened to the spore wall? Among several gametophytes, is there any pattern to what happened to the spore wall? The small plants emerging from the spore wall should be green. Why are they green? Locate the rhizoids which are clear elongated cells (spaghetti-like) that stick out from the base of the germinated spore. They are commonly near the spore wall. What could be the function of rhizoids in such a plant that contains so few cells? Identify cells that contain *chloroplasts*. What are the differences among the young plants that you observe? Draw several of these young plants and label the spore wall, rhizoids and photosynthetic cells with chloroplasts.

Third Period (Day 8- 14)

Remove only one of your two dishes at this time to make observations. How have the gametophytes changed from last week? By this time you should easily be able to identify the two distinct types of gametophytes (*prothallia*, singular *prothallus*) growing in your cultures. The larger mitten-shaped gametophytes have both male and female sex organs and are referred to as hermaphroditic gametophytes. A few male sex organs or *antheridia* (singular *antheridium*) are located on the edges of the plant and near the rhizoids at the lower part of the plant. The female sex organs or *archegonia* (singular *archegonium*) are found only just behind the growing notch or notch meristem. Why are archegonia found only here? Among these larger gametophytes should be numerous small, tongue-shaped male plants. Males have many antheridia. Do these male gametophytes have a meristem like female gametophytes? If not, what might be the consequences of not having a meristem? Males develop in response to the presence of a special chemical

(*antheridiogen*) in the medium that is secreted by developing gametophytes. What might be the ecological or genetic benefits for such a condition?

Prepare a wet mount that includes both types of gametophytes and observe them under the compound microscope. Draw one of each type of gametophyte. Label the spore wall, rhizoids, photosynthetic cells, sex organs, and notch meristem.

For your next set of observations, you should use a dish that has remained under the Culture Dome until just before use. Read the following set of directions and be prepared to work quickly so that you can see the male *gametes* in action -- the swimming sperm cells (*spermatozoids*). Depending on the available microscopes, you can make these observations with a dissecting or compound microscope or both.

For the dissecting scope, observations can be made after watering the culture with 10 drops of sterile distilled water. It is best to put the water on only one portion of the dish so that you will have some unwatered gametophytes for additional watering and observations, if necessary. For the compound microscope, prepare a wet mount of 4-10 older male gametophytes. Include one or two of the larger heart-shaped (cordate) gametophytes that have one to three archegonia. Try to place the side with archegonia so that it is facing up. Group the gametophytes on the slide so that you can carefully observe what happens to their antheridia and archegonia. Add 1 drop of water and place a coverslip over them.

Be persistent with your observations of the sex organs over the next 5-15 minutes as you scrutinize the different plants. Set the power on you microscope so that you can clearly observe several males at one time. Carefully scan all the male gametophytes present. Watch for changes in the antheridia themselves. When you see movement, you may want to increase the magnification. What happens to antheridia? What does water do? How can sperm swim in distilled water? Where do spermatozoids come from? The rapid movement of spermatozoids can make observation difficult unless they are caught on something or slow down. How could you determine how long (time and distance) sperm cells can swim?

Once you have seen spermatozoids, try to follow the general direction of their movement. If you have selected a mature female, at least some of the sperm should be swimming toward archegonia on another gametophyte. You may be able to see the sperm cells struggling down the neck of the archegonium, attempting to fertilize the egg. How can the sperm cells find their way to the archegonia and on to the egg cell? What happens when a spermatozoid finds its way successfully to an egg?

Before you return your cultures to the Culture Dome, flood the surface of the dish with sterile distilled water, about 1-2 ml or 1/2 of a Pasteur pipette full. This will help insure that many fertilizations take place to provide material for further observations.

Fourth Period (Day 14-21)

Prepare a wet mount of several different gametophytes. Be sure to include a few older cordate gametophytes from the dish. Carefully observe the region just behind the notch meristem. If fertilization was successful last week, you may be able to see bumps at the base of older 'pregnant' archegonia! What do you think these bumps will grow into? Where have these bumps come from? Over the next week or two you may want to observe your developing sporophytes. Soon some of the sporophytes may be hardy enough to transplant to a terrarium containing potting soil. Choose young sporophytes with well-developed roots, try not to take a lot of agar and make sure that you separate individual plants. A 2 liter clear plastic beverage bottle can make a small, inexpensive terrarium. If you watch your fern grow long enough (50 or more days) you should find sporangia that develop on the backside of specialized leaves. At that point, you may wish to grow spores and begin yet another life cycle generation of *C-Fern* .

Investigation 2. Quantitative Measurement -- Serial Dilution And The Mass Of An Individual C-Fern Spore

Background

A *C-Fern* spore is a very small single-celled structure that will develop into the sexual phase of the life cycle, the *gametophyte*. How small is very small? How much does a spore weigh? It would be very difficult to weigh an individual spore unless you had very sophisticated, specialized and expensive equipment. This investigation uses some careful manipulation and a commonly used laboratory procedure, serial dilution, to estimate the mass of a spore.

Objectives

To estimate the 'best' average mass of an individual spore by using a serial dilution technique.

Materials

- surface sterilized wild type *C-Fern* spores (unsterilized bulk or presterilized, preweighed in screw cap vial), 10 mg is sufficient to inoculate 30+ dishes
- Petri dishes (60 mm diameter) containing agar-solidified *C-Fern* nutrient medium, 6 per student or group
- volumetric pipettes (one each of 1 and 5 ml), sterile
- about 40 ml distilled water, sterile (SD-water)
- If spores are unsterilized, 6 sterile conical bottom centrifuge tubes and spore sterilization supplies (see Appendix B)
- If spores are presterilized - 6 sterile test tubes or sterile screw cap vials
- Pasteur pipette, sterile, with bulb
- spore spreader, sterile
- alcohol (70%)
- stereomicroscope (dissecting microscope)
- micrometer or plastic ruler

Procedures and Observations

Experiment setup

1. Suspend sterile spores in a known volume of water.
 - a. Option A. (Use this option only if appropriate balance is available and your sterilizing technique is excellent). Weigh out 10 mg wild type spores. Sterilize as usual (see Appendix A) taking extreme care to not lose spores. Suspend spores in 3 ml sterile water. Label this tube A.
 - b. Option B. Suspend wild type (10 mg preweighed, presterilized) spores in 3 ml sterile water.
2. Serially dilute the spore suspension as follows. Be sure to suspend spores thoroughly before transfer.
 - a. Remove 1 ml to a new tube (Tube B), add 2 ml water, suspend
 - b. Remove 1 ml to a new tube (Tube C), add 2 ml water, suspend
 - c. Remove 1 ml to a new tube (Tube D), add 2 ml water, suspend
 - d. Remove 1 ml to a new tube (Tube E), add 2 ml water, suspend
 - e. Remove 1 ml to a new tube (Tube F), add 2 ml water, suspend
3. Enter the appropriate information concerning these serial dilutions into Table 10.2.1.
4. Sow Petri dishes

- a. Label one small dish per tube with your name, date and treatment code.
- b. Carefully and completely suspend spores in each tube. Sow exactly 0.25 ml of the spore suspension from each tube onto a small dish.
- c. Carefully spread spores to as uniform distribution (across the whole plate) as possible using the technique described in Appendix A.

