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Plants-Don't Just Sit There, Do Something!!!

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Many botany labs involve having students look at flowers, seeds, and fruits, but often omit some of the "neat things" that are more hidden from view. Some of the most fascinating botanical phenomena are rarely included in the introductory laboratory, and yet they are easily seen if appropriate techniques and plants are used. These aspects of plant biology offer interesting questions for investigation, but without proper techniques they are often ignored. In this workshop we show how to prepare simple setups to view pollen tube germination, to see endosperm and early embryos of dicots, and to make preparations of chromosomes in broad beans. All of the techniques have the potential for use in a variety of student exercises and research projects.

Keywords: plants, botany, pollen tubes, chromosomes, embryos

Introduction

Students often have a hard time visualizing all of the steps in the typical life cycle of flowering plants. While many botany labs involve having students look at flowers, seeds, and fruits, they often omit some of the "neat things" that are more hidden from view, and that students seem to have a hard time understanding. Some of the most fascinating botanical phenomena are rarely included in the introductory laboratory, and yet they are easily seen if appropriate techniques and plants are used.

In this lab, students learn how to use simple setups to view pollen tube germination, to see endosperm and early embryos of dicots, and to make preparations of chromosomes in broad beans. Each of these techniques can be used as a part of a traditional botany lab, or all three of the techniques can be incorporated into a single, very full, three-hour lab session. Before the laboratory, students should have already become familiar with the use of microscopes, know the basic parts of a plant and have studied the typical life cycle of a flowering plant.

The techniques studied in this lab can be mastered by freshmen but are also appropriate for upper level students. Each has the potential for use in a variety of student exercises and research projects and lead to interesting questions for investigation.

Learning goals for this set of exercises

Students who have completed these exercises will be able to:

- Relate cell division, pollen tube growth, and embryogenesis to the entire plant life cycle.
- Make pollen preparations for observations of pollen tubes
- Dissect plant embryos from developing seeds of dicots
- Describe and identify stages of mitosis
- Make root tip preparations for observations of chromosomes

Student Outline

Introduction

When you think of plants you probably immediately think of the flowers. We appreciate flowers for their beauty, but their primary function is to serve as the reproductive organs of higher plants. The mature plant body typically consists of several organs: stems, roots and leaves, and at certain times, *flowers*. In order for a new plant to arise, there must be pollination followed by pollen tube growth and then double fertilization. The development of the embryo in the seed occurs within the fruit, which matures and eventually releases those seeds. They proceed, sequentially, through seed germination, and development and growth of stems, leaves and roots. Cell division followed by cell growth and differentiation, are the major processes occurring at this time. Eventually this leads once again to the production of flowers, fruits and seeds (Fig. 1).

When we study the plant life cycle, we usually focus on three major aspects: plant parts such as roots, stems, and flowers, flower parts such as petals, sepals, pistils and stamens, and seed anatomy and seed germination. However, in this exercise we will focus on pollination and fertilization, specifically pollen tube germination, embryogenesis within developing seeds and mitosis in the root apical meristem.

Have you ever wondered if these things are "real"? For example, have you actually seen chromosomes from a plant that you grew, or endosperm and embryos connected with a suspensor in an ovule? Have you actually seen a pollen tube? This lab will help convince you that these and other phenomena really do exist, and will allow you to think of ways to explore interesting biological questions in the process.

As you learn the different techniques, you will be asked to investigate several experimental questions. Once you have finished learning the procedures, see if you can formulate an interesting question of your own.

Part I. Pollination

Flowering plant reproduction begins with the process of pollination. It occurs when pollen grains released from anthers and carried by wind or animals land on the sticky stigmas of the same (*self-pollination*) or different flower (*cross-pollination*). The type of pollinator may influence the type of pollen produced; some of the major types are described here.

