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Sex in the Garden: Pollen Germination

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In nature, pollen germination occurs on the stigma of a flower and the pollen tube elongates towards the flower's ovule. Pollen germination and elongation is a dynamic process during which pollen must respond to different signals and develop a complex structure to deliver sperm cells for fertilization. Pollen germination can be easily visualized using an *in vitro* setup and a compound microscope. Students learn about the advantages and disadvantages of different *in vitro* assay methods, test multiple flower species, modify basic germination media and design an experiment related to pollen germination that integrates evolution and cell biology.

Keywords: pollen germination, scientific method, *in vitro* assay

Introduction

This lab exercise is easy to set up and inexpensive. The lab can be set up in a couple of hours using common lab equipment. Many flower pollens will germinate *in vitro* allowing students to investigate flowers of interest to them. *Nicotiana tabacum* and *Hemerocallis* can be used as starting species. Extensions to the lab can include comparing different flower species, modifying the basic germination media and having students design their own experiment. The experiment itself does not require sophisticated content background, allowing the experiment to focus on the scientific method.

Student Outline

Students will:

- Critically read a lab procedure
- Compare the germination of pollen in two different in vitro methods (hanging drop method and agarose pad method
- Observe pollen tube germination in a variety of flowers
- Calibrate the ocular radical in the microscope for different objective lens.
- Measure the length of pollen tubes using Image J.

Figure 1. Anthers releasing pollen.

Basic Background Information on Pollen Germination

Pollen germination occurs when a pollen grain develops a pollen tube. In nature, pollen germination occurs on the stigma of a flower and the pollen tube elongates towards the flower's ovule. Sperm will travel through the pollen tube to fertilize the ovule. The evolution of pollen eliminated the dependence on standing water for fertilization in seed plants.

Pollen develops in the anthers of the stamen of the flower. The anther has four pollen sacs held together by connective tissue. Each pollen sac has microsporocytes (microscope mother cells), which divide by meiosis to produce microspores. Each microspore divides by mitosis to produce either a 2-cell microspore or a binucleated microspore. Each microspore differentiates into a pollen grain.

Pollen grains range from 6 to 100 micrometers and have species-specific morphology. The wall of the pollen has three layers: the inner intine wall, the outer exine wall and the pollen coat. The pollen walls have modifications to allow for shrinking and swelling of the grain and thinner areas (apertures) where the pollen tube can break though the pollen wall.

Pollen germination and elongation is a dynamic process during which pollen must respond to different signals and develop a complex structure to deliver sperm cells for fertilization. Pollen germination can be visualized easily. This system is a simple model to study cell to cell interactions and environmental effects on development.

Pollen Germination Assays

Hanging Drop Procedure

The hanging drop procedure is used to observe living organisms over time where desiccation could be an issue. The surface tension of the drop concentrates the cells to the edge of the drop. Oil immersion cannot be used with a hanging drop preparation.

1. Prepare humidity chamber by placing filter paper in Petri dish.
2. Moisten paper with water.
3. Clean microscope slides and cover slides using ethanol. Rinse with water and dry using a Kimwipe.
4. Prepare a slide with a slide chamber where the hanging drop can be inverted onto the chamber.
5. Lightly grease the bottom of a gasket. Place the gasket onto a regular microscope slide or a depression slide.

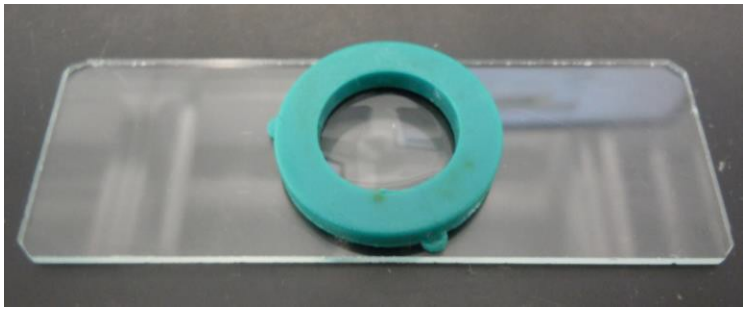

Figure 2. Greased gasket placed on microscope slide.

6. Determine the amount of media you will need to add to the cover slip to get a hanging drop (20 to 100 micrometers). The larger the drop, the less likely the drop will evaporate.
7. Flip the coverslip over and place onto the gasket.