Data Collection

5. Determine the number of spores on a dish for each dish sown. Set microscope to a magnification that allows you easily to keep track of *all* the spores in the field of vision.
 - a. For low density dishes, count all spores on the dish. Enter data into Table 10.2.2.
 - b. For high density dishes, a sampling procedure is required to estimate the actual number of spores on the dish. To sample, use an objective power that permits easy observation and tracking of all spores in a field. Move the dish to a 'random' position and count all spores in that given field of view. Repeat the procedure on a different field of view until sufficient data are collected. Enter the appropriate data into Table 10.2.2.

Data Analysis

6. Complete calculations in Tables 10.2.1-10.2.3. Pay careful attention to the headings in each column.
7. Calculate an estimate of the average individual spore mass for each dilution treatment. Use Figure 10.2.1 to graph the effective dilution vs. the calculated average individual spore mass.
8. Complete analysis of your data first, then analyze combined data from all groups. (Each group should provide average (mean) values for combined use.) Add the data from each different group in your class to Figure 10.2.1.

Table 10.2.1. Effective dilution and mass of spores per ml in tube.

Tube #	Starting volume in tube (ml)	Starting mass of spores in tube (mg)	Total effective dilution volume (ml)	Mass of spores per volume in tube (mg/ml)
A				
B				
C				
D				
E				
F				

Table 10.2.2. Total number of spores on dish.

Dish(es) from Tube	Total area of dish (cm ²)	Area of field(s) sampled on dish (cm ²)	Number of spores in sampled fields	Total number of spores on dish
A				
B				
C				
D				
E				
F				

Table 10.2.3. Average mass of spore and number of spores per mg.

Dish(es) from Tube	Volume sown on dish (ml)	Mass of spores in volume sown (mg)	Number of spores per mg	Average mass of a spore (mg)
A				
B				
C				
D				
E				
F				
Mean SD:				

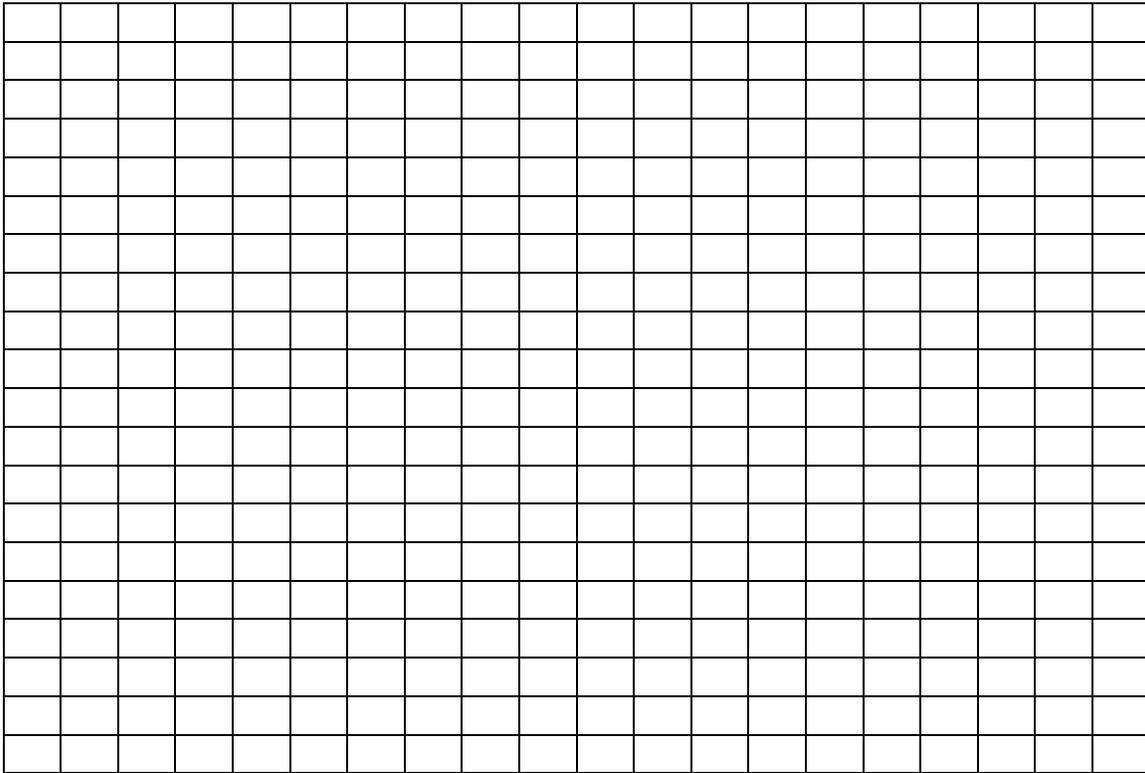


Figure 10.2.1. Effective dilution vs. average individual spore mass.

Discussion

1. Why is it necessary to be extremely careful when sterilizing the spores yourself?
2. Why is there variation in the average mass of a spore and number of spores per mg for the different dishes? How can this variation be reduced?
3. Why is it necessary to sample some dishes? Compare the sources of variations between dishes that were sampled and those that were not.
4. Is this investigation an *experiment*? If not, what are some things that could be done in order to “make” it into an experiment?
5. Can you think of another way to measure the average mass of a *C-Fern* spore that would require the use of a microbalance?
6. Describe the graph you completed (Figure 10.2.1) that shows the effective dilution vs. average mass of a spore.