Wind pollination The simplest method of cross-pollination, and the most inefficient, occurs by wind. The main features of wind-pollinated angiosperms are as follows: they produce enormous amounts of lightweight, non-sticky pollen; they lack showy floral parts or strong fragrances; they have well-exposed stamens and large stigmas; they have a single ovule in each ovary; and they have many flowers packed into each inflorescence.

Animal pollination Some flowers are pollinated by a specific animal, such as a particular type of bee, beetle, bird or bat. In most cases, relationships between plants and their pollinators are less specific than this. For example, the flowers of a particular plant species may be adapted for attracting insects rather than birds, but many different insect species may serve as pollinators. Conversely, a single animal species--a honeybee species, for example--may pollinate many different plant species.

Insects are the most common group of animals that pollinate flowers. There is no single set of characteristics for insect-pollinated flowers, because insects are such a large and diverse group of animals. *Vertebrate* pollinators are found primarily, if not entirely, among birds and mammals. Relatively few representatives of each group are involved in pollination, and as with insects, plants that birds and mammals pollinate may have a set of reproductive features that attracts mostly one kind of pollinator.

Double Fertilization and Embryo Development

Once pollen grains have been deposited on the stigma, sugars and hormones on the surface stimulate the grains to grow. The tube nucleus causes a *tube* to grow down the style toward the ovules in the ovary. Within the tube is the generative nucleus. When the tube reaches the embryo sac the *generative nucleus* divides to produce two sperm. One fertilizes the egg nucleus to produce the *diploid embryo*; the other fertilizes the two endosperm nuclei to produce the *triploid endosperm*, the food supply for the embryo. In this **double** fertilization both the embryo and the food supply are fertilized. Double fertilization is restricted to the Angiosperms, or flowering plants. How might this process confer an evolutionary advantage on this group of plants?

Part I. Pollen tube germination methods

1. Pollen grains may be stimulated to germinate by adding them to a pollination /germination medium which contains sugars.
2. Add a few drops of germination medium to a microscope slide and add pollen grains to it by shaking a stamen over it or touching it to the liquid. (This process works best if there is not too much pollen.)
3. Add a cover slip—not in the proper way, but by merely dropping it onto the liquid on the slide. This will trap air bubbles in the liquid, which seems to increase the percentage of pollen grains that germinate.

4. Make note of the name of the plant from which your pollen came, and the time at which you added it to the pollination medium. (Note on slide with permanent marker.)
5. Observe the pollen under the microscope, first under low, then under high power. Set the scope aside and return every 5 minutes to check for pollen tubes.

How long did it take for tubes to form? Can you see nuclei in them? (It may be necessary to adjust the light, focus and condenser settings to maximize detail.) If your pollen grains do not germinate, observe those of another student. Observe of a variety of types of pollen from freshly collected flowers and from "Bee pollen".