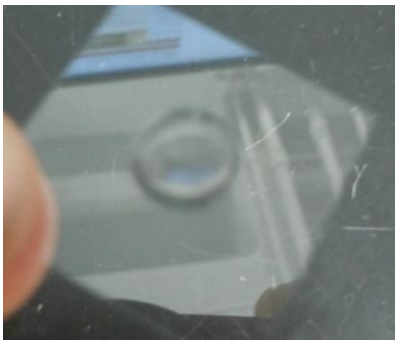

Figure 3. Flipped coverslip

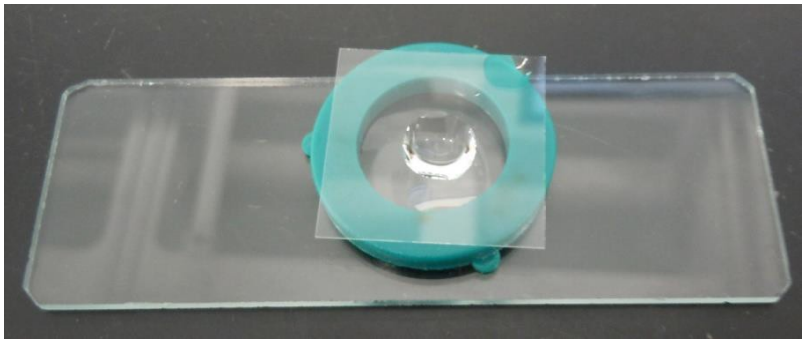

Figure 4. Coverslip on top of gasket.

8. Observe the drop under the low power objective.
9. Use the edge of the drop to focus the microscope.
10. Change the objective lens to 10X and refocus. You cannot use the 40X objective lens with this procedure.

Obtaining Pollen

1. Observe the flower.
2. Identify the different parts of the flower.
3. Find the anthers.
    - The best pollen to collect for germination comes from anthers that have burst or split open. The process is referred to as dehisce. You can quickly tell if the pollen is mature by gently brushing your finger across the anther. If you see a residue of pollen on your finger, the pollen is most likely mature.
4. Remove a pipette tip using the P200 micropipettor.
5. Eject the tip into your fingers.
6. **Gently** rub the anther with the pipette tip until a small amount of pollen is transferred to the needle.
7. Transfer the pollen to the drop of media on the cover slip by tapping the tip with your finger.
8. Invert the cover slip over your slide.
9. Record the time.

Observing Pollen Tube Growth

1. Observe the pollen under 100 x magnification
   (10 x objective lens and 10x ocular lens)
2. Record the shape, size and any unique features of the pollen grains. You may draw the pollen shape on a piece of paper and then take a photo using your cell phone (will be demonstrated in class).
3. Check the pollen every 30 minutes until the pollen tubes begin to grow. Keep the slide in the humidity chamber between observations. You may search through the fields of views until you find a field of pollen with a good density of pollen.
4. Once the pollen tubes have begun to germinate, record the time, percentage of pollen that has germinated and the length of five pollen tubes every 10 minutes for 30 minutes.

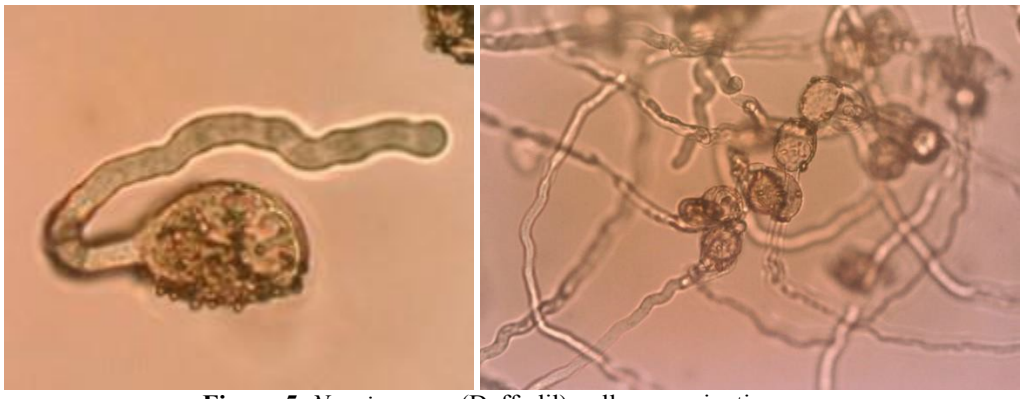

Figure 5. *Narcissus sp.* (Daffodil) pollen germination.

Agarose Method

Agarose gels are transparent, easy to make and can be made with a variety of buffers.

1. 1% molten agarose can be found on a hot plate.
2. Clean a microscope slide.
3. Warm the microscope slides by placing the slides on the edge of the hot plate.
4. Using a glass pipette, add 1 drop of agarose to the warm slide.
5. Allow the agarose to spread over the warm slide for 15 seconds.
6. Place a second slide perpendicular to the first slide.
7. Remove the slide from the hot plate and allow the slides to cool.
8. Prepare humidity chamber by placing filter paper in Petri dish.
9. Store your agarose slide in the humidity chamber when not in use.