Investigation 3. Environmental Controls On Sex Determination - Gametophytic Density And Sex Expression in *C-Fern*

Background

A mature *C-Fern* spore is a haploid, single cell encased in a spore wall. Upon germination, mitotic divisions give rise to a mature haploid *gametophyte* or *prothallium*. When grown in multispore cultures, two types of gametophytes are produced that bear male and/or female sex organs (*gametangia -ium*), the *antheridia* (ium) and *archegonia* (ium), respectively. The hermaphroditic gametophyte is the larger of the two and is characterized by a localized notch *meristem* that results in a heart or *cordate* shape. The hermaphrodite initially produces the female sex organs and subsequently produces a small number of male sex organs. The tongue-shaped male gametophyte is smaller, lacks a meristem, and only produces male sex organs. This investigation explores how these two types of gametophytes are produced.

Objectives

Observe effects of spore sowing and gametophyte density on sex expression of gametophytes.

Materials

- surface sterilized wild type *C-Fern* spores (unsterilized bulk or presterilized, preweighed in screw cap vial), 10 mg is sufficient to inoculate 36+ dishes of various densities
- Petri dishes (60 mm diameter) containing agar-solidified *C-Fern* nutrient medium, 6 per group, 6 groups
- about 40 ml distilled water, sterile (SD-water)
- If spores are unsterilized, 2 sterile conical bottom centrifuge tubes and spore sterilization supplies (see Appendix B)
- If spores are sterilized - 2 sterile test tubes or sterile screw cap vials
- volumetric pipettes (one each of 1 and 5 ml), sterile (optional)
- Pasteur pipette with bulb, sterile
- spore spreader, sterile
- alcohol (70%)
- stereomicroscope
- micrometer or plastic rule
- Culture Dome

Procedures and Observations

Experiment setup (Day 1)

1. To establish a series of cultures with various spore densities, use one of the following Options.
 - a. Option A. Use culture dishes established in Investigation 2, go to Step 6.
 - b. Option B. Use wild type (10 mg preweighed, presterilized) spores, go to Step 2
 - c. Option C. Weigh out 10 mg wild type spores. Sterilize as usual taking care to not lose spores, go to Step 2.
2. Dilute spore suspension and sow dishes as given below. At each dilution, up to 6 dishes can be sown with a particular spore density. If fewer dishes are sown, the unused portion of the spore suspension should be discarded.

- a. Add 10 drops of SD-water to spores. Carefully and completely suspend spores in the tube. Sow 1 drop of suspension onto six dishes (dishes “A”).
 - b. Add 6 drops of SD-water and resuspend spores, sow 1 drop onto six dishes (dishes “B”).
 - c. Add 6 drops of SD-water and resuspend spores, sow 1 drop onto six dishes (dishes “C”).
 - d. Add 6 drops of SD-water and resuspend spores, sow 1 drop onto six dishes (dishes “D”).
 - e. Add 6 drops of SD-water and resuspend spores, sow 1 drop onto six dishes (dishes “E”).
 - f. Add 6 drops of SD-water and resuspend spores, sow 1 drop onto six dishes (dishes “F”).
3. Check dishes E, F, for sufficient spores, i.e. Dish E should have about 10-25 spores and Dish F should have about 5 spores. Adjust the number, if necessary with spores remaining in tube.
 4. Distribute spores and label dishes.
 - a. Carefully spread spores to as uniform a distribution as possible using the sterile spore spreader.
 - b. Label the small dishes you have sown with your name, date and treatment code
 5. Carefully put dishes into Culture Dome and place under lights.

Data Collection (Day 8 or 10)

6. Count the number of *germinated gametophytes* on a dish for each dish sown. For low density dishes, count all gametophytes. For high density dishes, a sampling procedure is required to estimate the actual number of gametophytes on the dish. Determine the area of the Petri dish. Determine the area of the microscope field that you used to sample the dish. Enter all of this data into Table 10.3.1. Complete the Table to obtain the total number of gametophytes on a dish and their density.

Data Collection (Day 10 or 12)

7. Determine the number of each sexual type for each dish sown. For low density dishes, count all individuals. For high density dishes, a sampling procedure is required to estimate the actual number. Determine the area of the Petri dish. Determine the area of the microscope field that you used to sample the dish. Enter all of this data into Table 10.3.2. Complete the calculation in Table 10.3.2 to obtain the percentage of each sexual type.
Graph the density of gametophytes vs. the percentage of a sexual type (male, hermaphrodite or both!) on the linear graph (Figure 10.3.1) and semi-log graph (Figure 10.3.2).

Table 10.3.1. Total number of gametophytes on dish.

Dish	Total area of dish	Area of field(s) sampled on dish	Number of gametophytes in	Total number of gametophytes on	Number of gametophytes per
A					
B					
C					
D					
E					
F					

Table 10.3.2. Number and percentage of gametophytic sexual types.

Dish(es)	Number of male gametophytes	Number of female gametophytes	Total number of gametophytes	Percentage of male gametophytes	Percentage of female gametophytes
A					
B					
C					
D					
E					
F					