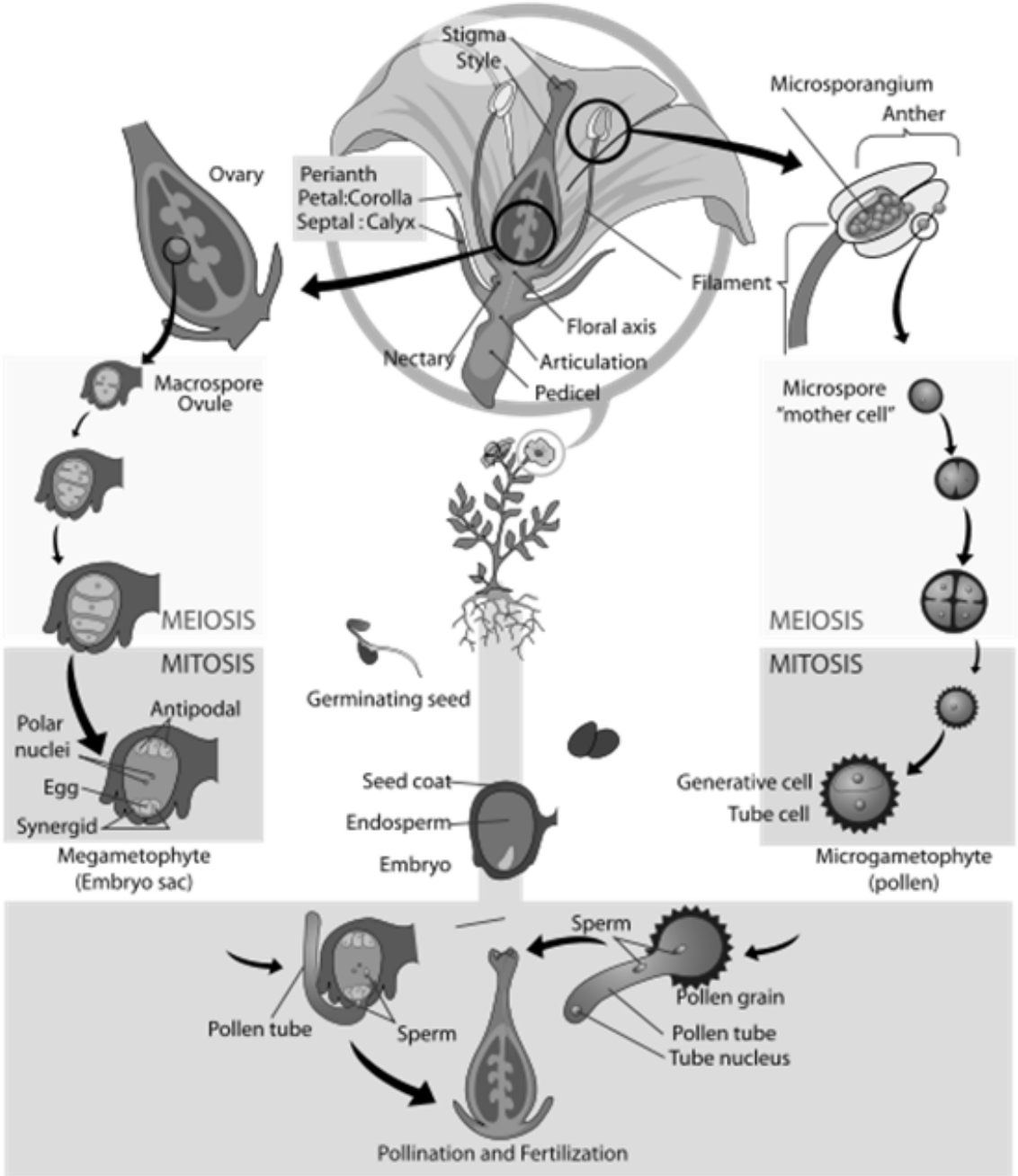

Figure 1. Flowering plant life cycle. (LadyofHats Mariana Ruiz [Public domain], via Wikimedia Commons)

Questions for Investigation:

1. What types of plants had the greatest success of pollen tube germination?
2. Do plants pollinated by wind show a higher or lower percent germination than those pollinated by insects?
3. Do "weed species" have a higher percentage of pollen tube germination than "cultivated species"?
4. Do "polyploid species" have a higher percentage of pollen tube germination than "diploid species"?
5. Do those pollen grains near air bubbles germinate better than those away from an oxygen source?
6. Is pollen tube germination inhibited by the presence of other pollen grains?
7. Does the direction of tube growth differ if a stigma of the same species is nearby?
8. Does fresh "bee pollen" from the health food store retain its ability to germinate?
9. Does the germination media "recipe" make a difference in germination of different species?

Part II. Ovule and Seed Development

Following pollination and double fertilization, which takes place within an ovule and embryo sac, a zygote develops into a multi cellular embryo, and the 3N endosperm proliferates. Over a period of days, the ovary swells and develops into the fruit and the fertilized ovules develop into seeds. We can observe this process in Wisconsin fast plant seed pods.