Observing Pollen Tube Growth

1. Remove the slide from the humidity chamber.
2. Dry the bottom of the slide using a Kimwipe.
3. Observe the pollen under 100X and 400X magnification
4. Record the shape, size and any unique features of the pollen grains. You may draw the pollen shape on a piece of paper and then take a photo.
5. Continue to check the pollen.

Compare the Pollen Growth between Different Flowers

1. Collect pollen from two different flowers.
2. Use either the hanging drop or the agarose method assay to determine pollen growth.
3. Decide on a variable to measure.
    - Time it take for the pollen tube to reach one pollen grain diameter
    - How long the pollen tube will be after a pre-determined time
    - Percentage of pollen that germinates
4. Observe the pollen under 100X and 400X magnification and record data. Take a picture of the pollen with the eyepiece reticule present in the view.
5. Calibrate your eyepiece reticule using a stage micrometer (See Appendix A).
6. Using Image J, calculate the length of a typical pollen tube from the image you took on your cell phone. (See Appendix B).

Materials

A List of Equipment and Supplies Needed for a Class of 25 Students

Basic Germination Media:

1mM KCl
0.1 mM $CaCl_2$
1.6 mM H_3B0_3
10% glucose
1% agarose

Regular glass slides
Cover slips
Petri dish (large enough to up a glass slide in)
Flowers
Compound microscopes
Micropipettors (200ul)
Slide warmer
Stage micrometer
Microscope
Eyepiece reticule

Notes for the Instructor

This manuscript is based on an undergraduate biology lab at University of California Irvine. Students work with pollen germination for multiple weeks. Initially, they explore the two different assays which will be presented in the workshop. Later students expand on these assays by bringing their own flowers, modifying the germination media components and finally designing their own experiments.

Image J is relatively easy to use by students with little supervision. Students are given a PowerPoint to help them work through the set of images using the screen shots as part of an introduction to data analysis. Students are given a practice quiz to help them determine if they are having any problems using Image J, before they are given a class quiz. The most common student error is reporting an area measurement when doing linear measurements. Image J reports both a length and area value for each line measurement, because the line has both a width and a length. The results from the quizzes are used as data to illustrate variability. Student routinely use Image J for every lab activity in the UC Irvine Cell and Developmental laboratory. Students report using Image J in microbiology, molecular biology labs and with their own independent research. Different sets of images could be designed to be more closely related to other laboratory experiments.

Easter Eggs: Errors Intentionally Introduced to Encourage Students to Carefully Read the Lab Procedures

Before a lab, students are told the procedures may contain errors, which I call Easter Eggs. These errors help student learn the skill of trouble shooting an experiment. The following are a few of the common Easter Eggs I include in the procedure. I usually include additional errors and continue the inclusion of Easter Eggs in all future procedures.

The students are asked to "**Gently** rub the anther with the pipette tip until a small amount of pollen is transferred to the needle". The step before asked the students to use a pipette tip.

In the agarose method, students are asked to "Place a second slide perpendicular to the first slide, after they have added a few drops of agarose to a warm slide". If students place the additional slide on the agarose and then try to remove the second slide after the agarose has dried, they will not get a nice even agarose pad. Frequently students will keep repeating this step over and over, with the same results. You can guide the student by asking what physical properties the agarose pad should have. The pad should be flat but also thick enough to not dry quickly. The best way to achieve a flat pad is to not place a second slide perpendicular to the first slide.

Also, no instruction indicates when the pollen needs to be added to the agarose. Sometimes students will add the pollen while the agarose is still hot.

The students are given an image of *Narcissus sp.* (Daffodil) Pollen Germination. Although the flowers are not *Narcissus sp.* When asked to report the genus species of the plant they are working with, many students report *Narcissus sp.* You can add additional "Easter Eggs" to the procedure.

When students were asked "what were the 3 most important skills learned from this laboratory exercise?", the most frequently reported are: critical thinking, scientific doubting, results analysis results and experimental design modifications

Student Comments on the Pollen Experiment:

- I learned to challenge protocols; there may be a better alternative to a method. 2) I learned to work together in a group with other individuals, this is especially important since science requires collaborations. 3) I learned that it's more important to understand why the results occurred rather then just accepting the results.
- Thinking critically, having a plan, questioning everything that even someone of authority tells you (Easter eggs in protocols)
- The ability to read scientific papers. 2) Preparing for lab by analyzing the protocol. 3) Asking for help when needed.

- During an experiment, it is important to constantly be thinking of why certain

things in the lab are done the way they are, why certain reagents are added and their importance, and explanations for results that are obtained. 2. It is also important to have the skill of constantly be documenting what is being done during the lab and taking pictures for future use. 3. I also learned the important skill of keeping an open mind set when doing a new experiment as it can help me learn new perspective on things.