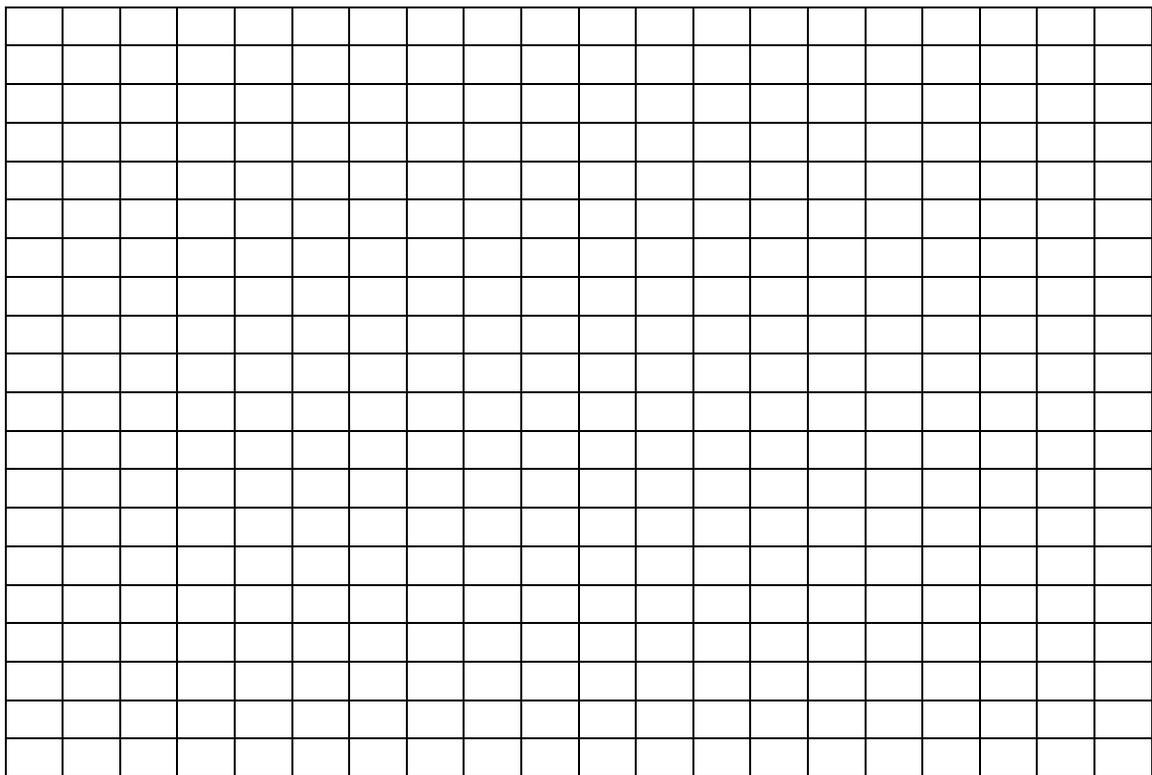


Figure 10.3.1. Density of gametophytes vs. the percentage of sexual type.

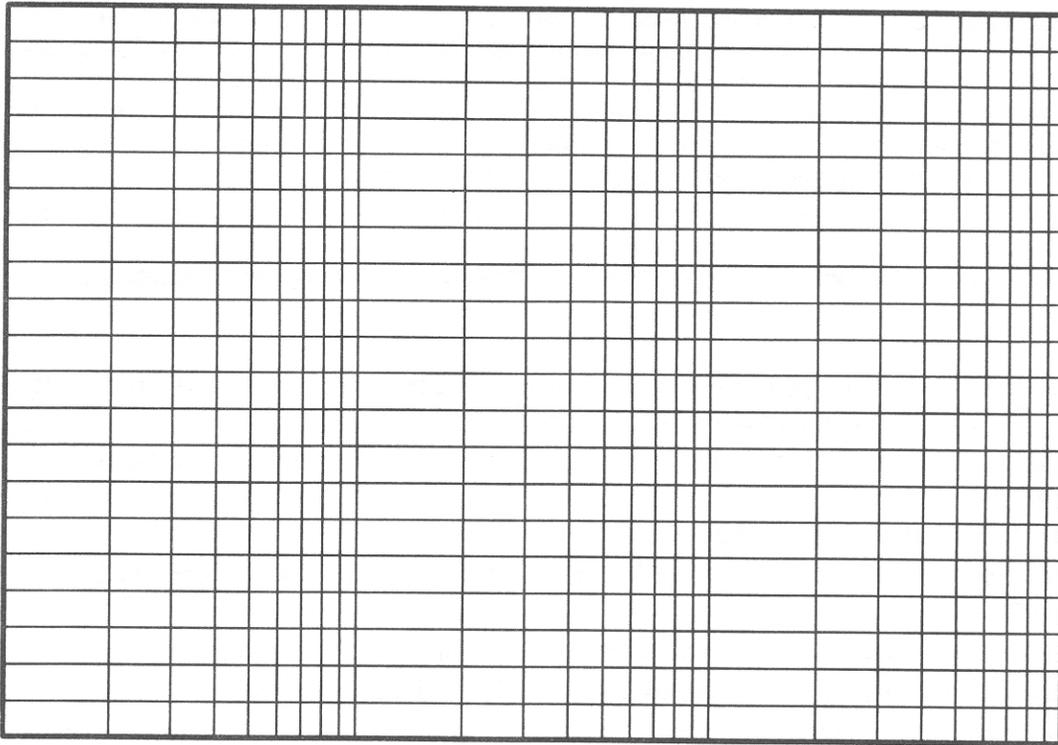


Figure 10.3.2. Density of gametophytes vs. the percentage of sexual type.

Discussion

1. Do spores appear to change before germination occurs?
2. In what sequence do structure(s) or cell types appear during gametophyte development?
3. Why do some spores not germinate?
4. Do all structures of the gametophyte contain chlorophyll?
5. Why should spores be spread to a uniform distribution?
6. Describe the relationship between gametophyte density and sex expression?
7. From an evolutionary and/or breeding system perspective, why would such a relationship exist between gametophyte density and sex expression?
8. What does the relationship between gametophyte density and sex expression tell us about how the sex expression response occurs?
9. Why does variation occur?
10. Is the investigation here considered an experiment? If so, what would be considered the control? the experimental treatments? If not, why not?

Investigation 4. Ecology in a dish - Environmental Controls Of Sporophyte Development - Population Density And Growth of *C-Fern* Sporophytes

Background

Organisms do not exist independent of their environment! An assortment of physical and chemical factors (*abiotic*) and the organisms themselves (*biotic*) influence the distribution, abundance and productivity of organisms. What are some important abiotic factors that could influence the growth of organisms? How do organisms interact with these abiotic and biotic factors? What is the result of these interactions? This investigation examines the influence of gametophytic and sporophytic density on the growth and development of sporophytes.

Objectives

Observe effects of density on the growth and development of *C-Fern* sporophytes.

Materials

- surface sterilized wild type *C-Fern* spores (unsterilized bulk or presterilized, preweighed in screw cap vial), 10 mg is sufficient to inoculate 36+ dishes of various densities
- Petri dishes (60 mm diameter) containing agar-solidified *C-Fern* nutrient medium, 6 per group, 6 groups
- about 40 ml distilled water, sterile
- If spores are unsterilized, 2 sterile conical bottom centrifuge tubes and spore sterilization supplies (see Appendix B)
- If spores are sterilized - 2 sterile test tubes or screw cap vials
- volumetric pipettes (one each of 1 and 5 ml), sterile (optional)
- Pasteur pipette with bulb, sterile
- spore spreader, sterile
- alcohol (70%)
- stereomicroscope
- micrometer or plastic rule
- Culture Dome

Procedures and Observations

Experiment setup (Day 0)

1. To establish a series of cultures with various spore densities, use one of the following Options.
 - a. Option A. Use cultures established from Investigation 2 - skip to step 6
 - b. Option B. Use culture dishes established from Investigation 3- skip to step 6
 - c. Option C. Use wild type (preweighed, presterilized) spores, go to step 2
 - d. Option D. Weigh out 10 mg wild type spores. Sterilize as usual taking care to not lose spores, go to Step 2.
2. Dilute spore suspension and sow dishes as given below. At each dilution, up to 6 dishes can be sown with a particular spore density. If fewer dishes are sown, the unused portion of the spore suspension should be discarded.