Fast plants: Endosperm and Early Embryos of Dicots

From approximately 22-30 day old Fast Plants remove several seed pods (fruit), place one on a glass slide and observe it with the dissecting microscope. Using two teasing needles, or needles of tuberculin syringes, remove ovules from different size pods and place them on the stage of the microscope with a small drop of water. Use the needles to pop open the ovule while looking through the microscope. Observe and draw several embryos, and determine their stage(s). Share your findings with others in the lab to observe as many stages as possible.

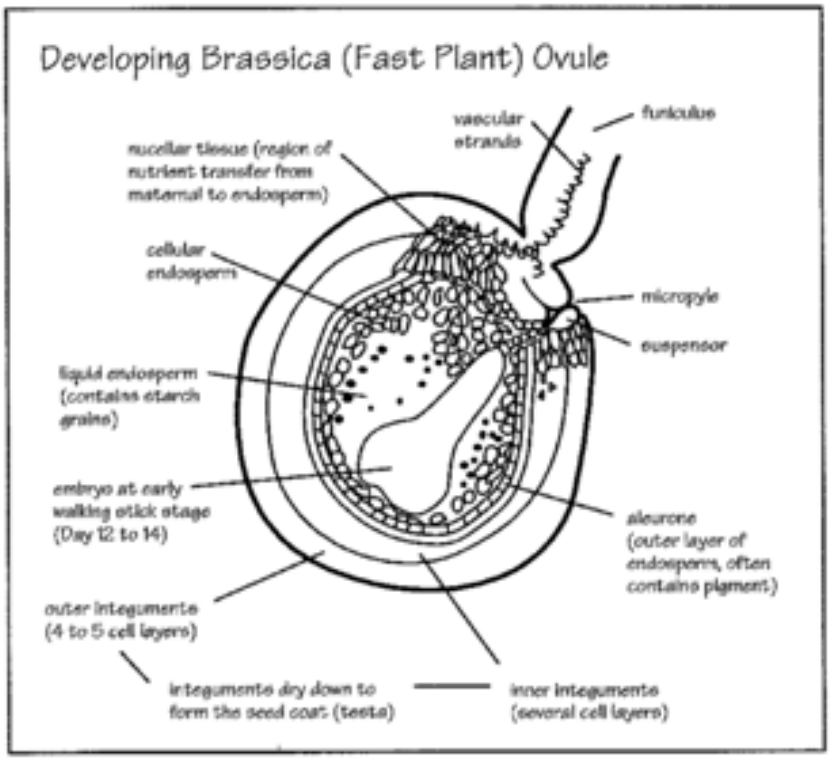

Figure 2. Developing *Brassica* ovule. (Lauffer D., Williams, P., (2007). Wisconsin Fast Plants®. Retrieved (Sept 16, 2011), from Wisconsin Fast Plants Web site: **www.fastplants.org**)