- Thinking CRITICALLY about what I am doing and WHY. I believe this was emphasized MUCH more in this lab compared to previous labs I have taken, and that's awesome. 2. Working in groups. Previous labs I have taken also focused on group work, but for some reason, I feel like I learned more about how to work well in a group compared to other labs. 3. Always have aback-up plan. There have definitely been times where things could have gone (or did go) wrong. Those times have taught me to always have a plan B.

Sample Results

Depending on the flower, pollen tubes can be seen within 15 to 30 minutes after they have been exposed to germination media.

Outline to Present the Laboratory

1. Present the background of pollen germination.
2. Demonstrate the set-up of the two assays.
3. Allow time for the participants to set up their own germination assays.
4. Demonstrate how to calibrate the microscope using a stage micrometer.
5. Demonstrate how to take picture of the pollen using a cell phone.
6. Demonstrate how to use Image J and the photos taken from the cell phone to calculate the length of a pollen tube.
7. Allow time for participants to complete the activities.
8. Reflect on the experiment,
9. Show examples of student work.

Acknowledgments

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Cited References

Mauzy-Melitz, D. Image J. In K. McMahon (Ed.), *Tested Studies for Laboratory Teaching Proceedings of the Association for Biology Laboratory Education. ABLE Eugene 2014*, Vol. 36.

About the Author

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Debra received the TA Professional Development Award from the University of California and became a fellow of the National Academy of Sciences Educators in the Life Sciences in 2011.

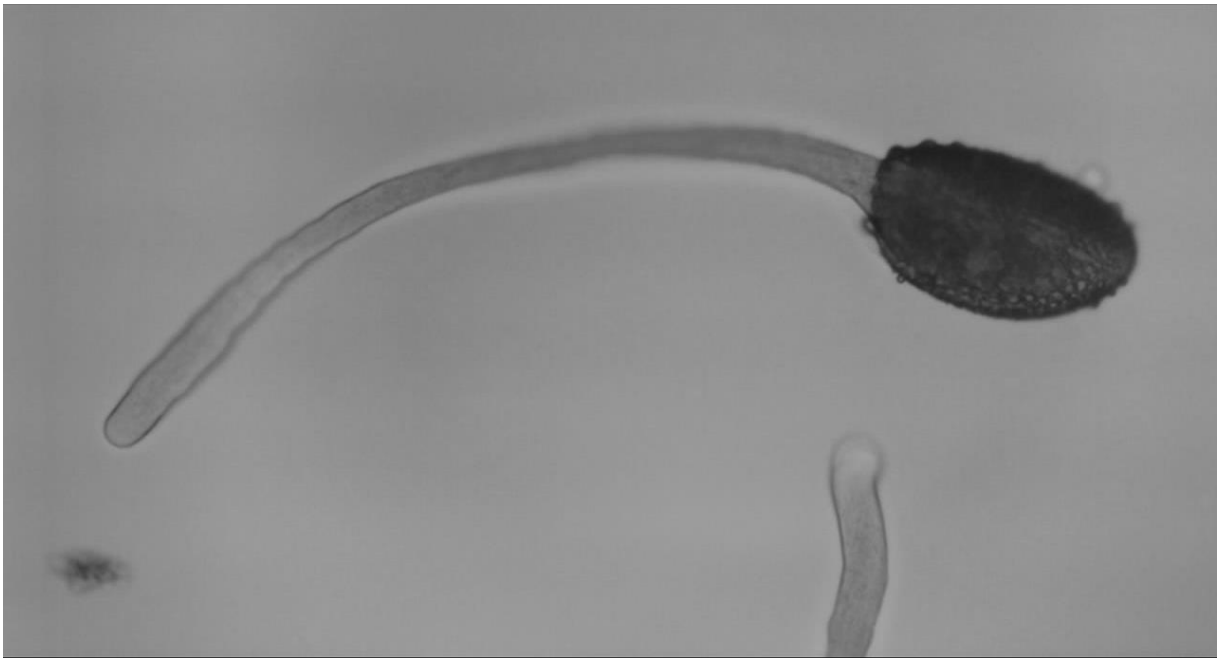


Figure 6. Day lily pollen (*Hemerocallis* sp.) one hour after pollen was added to germination media (hanging drop method).

Appendix A
Microscope Magnification Calibration

Questions to be Answered

What was the conversion factor for your 10x and 40x objectives?