Table 10.4.2. Size of sporophytes on dish.

	Length (mm)	Length (mm)	Length (mm)	Length (mm)	Length (mm)	Length (mm)
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
Mean						
SD						

Discussion

1. Do gametophytes appear to change after fertilization occurs?
2. In what sequence do structure(s) or cell types appear during sporophyte development?
3. Why do some female gametophytes not produce sporophytes?
4. Do all structures of the sporophyte contain chlorophyll?
5. Describe the relationships among gametophyte density, sporophyte density and sporophyte size?
6. From an evolutionary and/or breeding system perspective, why would such a relationship exist between gametophyte density and sporophyte production/density?
7. What does the relationship between gametophyte density and sporophyte production/density tell us about how sporophytes interact?
8. Why does variation occur?
9. How could this investigation be redesigned to discriminate between direct and indirect interaction among the sporophytes?

166 C-Fern™

- Petri dishes (60 mm diameter) containing agar-solidified *C-Fern* nutrient medium, 2 per student
- If spores are unsterilized, 1 sterile conical bottom centrifuge tubes and spore sterilization supplies (see Appendix B)
- 150 ml distilled water, sterile (SD-water)
- Pasteur pipettes and pipette bulbs, sterile
- spore spreader, sterile
- stereomicroscope
- Culture Dome

Procedures and Observations

First Period (Week 1)

1. Prior to this class, Petri dishes containing agar-solidified mineral nutrient medium were inoculated with spores from an F1 hybrid *C-Fern* plant. *If this was not done for you, your teacher will give instructions on how to sow your own plates.* Label the plates provided to you with your name and the inoculation date. Using the highest magnification on a stereomicroscope with transmitted light (i.e., from below), observe the cultures.
2. What do you observe?
3. Do some of the spores show signs of germination? What is the first visible structure in germinated spores?
4. What type of cell division is occurring during the germination process?
5. What type of cell division took place in the F1 sporophyte to produce these spores?
6. Are the spores haploid or diploid?
7. If the F1 hybrid was heterozygous for a single mutant trait, what genotypes would be present in the spores? What is the expected ratio of genotypes?
8. What will develop from the spores after they germinate? Will they be haploid or diploid?
9. When you are finished with your observations place the cultures back in the Culture Dome under the lights.

Second Period (Week 2)

1. Observe the cultures under a stereomicroscope. What things are different from last week?
2. What type of cell division took place during the growth of gametophytes?
3. While you are observing the culture tilt the lid up and add 1-2 ml SD-water. Lower the lid and tilt the plates back and forth to cover all of the gametophytes with water. Observe the release of swimming sperm from antheridia and their attempts to find mature eggs within archegonia.
4. Do all the gametophytes have the same phenotype? Describe any differences you observe.
5. Did you observe differences in sexual phenotypes as well as in another phenotype?

For this experiment we will ignore differences in sexual phenotype and focus only on the larger heart-shaped hermaphroditic gametophytes. The smaller tongue-shaped ones are specialized males.

6. Which of the phenotypes would you designate as a mutant? Why?

7. Take a random sample of the gametophyte population in each dish by counting up to 50 individuals and identifying their phenotype. Record your data in Table 10.5.1. During this procedure, the lid should be left on the Petri dish if possible. If it becomes fogged, a 'clean' lid from an unused dish may be quickly exchanged for the old one. After scoring, replace the old lid over the culture.
8. Why is it important to take a random sample from the cultures?
9. Suggest a method of taking data that would insure a random sample?

Table 10.5.1. Gametophyte phenotypes.

Date data recorded:	Age of culture:		
Description	plate 1	Number plate 2	Total

10. Can you determine anything about the pattern of inheritance from data in Table 10.5.1?
11. Are the plants in the culture haploid or diploid?
12. Can you identify the reproductive structures that are present?
13. Make a drawing and use these terms to label structures that you observe in the cultures: antheridium, archegonium, meristem, rhizoid and spore coat.
14. When you are finished with your observations, remove any excess water from the culture by lifting the lid slightly and pouring off the excess. When you are finished with the culture, place it back into the Culture Dome under the lights. Be sure the lid is in place.
15. Did the fertilization events that you observed appear to be random?
16. What products will result from these fertilizations? Will they be haploid or diploid?
17. Predict the genetic outcome of the fertilizations by formulating a hypothesis to explain the inheritance of the trait. Assign symbols for the mutant and wild type states and, based on your hypothesis, diagram the cross from the F1 to the F2 generation. Indicate expected ratios for both the gametophyte and F2 sporophyte generations. When and how could your hypothesis be tested?

168 C-Fern™

Third Period (Week 3 OR 4)

1. Observe your cultures under the microscope. What things are different from last week? Can you observe any new structures showing the mutant and wild type phenotypes?
2. Are the young sporophytes haploid or diploid? Why?
3. Make a simple drawing of what you are observing and label it with the following terms: gametophyte, sporophyte leaf, sporophyte root.
4. Take a random sample of the sporophyte population in each dish by counting up to 50 individuals and identifying their phenotype. For this part of the experiment, the lid may be removed from the culture. It may be easier to score the phenotype by gently and randomly pulling up individual sporophytes with a dissecting needle and laying them out in a row on empty areas of the culture plate. Sporophyte leaves are thicker than gametophytes, so it will take some careful observations to correctly identify each phenotype. Use of only reflected light (from above or the side) is best. Having both wild type and mutant types lying side by side greatly simplifies this process. Record your data in Table 10.5.2.

Table 10.5.2. Sporophyte phenotypes.

Date data recorded:	Age of culture:		
Description	plate 1	Number plate 2	Total

5. Following scoring of phenotypes, the lid may be placed back on the plate and the culture returned to the Culture Dome or taken home for observations over the next several weeks to determine if the phenotype is apparent without the use of a microscope on mature sporophytes.
6. If time permitted, this experiment could be carried out through the gametophyte generation produced by spores from the F2 sporophytes. What predictions would you make concerning such extended observations?