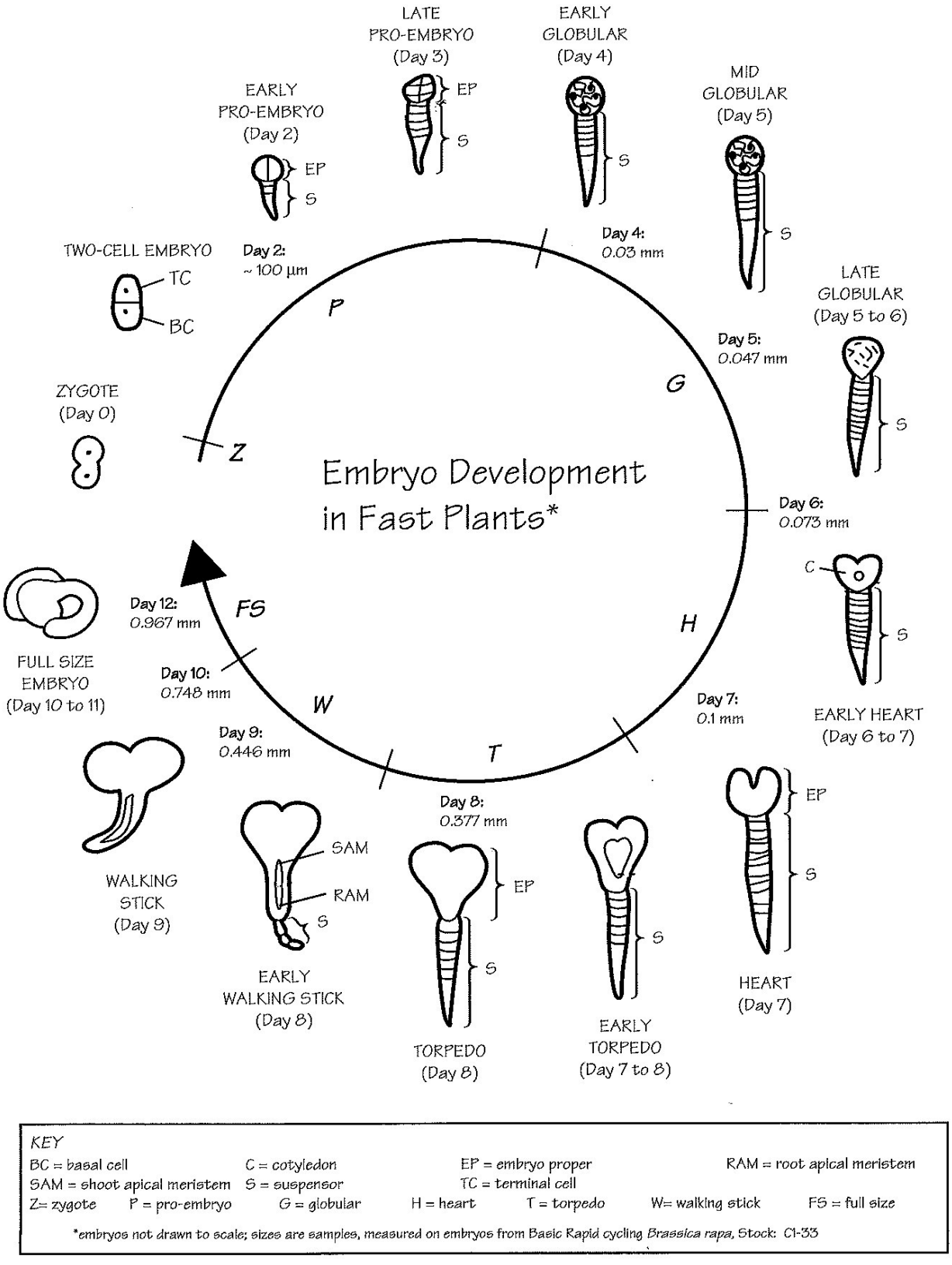

Figure 3. Embryo development in Fast Plants. (Lauffer D., Williams, P., (2007). Wisconsin Fast Plants®. Retrieved (Sept 16, 2011), from Wisconsin Fast Plants Web site: **www.fastplants.org**)

Step by step directions for embryo dissection adapted from Fast Plant manual

(**http://www.fastplants.org/intro.lifecycle.php**)