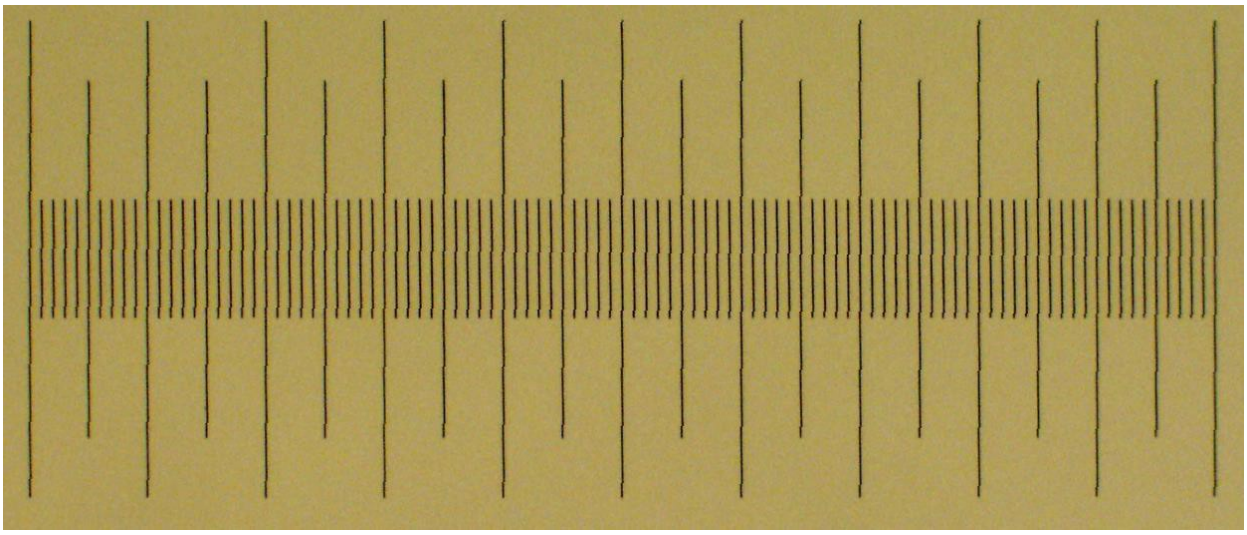
Materials

Stage micrometer
Microscope slide with a ruler etched on the glass
A typical stage micrometer is 1 mm long with 10 major divisions and 10 minor divisions (100 total divisions).
Each division is 10 micrometers or microns (μm)
Confirm the micrometer scale, because some micrometers can be 2 mm long.
Eyepiece reticule
(A small piece of glass, fitting in the ocular lens, with a ruler etched on the glass)

Procedure

Calibration of the Microscope

1. Focus the stage micrometer under the 10X objective lens. The lines on the stage micrometer represent **REAL** units. Each division has a length of 0.01 mm (10 μm).



2. Focus the eyepiece reticule using the focus ring on the ocular lens. The eyepiece reticule lines represent **ARBITRARY** units (au).

3. Adjust the distance between the ocular lenses until the interpupil distance is correct for you. Move the ocular lens apart and gradually bring the lens closer together until you only see one image through both of the lens. If you do not correctly adjust your interpupil distance, you can get a headache when looking though the microscope.

4. Rotate the eyepiece reticule until the ruler is parallel to the stage micrometer.

5. Move the stage micrometer using the stage controls until the two "0" points are aligned.

6. Find another point where the two ruler lines align. Record the number of lines on the stage micrometer. Record the number of lines on the eyepiece reticule.

7. Multiply the number the number of lines on the stage micrometer by 0.01mm to determine a REAL length in mm.

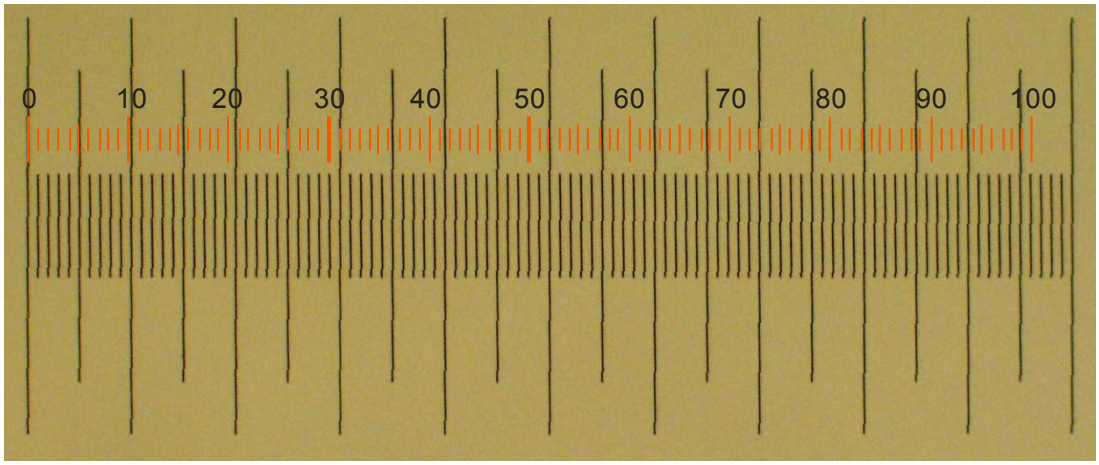
8. The conversion factor will be the real length divided by the number of lines on the eyepiece reticule.