Data Analysis

You should now test the hypotheses concerning the method of inheritance that you made in Week 2 of the experiment to determine if the data you collected support or do not support your

model of inheritance and expression. To do this, geneticists typically use the Chi-square statistical test. Refer to your textbook for a full explanation of this test. In short, this test expresses the difference between expected (hypothetical) and observed (collected) numbers as a single value (χ^2). If the difference between observed and expected is large, a large χ^2 value results, while a small difference results in a small value of χ^2 . Chi-square is calculated according to the following formula:

$$\chi^2 = \sum \frac{(Ob - Ex)^2}{Ex}$$

Where **Ob** and **Ex** are the observed and expected values, respectively, for the numbers of individuals in each particular class or grouping. The Expected number is determined from a specific hypothesis. For each class the Expected value is subtracted from the Observed value, and the result is then squared and divided by the Expected value. Results from these operations are summed for all classes to give the Chi-Square value.

In an experiment, the observed numbers may deviate from the expected numbers either because of random chance associated with sample error or because the hypothesis was incorrect. Chi-square analysis helps us to decide between these two possibilities by relating the χ^2 value to probability. To do this, statisticians have constructed tables of Chi-square values with their associated probabilities. Because probabilities differ as the number of phenotypic classes changes, probabilities are also based upon degrees of freedom. The number of degrees of freedom is always one less than the number of phenotypic classes in any experiment. Geneticists have generally agreed upon a probability value of 0.05 as the lowest acceptable value derived from the χ^2 test. This number indicates that if the experiment was repeated many times, the deviations from expected due to chance alone would be as large or larger than those observed about 5% of the time. Probabilities equal to or greater than 0.05 support the hypothesis while probabilities lower than 0.05 do not support the hypothesis. In such cases either the experimental design or the hypothesis, or both, need to be re-examined.

Transfer your data from Week 2 and 3 into the following Tables to calculate χ^2 .

Table 10.5.3. Calculation of χ^2 from Week 2 gametophyte data.

Phenotype	Observed (O)	Expected (E)*	O - E	(O - E) ²	(O - E) ² /E
Totals					$\chi^2 =$

*This number should be based upon your hypothesis made on Week 2.

Use Table 10.5.4 to determine the probability of obtaining the χ^2 value that you calculated. How many degrees of freedom are there? What is the approximate probability? Is your hypothesis supported or not supported? If it is not supported, what might be changed in your hypothesis or in the experimental design?

Table 10.5.4. Distribution of χ^2 (Chi -Square).

Degrees of Freedom	Probability of obtaining a χ^2 value as large or larger			
	.10	.05	.01	.001
1	2.71	3.84	6.63	10.83
2	4.61	5.99	9.21	13.82
3	6.25	7.82	11.35	16.27
4	7.78	9.49	13.28	18.47
5	9.26	11.07	15.08	20.52

Table 10.5.5. Calculation of χ^2 from Third Period (Week 3 or 4) sporophyte data.

Phenotype	Observed (O)	Expected (E)*	O - E	(O - E) ²	(O - E) ² /E
Totals					$\chi^2 =$

*This number should be based upon your hypothesis made on Week 2.

Use Table 10.5.4 to determine the probability of obtaining the χ^2 value that you calculated. How many degrees of freedom are there? What is the approximate probability? Is your hypothesis supported or not supported? If it is not supported, what might be changed in your hypothesis or in the experimental design?

Discussion

The purpose of this exercise was to demonstrate some basic principles of inheritance. One of the principles derived from Mendel's work was the Law of Segregation. Did you observe segregation in this experiment? At what stage did it occur?

Most genetic predictions are based on the expectation that gametes unite at random to form zygotes. Did you observe what appeared to be random fertilization? When did it occur?

Mendel also observed phenotypic characters that were dominant or recessive. Were these observed in your experiment? Were they observed equally in both gametophytes and sporophytes? How did you determine which character was dominant and which was recessive?

If gametophytes had not expressed the phenotype, would you have been able to form a hypothesis from observations of the gametophyte generation? Why or why not?

Acknowledgments

Development and dissemination of the *C-Fern* has been supported, in part, by the National Science Foundation and the University of Tennessee. We gratefully acknowledge Stephenie Baxter for her superb laboratory assistance and the illustration of the C-Fern life cycle.

Literature Cited

- Chasan, R. (1992). *Ceratopteris*: A model plant for the 90s. *The Plant Cell* 2, 113-115.
- Copeland, E. B. (1942). Edible ferns. *American Fern Journal* 32, 121-126.
- Hickok, L. G. (1977). The cytology and derivation of a temperature-sensitive meiotic mutant in the fern *Ceratopteris*. *American Journal of Botany* 64, 552-563.
- Hickok, L. G. (1979). A cytological study of intraspecific variation in *Ceratopteris thalictroides*. *Canadian Journal of Botany* 57, 1694-1700.
- Hickok, L. G. (1987). Applications of in vitro selection systems: whole plant selection using the haploid phase of the fern *Ceratopteris*. In *Biotechnology in agricultural chemistry* (H. M. LeBaron, R. O. Mumma, R. C. Honeycutt and J. H. Duesing, eds.), Vol. American Chemical Society Symposium Series 334, pp. 53-65. American Chemical Society.
- Hickok, L. G., Warne, T. R., and Fribourg, R. S. (1995). The biology of the fern *Ceratopteris* and its use as a model system. *International Journal of Plant Science* 156, 332-345.
- Hickok, L. G., Warne, T. R., and Slocum, M. K. (1987). *Ceratopteris richardii*: Applications for experimental plant biology. *American Journal of Botany* 74, 1304-1316.
- Klekowski, E. J., Jr. (1969). Reproductive biology of the Pteridophyta. III. A study of the Blechnaceae. *Botanical Journal of the Linnean Society* 62, 361-377.
- Lloyd, R. M. (1973). Systematics of the genus *Ceratopteris* (Parkeriaceae), I. Sexual and vegetative reproduction in Hawaiian *Ceratopteris thalictroides*. *American Fern Journal* 63, 12-18.
- Lloyd, R. M. (1974). Systematics of the genus *Ceratopteris* Brongn. (Parkeriaceae) II. Taxonomy. *Brittonia* 26, 139-160.
- Lloyd, R. M., ed. (1993). *Parkeriaceae* Hooker, water fern family, Vol. 2 Pteridophytes and Gymnosperms. Oxford University Press, New York.
- Warne, T. R., and Hickok, L. G. (1989). Evidence for a gibberellin-biosynthetic origin for the antheridiogen of *Ceratopteris*. *Plant Physiology* 89, 535-538.
- Warne, T. R., Walker, G. L., and Hickok, L. G. (1986). A novel method for surface-sterilizing and sowing fern spores. *American Fern Journal* 76, 187-188.