1. At the desired day after pollination (dap), students should use fine scissors to remove one pod from one of their two plants
2. Using the dissection needles or a sharp blade, cut along one seam of the pod where the two carpels are fused. Pry open the pod to reveal the ovules aligned within the carpel; each ovule is attached to the vascular strands of the placenta by its funiculus. You will also see a thin paper-like septum separating the ovules in each carpel.
3. Observe the opened pod with a dissecting microscope.
4. Remove an ovule from the opened pod with fine forceps or dissecting needles, keeping a portion of the funiculus attached to the ovule.
5. With a pipette, transfer a small drop of water to cover the ovule on the slide.
6. Place the opened pod with remaining ovules on moist paper toweling in a covered petri dish. This will keep it fresh for further sampling.
7. Place the slide with ovule on a dissecting microscope and observe the ovule, noting the funiculus attachment and the micropyle (opening through which the pollen tube entered the ovule). If the ovule is illuminated from below, students may be able to see the indistinct embryo within the ovule. This will depend on the stage of embryo development.
8. With needles make an incision across the ovule at the end opposite the funiculus. As this cut is made, the embryo may float out into the water along with the cloudy starchy liquid endosperm. Make a second incision perpendicular to the first and using dissection needles gently pull open the integuments which will develop into the seed coat. Embryos at 9 dap will generally be visible once the seed coat is open.
9. If the embryo is not visible, slowly and carefully remove small pieces of the integuments, working toward the micropylar end. Young embryos in the heart and globular stages are found surrounded by a funnel of aleurone and nucellar tissue from the torn embryo sac. The young embryo is immersed in cellular endosperm and is anchored by the suspensor in the integuments at the base of the funnel. Continue to carefully tease out the embryo and, if possible, its attached suspensor.
10. Once the embryo has been removed, increase the magnification of the microscope, or transfer the preparation to a compound scope for viewing and drawing.
11. Identify and record the stage of embryo development (globular, heart, torpedo, etc.).
12. Dissect ovules from pods of different dap. Share "spare" ovules with other students or exchange them for ovules at different stages of development.

    Suggested dissection times include 6,9,12, and 17 to 20 dap (days after pollination).

Questions for Investigation:

1. Are all of the embryos within a single seed pod at the same stage of development? What might this tell us about the success of the pollinator? If they are not, what might this indicate?
2. Does the amount of light the plant receives influence the rate of embryo maturation?
3. Does the temperature at which the plant is grown influence the rate of embryo maturation?

Part III. Observing Mitosis

Purpose

The purpose of this part of the laboratory is to identify the stages of mitosis and to identify the chromosome types according to centromeric position.

Background

Chromosomes are the vehicles of inheritance in all higher organisms. Within the cells of each organism can be found a basic number of chromosomes, which is called the genome. An organism containing one genome is said to have the haploid or 1N number of chromosomes. Organisms originating through sexual reproduction receive a single genome from each parent (one from the paternal and one from the maternal parent). Such organisms have two genomes per nucleus and are thus diploid. Occasionally organisms are found with 3, 4, 5 or more genomes per cell nucleus. Collectively we call these organisms polyploids. Since a diploid organism is represented by two sets of chromosomes, one set from each parent, we assume that each chromosome has a corresponding partner. Such pairs are homologues and are thus said to be homologous.

Chromosomes are composed chemically of a complex arrangement of DNA and protein. Located at some point along the length of the chromosome is a constriction called the centromere, which divides the chromosome into two portions, which may or may not be of equal size. Functionally, the centromere acts as an attachment site for the microtubule fibers, which make up the spindle. These fibers aid in the movement and distribution of chromosomes during nuclear division.

You will observe the chromosomes of the broad bean (root tip) during this laboratory period. Since chromosomes cannot generally be visualized in living cells without relatively sophisticated equipment, it will be necessary for us to kill and stain the chromosomes. Your instructor may ask you to do the staining, or root tips may have been stained for you.

Procedure

A. For this part of the exercise, use tips from the vial labeled A.

1. Pick up a single root and place on a slide, then using another slide "cut" off all but the stained tip (approx. 1-3 mm). Add a drop of 45% acetic acid and a cover slip, being careful to prevent air bubbles.

2. Tap root with a pencil eraser, until the "spot" is much paler and about 8-10 mm in diameter. Then, with a folded Kimwipe on top, mash down firmly with your thumb.

3. Observe the squash under low power (10X). If the cells are in a monolayer (none or very few overlapping), continue. If cells are still stacked 2 or more deep, continue tapping with the pencil eraser until the squash is light pink and well spread, or lift the cover slip and add another drop of acetic acid.