9. The number will be the conversion factor you will use when measuring an object on the stage. The conversion factor units are mm per au (arbitrary unit).

10. You can convert to mm to µm by multiplying the conversion factor by 1000.

11. Repeat the calibration for the 40X objective (steps 1-8)

Example
(The reticule lines were colored red to help distinguish the reticule lines from the micrometer line.).



73 arbitrary lines (red) on the ocular reticle line up with 75 lines on the stage micrometer. Each line on the stage micrometer equals 0.01mm or 10 microns (µm)
75 lines on the stage micrometer are equal to 750 microns. The conversion factor is 750 microns divided by 73 au.

Measuring an Object under the Microscope

1) Align the eyepiece reticule along the length you wish to measure.

2) Count the number of divisions on the eyepiece reticule.

3) Multiple the number of divisions by the conversion factor for the objective lens used.

4) Remember to include all units.

Appendix B
Using Image J to Measure Pollen Tubes

From:
Mauzy-Melitz, D. Image J. In K. McMahon (Ed.), *Tested Studies for Laboratory Teaching Proceedings of the Association for Biology Laboratory Education. ABLE Eugene 2014*, Vol. 36.

Introduction

An image is more than just a beautiful picture. For scientists, images contain a wealth of information. Image processing can complement many of the current biology laboratories. Image J is a free, easy to use image processing program, developed by the National Institutes of Health, widely used in research applications. Image J also supports standard imaging processing functions allowing students to crop, manipulate contrast or subtract background. Java plugins and macros have been developed by Image J users to expand the functionality of the program. Multiple tutorials as well as YouTube videos are available to help users. With almost every aspect of Image J. At the University of California Irvine, Image J is used in the Developmental and Cell biology labs to give students an appreciation for microscopic quantitative techniques. Students analyze their own images and images obtained from research. Measurements, conversion from pixels to micrometers, area determinations, density profiles, histograms and particle counts are typical Image J applications. The mini-workshop presented during the 2014 ABLE conference is similar to material presented to undergraduate students in a scientific writing course that is a perquisite to all biology laboratories.

Objective

Students in biology laboratory courses frequently use microscopes to visualize experimental results but rarely analyze the results. Introducing students to Image J, a free simple image processing program, can promote quantitative skills into the visualization process. These skills can be later used by the students in research laboratories.

Materials

Image J program downloaded from http://rsbweb.nih.gov/ij/. The Image J program can be downloaded into a flash drive to facilitate distribution.

Set of practice images. Practice images should include an image of an object with a centimeter ruler (Figure 1), a microscope image of a stage micrometer with a scale bar included, (Figure 2) images of objects seen under the microscope at the same magnification as the stage micrometer slide (Figure 3).

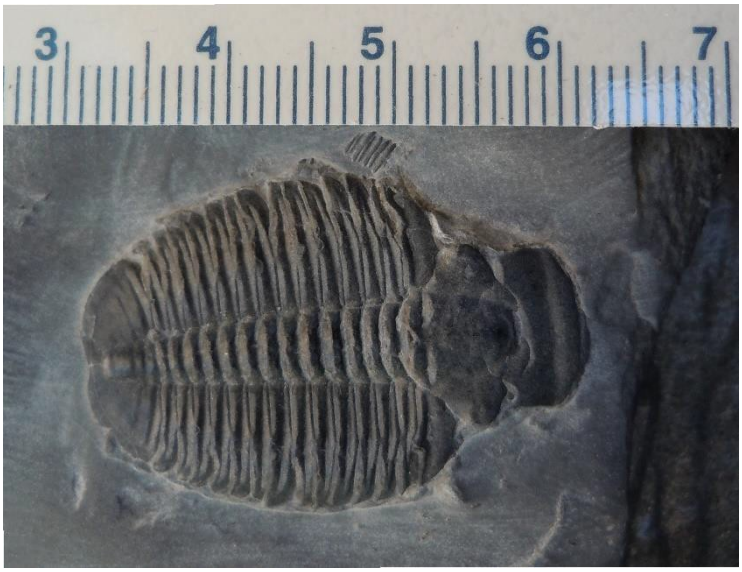


Figure 1. Photograph of a trilobite fossil with a centimeter ruler.