Appendix A. Surface Sterilization and Sowing of *C-Fern* Spores

For best results, spore sterilization and sowing can be conveniently performed on a sterile bench or laminar flow hood. However, if such equipment is not available, any clean area free of

172 C-Fern™

drafts can be successfully used. Observe all standard sterile technique precautions. This method is adapted from Warne et al. (1986).

Materials:

- *C-Fern* spores
- Sterile 15 ml conical bottom centrifuge tubes. Foil covered and dry sterilized at 120°C for 2 hours or autoclaved.
- Sterile Pasteur pipettes. Autoclaved.
- Sterile distilled water
- Sterile pipette bulbs
- Diluted standard laundry bleach or equivalent 0.825% sodium hypochlorite solution, i.e. 1 part bleach (5.25% sodium hypochlorite) to 5 parts distilled water. This solution should be prepared fresh immediately before use.
- Waste container

Note: There are approximately 1250 *C-Fern* spores per mg dry weight. 10 mg of spores can be used to inoculate 33+ Petri dishes at a standard density of about 300 spores per dish. For this density and number of dishes, 4 ml water should be used to suspend 10 mg spores and three *drops* (not squirts!) of spore suspension from a Pasteur pipette should be delivered to each dish. Adjust the water volume according to the specific average drop volume for the pipettes used.

Procedure:

- 1. Weigh spores.** Weigh out spores onto glassine weigh paper and transfer them to a sterile 15 ml centrifuge tube.
- 2. Presoak spores.** Cover spores with glass distilled water and let stand for 5 minute to 24 hours. Be sure all spores are wetted. Water for soaking spores does not need to be sterile.
- 3. Remove presoak water.** Remove water from the spores using a sterile Pasteur pipette (most spores will have settled to the bottom of the centrifuge tube) using the technique described below. This procedure for removing liquids from centrifuge tubes will be used also in spore sterilization and rinsing.

Select a sterile Pasteur pipette with a tip that is not cracked or chipped and attach a rubber pipette bulb on the pipette. Insert pipette into centrifuge tube and suspend spores by bubbling a small amount of air into the water. While air is slowly bubbling out of the pipette, gently but securely seat the pipette onto the base of centrifuge tube. Sometimes it helps to rotate the pipette tip gently, but don't press too hard on the pipette or you will crush the tip. Squeeze the bulb to force additional air out of the pipette. When the bulb is released, water should enter the pipette and the spores should collect around the outside of the base of the pipette tip, provided the pipette is securely seated on tube bottom. If you can't remove liquid without bringing the spores along, try another pipette. Remember that time is critical when the sterilizing solution (sodium hypochlorite) is in the tube! It advisable to practice this technique with water prior to sterilizing the spores. With practice you should be able to remove liquids free of spores, in about ten seconds. When the presoak water is successfully removed, discard the pipette.

- 4. Surface sterilize spores.** To sterilize, suspend spores in one full Pasteur pipette (about 1-2 ml) of 0.875% sodium hypochlorite (1 part bleach (5.25% sodium hypochlorite) to 5 parts distilled water). Rinse down the lip and sides of the centrifuge tube with sodium hypochlorite solution. To insure that the spore mass becomes evenly suspended it may be

necessary to bubble air through a clean, sterile pipette. Sterilize spores for three minutes. Remove sodium hypochlorite solution with clean, sterile pipette using the technique described above. For precise timing of surface sterilization, the time needed to remove sodium hypochlorite must be factored in.

5. **Rinse spores.** To rinse spores, add one full pipette of sterile glass distilled water. The pipette used to add sterile distilled water may be used repeatedly as long as care is taken to prevent contamination with foreign spores. Remove rinse water with clean, sterile pipette. Repeat the rinses two more times. The pipette used to remove sterile distilled water may be left in tube for use in sowing spores.
6. **Sow spores.** Add the appropriate amount of sterile glass distilled water to sow a given number of plates. Thoroughly suspend spores in water and dispense drops (not squirts!) of spores to plates. Distribute spores across the agar surface by gently spreading with a sterile wire (such as a standard paper clip) bent in the shape of a small coat hanger.

Appendix B

C-Fern Nutrient Medium - Composition And Preparation

Preparation of Agar-Solidified Basic C-Fern Nutrient Medium From Stock Solutions

Materials for Preparation of Stock Solutions:

- Macro-, micronutrient and Fe salts (Refer to Table 10.A.1 for listing)
- Distilled or deionized water
- volumetric flask(s)
- Microbalance
- Magnetic stir plate and stir bars
- For Fe stock solution: hot plate, 2 L Erlenmeyer flask, watch glass
- Storage bottle(s)

All components of basic *C-Fern* nutrient medium should be prepared using high quality distilled and/or deionized water.

Prepare macronutrient and micronutrient stock solutions separately by dissolving all listed quantities of components (Table 10.A.1), individually, in sequence, into about 800 ml distilled water; bring to a 1 L final volume. Stock solutions will keep for over 6 months and should be stored in glass at 4 °C.

Prepare Chelated Fe-EDTA stock solution by dissolving each component separately into about 450 ml water. On a hot plate, heat EDTA solution to boiling, add hot EDTA solution **TO** the solution. Cover with a watchglass and boil combined solutions for 1 hour, cool completely, then bring to 1 liter volume.

Table 10.A.1. Composition of Basic *C-Fern* Medium Stock Solution and Final Medium

Nutrient components	Stock solution (g/L)	Final Medium (ml Stock/L)	Final Medium Composition (mg/l)
1 10× Macronutrients		100	
NH ₄ NO ₃	1.25		125
K ₂ HPO ₄	5.00		500
MgSO ₄ ×7H ₂ O	1.20		120
CaCl ₂ ×2H ₂ O	0.26		26
2 200× Micronutrients		5	
MnSO ₄ ×H ₂ O	0.0500		0.25
CuSO ₄ ×5H ₂ O	0.0740		0.37
ZnSO ₄ ×7H ₂ O	0.1040		0.52
H ₃ BO ₃	0.3720		1.86
(NH ₄) ₆ Mo ₇ O ₂₄ ×4 H ₂ O	0.0074		0.037
3 100× Chelated Iron Solution		10	
FeSO ₄ ×7H ₂ O	2.78		27.8
Disodium EDTA×2H ₂ O	3.73		37.3

^aThis formulation is based on a medium described in Klekowski 1969 (*Botanical Journal of the Linnean Society* 62: 361-377). Higher concentrations of macronutrients in the stock solution are unstable and may form precipitates as will most combinations of macronutrient, micronutrient and chelated iron stock solutions.