4. Locate some cuboidal cells under low, then high power. Look for actively dividing cells.

A. What stages of mitosis are the cells in?

B. How many different cells are actively dividing relative to the number in interphase?

C. Can you determine the number of chromosomes? Why are why not?

B. *Vicia faba* Chromosome Morphology

1. Obtain a root tip from vial **B** on the front desk. These roots have been pre-treated with colchicine to arrest mitosis at metaphase. (Colchicine prevents subunits of spindle from forming.)
2. Repeat steps l-4 as in the section above.
3. If the slide is satisfactory and chromosomes are well scattered within the cells, observe. If, however, the cells are not well scattered, quickly but carefully lift the coverslip and add another drop of acetic acid. Replace cover slip and spread the cell with pressure

You should be able to accomplish the following by observing the cells on the slide:

1. How many chromosomes are in this genome?
2. Identify the centromeres.
3. Identify the chromatids.
4. Determine how many satellite chromosomes are present.
5. How many pairs of chromosomes are present?

   Note: Most *Vicia faba* chromosomes are acrocentric; there is 1 pair of great big, metacentric chromosomes, with 2 constrictions.

Questions for Investigation:

1. Which stage is the longest in duration of time? Which stage is the shortest in duration of time? How did you arrive at this conclusion?
2. Does the time of day that you collect the cells make a difference in the number of dividing cells?
3. What is the effect of colchicine?
4. Can you find any evidence of nucleoli? Why are there no "typical" nucleoli in these cells?
5. Why was it easier to count the number of chromosomes in Vial B than in Vial A?

Notes for the Instructor

Part I. Pollination

Many studies have shown that the pollination media that is used makes a difference in the results. The basic media that we use is:

Germination medium

100 g sucrose (table sugar)
0.1 g boric acid (H_3BO_3)
0.3 g calcium nitrate ($Ca(N0_3)_2 \cdot 4H_20$)
1 liter distilled water (This works better if mixed as an entire liter, instead of in smaller quantities. It may be covered and refrigerated for a few days.)

Notes

We have found that we have the best luck when collecting pollen from plants "in the wild" from both native and introduced plants, as opposed to indoor or newer varieties of nursery plants. We have had good luck in the winter/early spring with Camellias and with Lenten Roses (*Helleborus* spp.) Sometimes Impatiens work well. The presence of air bubbles seems to make a difference, though we have not seen anything about this in the literature.

Part II. Embryos

Look at the fast plant website **http://www.fastplants.org** for additional information on using fast plants to observe young embryos. It takes practice to get good at this, and a little luck. Note that embryos can be preserved in glycerol for later dissection by harvesting and fixing the pods in Farmer's solution (3 parts glacial acetic acid: 1 part 95% ethanol) and storing frozen in glycerol. This works best with later stages.

Part III. Mitosis

Note: Place absorbent paper on countertop to collect excess stain and acid. This stain will stain clothing and fingers bright pink. A small amount of 45% acetic acid will remove stain from fingers.

Methods for Observing Mitosis in Broad Beans (Vicia faba) root tips:

Plant broad beans which have been soaked in water overnight in vermiculite (1-2 inches deep) in a plant tray ten days before needed for lab. Place under grow lights, and water as needed.