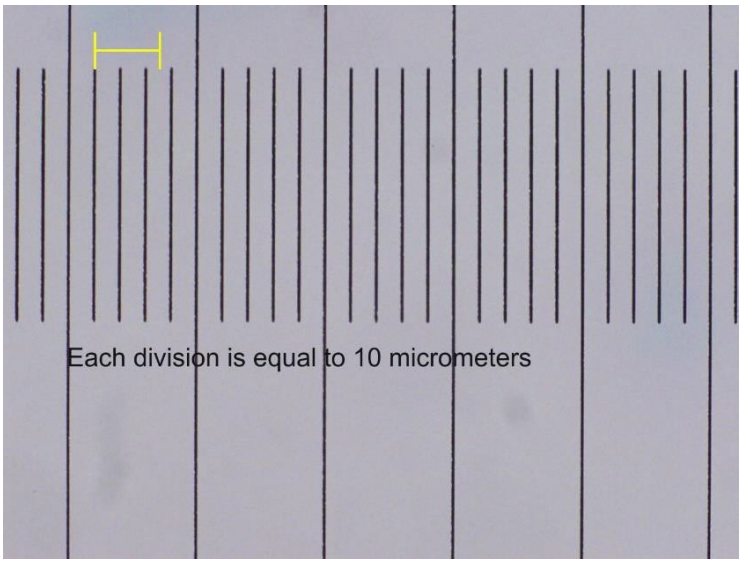


Figure 2. Microscope image of a stage micrometer taken with a 40x objective lens. Each division of the stage micrometer is equal to 10 micrometers. Scale bar is equal to 250 micrometers.

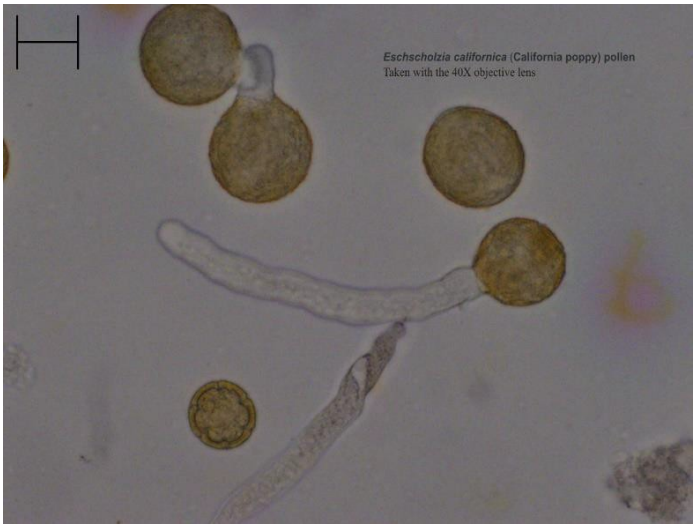


Figure 3. Microscope image of *Echscholzia californium* pollen germinating. The image was taken with the same objective lens and microscope as the image in Figure 2.

Methods

The Image J commands for each of the process are outlined below. An image must be opened in Image J for the program to function correctly. The Image J program window consists of two bars, a menu command bar with pull downs and a tool bar. The menu commands bar consists of the major commands: File, Edit, Image, Process, Analyze, Plugins, Window and Help. Mac based computers will have the Menu command bar at the top of the screen. PC based computers will have the command bar and the tool bar attached somewhere within the screen.

Change Line Color and Width

The default line width is one and the color is yellow. For some images, either the line width or the color may need to be change to allow for better visualization.

1. *Edit > Options > Line Width*
2. *Edit > Options > Colors > Selection*

Length Measurements

Length measurements are the most common measurements made in most biology laboratory activities. If the measurement is for a straight object, the Straight Line Tool should be used. The Segmented Line Tool is used for measurements of nonlinear objects. The Freehand Line Tool is available from the pull down menu of the Straight Line Tool with a icon of a wavy line instead of a straight line. Figures 1, 2, and 3 should be used to demonstrate length measurements. Figure 3 will require the use of the Freehand Line Tool.

1. Select Straight Line Tool.
2. Draw a line while holding down on the right hand mouse button. (Ctrl click for Mac)
3. Click on *Analyze* > *Measure* (Shortcut key: Ctrl or Command M)
4. Length in pixels will be displayed in a Results window (window may be hidden behind image)
5. Other Line tools are Segmented and Freehand line tools (Right click to see line choices)

Set Scale

The default measurement value is in pixels. If the image has a ruler, stage micrometer or scale bar, the pixel values can be converted into metric measurements. Figure 1 of the Trilobite includes a centimeter ruler. Figure 2 is of a stage micrometer with the distance between each interval equal to 10 micrometers. Figure 3 of the California poppy pollen germination was taken with the same objective lens as figure 2. The value for the scale bar can be determined for Figures 3, 4 and 5 from figure 2. It is important to have students confirm their measurements. A simple confirmation that the set scale is working correctly is to measure the distance between 4 and 5 on the centimeter ruler in figure 1.