Materials for Preparation of Final Medium:

- Macronutrient, micronutrient and Chelated Iron stock solutions
- Volumetric glassware: 100 ml graduated cylinder, 10 ml pipette, 1 L volumetric flask
- Distilled or deionized water
- 2 L Erlenmeyer flask (per 1 L media)
- Agar (BactoAgar)
- For pH adjustment: pH meter, magnetic stir plate, magnetic stir bar
- 0.1N NaOH solution
- Foil (to cover flask during autoclaving)
- Autoclave
- Sterile Petri dishes, 60x15 mm

To prepare Basic *C-Fern* nutrient medium, add appropriate volume of each stock solution **TO** about 800 ml distilled water in a volumetric flask and bring to a 1 L final volume. For agar-solidified medium, transfer nutrient solution to 2 L Erlenmeyer flask, add 10 g (1% w/v) agar (BactoAgar, Difco Laboratories). Some plant tissue culture grade agars and agar substitutes can result in inhibited or abnormal growth of gametophytes or sporophytes and should therefore be avoided. Adjust nutrient medium to pH 6.0 using 0.1 M NaOH. Autoclave nutrient medium at 175 C / 20 psi for 15 minutes. Dispense medium to Petri dishes. Dishes should be about 1/2 full -- about 15 ml in a 60 mm dish or 40 ml in 100 mm dish. This ensures an adequate nutrient and water supply through to the young sporophyte stage. One liter of nutrient medium should pour about 55 small dishes (60 mm) and 20 large dishes (100 mm). Allow agar dishes to cool completely undisturbed. Condensation on Petri dish lids is minimized if dishes are poured and cooled in stacks.

Appendix C

General Culture Instructions, Temperature, and Culture Domes

Culture temperature is the most critical element in achieving rapid and repeatable growth and development. The temperature optimum for spore germination and gametophytic development is about 28-30°C, which is somewhat higher than many other ferns. Lower temperatures will substantially alter development, for example at 21-22°C development times will be increased about two-fold. To achieve a stable temperature and hence predictable growth, as well as reduce excessive evaporation and contamination, culture dishes should be placed in 'Culture Domes' (clean plastic greenhouse trays covered with a transparent dome). These are available from Carolina Biological Supply Company. Note that the temperature inside the covered tray will likely be about 2°C higher than ambient temperature in the room. Temperature inside the Culture Dome can be regulated by varying the distance from the lights, anywhere from 20 – 45 cm.

pH

The pH optimum for gametophytic growth of the wild type is about pH 6.00. At pH below about 5.6, gametophytic growth is inhibited in a linear fashion. Nearly all standard media are typically unbuffered or very weakly buffered. The initial pH of the *C-Fern* nutrient medium is usually < 5.0 and must be carefully adjusted with a base, such as NaOH.

Light

We use continuous illumination by 40 W cool white fluorescent tubes covering a culture space width of about 50 cm at a distance of 45 cm or less (see Temperature regulation, above) from the cultures (about 80-85 mmoles photosynthetically active radiation (PAR) x m⁻²xsec⁻¹).

‘DarkStart’ - Synchronization of development

In order to enhance synchronization of spore germination and early gametophyte development, sterilized spores may be soaked water in complete darkness from 4 to 7 days, then sown as usual. Spores should only be presoaked to achieve wetting, i.e. for a few minutes, immediately sterilized if necessary and as soon as possible placed into complete darkness. Exposure to light during the sterilization procedure, if performed, should be as short as possible. ‘DarkStart’ soaking should take place in a sterile screw cap tube, either placed in a light tight box or carefully wrapped in aluminum foil (twice is best!). Once plates are inoculated and exposed to light, germination begins 24-36 hours after exposure to light and is complete in four to five days. In addition, early stages of development should be more synchronous and the frequency of male gametophytes should be reduced.

Appendix D**Sources of Supplies and Materials**

Carolina Biological carries all the appropriate culture supplies and equipment for C-Fern culture. These include: prepared media, powdered media, an extensive C-Fern Manual, Culture Domes, lighting fixtures, and spores of wild type, various mutants and F1 hybrids (see Table 10.A.2). In addition, versions of the exercises described here, as well as others, are available in various Kit forms. Please contact 1-800-334-5551 for further details and new products. The C-Fern Web Site (www.bio.utk.edu/cfern/) contains additional information and updates about the availability of new mutant and F1 spore stocks.

Table 10.A.2 C-Fern™ Spores Available from Carolina Biological Supply Co. premeasured and presterilized in Kit-sized vials (enough to sow 35 dishes) or unsterilized in bulk-sized vials (enough to sow 140 dishes).

Strain	Symbol	Description
wild type	RNWT1	Basic <i>C-Fern</i> genotype

Single Gene Nuclear Mutants

polka dot	<i>cp</i>	Chloroplasts form clumps that resemble polka dots in both gametophyte and sporophyte tissue. Recessive in sporophytes.
glyphosate tolerant	<i>glt 1</i>	Gametophytes and sporophytes tolerant to the active ingredient in glyphosate containing herbicides, e.g. RoundUp®(Monsanto). Incomplete dominance in sporophytes.
paraquat tolerant	<i>pq45</i>	Gametophytes and Sporophytes tolerant to the herbicide paraquat. Recessive in sporophytes.
dark germinator	<i>dkg</i>	Does not require light to initiate spore germination.
abscisic acid tolerant	<i>abr48</i>	Gametophytes tolerant to the plant growth regulator, abscisic acid.
maleless	<i>her1</i>	Does not form male gametophytes. Insensitive to the male-inducing pheromone.
non-etiolated	<i>det30</i>	Gametophytes do not show elongation response when grown in the dark.
salt tolerant	<i>stl2</i>	Gametophytes and sporophytes tolerant to Na ⁺ and Mg ²⁺ salts
FUDR tolerant	<i>fdr1</i>	Gametophytes tolerant to the nucleotide analog 2'-deoxy-5-fluorouridine.

F1 Stocks Single gene mutants X wild type (RNWT1)

F1 polka dot	cp/CP	F1 gametophytes segregate in a ratio of 1 wild type to 1 polka dot. F2 sporophytes segregate in a ratio of 3 wild type to 1 polka dot.
--------------	-------	--