1. The morning of lab, harvest roots. If possible this should be done around 8-9 AM since mitosis is diurnal, with the greatest number of cells in various stages of mitosis in the morning.
2. Remove a plant from the vermiculite, rinse it in water and use scissors to snip off a cm or two of all of the root tips into a small beaker of Farmer's solution (3 parts 95% ethanol to 1 part glacial acetic acid). Leave in Farmer's for 2-3 hours.
3. About 1-1.5 hours before lab remove roots from Farmer's, rinse in distilled water (dip in small beaker of water) then put in 5 N HCl for 30 minutes to hydrolyze the middle lamella and allow cells to separate.
4. After 30 minutes in 5 N (=5M) HCl, rinse again in fresh distilled water, and place in Schiff's reagent for approx 30 minutes to stain the nucleus. (Schiff's stains DNA but not RNA.) Remove root tips to a small beaker of distilled water after 30 minutes in order to prevent over-staining.
5. Pick up a single root and place on a slide, then using another slide "cut" off all but the stained tip (approx. 1-3 mm). Add a drop of 45% acetic acid and a cover slip, being careful to prevent air bubbles.
6. Tap root with a pencil eraser, until the "spot" is much paler and about 8-10 mm in diameter. Then, with a folded Kimwipe on top, mash down firmly with your thumb.
7. Observe the squash under low power (10X). If the cells are in a monolayer (none or very few overlapping), continue. If cells are still stacked 2 or more deep, continue tapping with the pencil eraser until the squash is light pink and well spread, or lift the cover slip and add another drop of acetic acid.
8. Locate some cuboidal cells under low, then high power. Look for actively dividing cells. Can you determine the number of chromosomes? What stages of mitosis are the cells in?

Treatment with Colchicine

1. Extract 10 day old broad bean plants from flats, wash roots to remove vermiculite and pull off cotyledons.
2. Submerge root system of intact plants in 0.05% colchicine solution at 9 AM and leave in for at least 2 hours.
3. Collect roots at 11 AM to 12 noon, cut off root tips (1-2 cm).
4. Fix in Farmers until 1-1 ½ hrs before lab time.
5. Proceed as in step 4 above.

Alternate directions for preparing root tips ahead of time

After roots have been collected and put into Farmer's solution for two to three hours, the roots can be rinsed in water, placed in glycerol, and frozen. When ready to use, rinse roots in distilled water 8 to 10 times, and then proceed as above with HCl and Schiff's.

Recipe for Schiff's Stain (Careful, this can get messy.)

1. Add one g of basic Fuchsin to 200 ml boiling distilled water and boil one minute while stirring. Be careful—this solution has a tendency to boil over!
2. Cool to 50°C in an ice bath.
3. Filter with aspirator using Buchner funnel and add 30 ml of 1N (=1M) HCl.
4. Add 3 g of potassium metabisulfite (K_2SO_5) or sodium metabisulfite (Na_2SO_5). Cover with Parafilm and allow to bleach 24 hrs in a dark place.
5. After 24 hrs, add 1 g of decolorizing charcoal (carbon) and shake 1 minute.
6. Filter using aspirator and store in clean amber colored bottle. Label and store in refrigerator. (Needs to be made fresh each semester.)

Or you can just order from Carolina Biological Item #887265 Schiff's Reagent, Laboratory Grade, 100 ml $13.25 or Wards Item #947V8806 Schiff's Reagent Lab Solution 500 ml $19.90

Literature Cited

Mulcahy, M.P. and M.B. Puterbaugh. 2004. Using ant and butterfly pollination to involve students in scientific exploration. Pages 135-158, in *Tested Studies for Laboratory Teaching,* Volume 25 (M. A. O'Donnell, Editor). Proceedings of the 25th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 414 pages **http://www.ableweb.org/volumes/vol-25/7-puterbaugh-mulcahy.pdf**

Wisconsin Fast plant web page: **http://www.fastplants.org** Going for the globular: embryogenesis.pdf

About the Authors

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Karen and Marsha have team taught numerous times, as well as collaborated in the oversight of numerous student research projects.

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The Association for Biology Laboratory Education (ABLE) was founded in 1979 to promote information exchange among university and college educators actively concerned with biology learning and teaching in a laboratory setting. The focus of ABLE is to improve the undergraduate biology laboratory experience by promoting the development and dissemination of interesting, innovative, and reliable laboratory exercises. For more information about ABLE, please visit **http://www.ableweb.org/**

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