1. Select Straight Line Tool
2. Draw a line while holding down on the right hand mouse button across a ruler or scale bar.
3. Click on Analyze > Set Scale
4. Click on Global (Need to confirm scale next time you open a new image with a different magnification)
5. Confirm scale is correctly set by re-measuring the ruler.

Area Measurements

Area measurements are similar to linear measurements. The most common tool used is the Freehand tool (icon is a heart shaped object). Figure 4 and 5 should be used for area measurements.

1. Use *Selection* tools (Rectangle, Oval, Polygon, Freehand)
2. Same procedure as line measurements.

Appendix C
Lab Report from a Student Designed Experiment Based on Pollen Germination

Effect of Pistil Length on Pollen Tube Length
by Audrey Park

Introduction

The stigma is typically sticky to allow pollen grains transferred by the wind, animals, or surrounding water to easily adhere. Its stigmatic tissues and aquaporin channels help rehydrate the pollen, thereby polarizing the cell and assisting in initial germination. Pollen germination occurs when the sperm cells in a pollen grain migrate through the pistil towards to ovule, where the embryo sac is located. Pollen grains start off as a diploid microspore mother cell that undergoes meiosis to form 4 haploid cells. As the flower develops, the haploid cells undergo mitosis and differentiate into pollen grains (Raven, 2011). The pollen grains consist of a tube cell nucleus and generative cell. The tube cell nucleus aids in the elongation of the pollen tube while the generative cell develops into sperm cells.

The pollen grain forms a very small aperture where the pollen tube protrudes and begins to elongate. Golgi vesicles that are present in abundant amounts in the apical tip of the pollen tube fuse and become a part of the tube cell membrane, ultimately expanding the wall. These vesicles are made up of pectin, which react with calcium to form cross-bridges, strengthening the apical tip to withstand the turgor pressure.

However, the acidic nature of pectin also triggers the release of cell wall-loosening agents to aid in the flexibility of the tip to allow elongation to occur. The pollen tubes receive signaling cues from the ovary to guide it downwards in the right direction. The sperm cell travels down the pistil through this pollen tube and penetrates the ovary at the base of the flower to form a zygote. The journey through the pistil of the flower is the most crucial part of germination because the stigma, style, and ovary interact and send signaling cues to the pollen grain (Kliwer, 2010).

The focus of the paper will address the relationship between pistil length of a flower and its pollen tube lengths. There have been several studies conducted on the flower size and the rate at which its pollen germinates. According to a previous experiment, longer pistils have larger chemical gradients and stronger signaling cues, thus allowing
the pollen tubes to continuously grow until the ovule has been penetrated (Hulskamp, 1995). This is crucial for plant reproduction because pollen on long pistils must be able to reach the ovary at the base of the flower in order for sperm to penetrate the embryo sac. In 1990, scientist E.G. Williams experimented with interspecific pollination with flowers of varying pistil lengths. His results showed that pollen from small flowers could not reach the ovule of flowers with long pistils. However, pollen tubes from a large flower would over-penetrate the ovule at the base of a flower with a short pistil (Williams, 1990).

In this experiment, I used 7 different flowers of varying pistil lengths: *Lonicera sempervirens, Tecoma campensis, Aloe arborescens, Cotyledon orbiculata, Penstemon centranthifolius, Tabebuia impetiginosa,* and *Digitalis purpurea.* These flowers are all tubular, fused corollas, ranging in length from 7.4 to 2.6 centimeters and thrive in partial to full sunlight. With this said, is there a correlation between the pistil length of a flower and its respective pollen tube lengths? I hypothesize that flowers with long pistils will produce longer pollen tube lengths because the sperm cells must travel a further distance to the embryo sac, located at the base of the plant.

Materials and Methods

Plant Material and Study Site

For this experiment, seven different tubular, fused corolla flowers were tested: *Lonicera sempervirens, Tecoma campensis, Aloe arborescens, Cotyledon orbiculata, Penstemon centranthifolius, Tabebuia impetiginosa, Digitalis purpurea. Tecoma campensis* should be watered only once a week to allow the soil to dry between waterings. These flowers bloom into bright red-orange flowers that attract many pollinators, such as hummingbirds.

Cotyledon orbiculata and *Penstemon centranthifolius* are both desert succulents, and therefore, thrive in dry, arid environments. *Aloe arborescens* has active and rest periods. During its active growth periods, it is essential that the plant has access to full sun exposure. On the other hand, *Digitalis purpurea* should be kept in partial shade and in cool environments. Lastly, *Lonicera sempervirens* should ideally be watered regularly to keep the soil rich and moist. These flowers were all picked 2-3 days prior to testing from the University of California, Irvine arboretum (33.6635° N, 117.8530° W).

Microscopy

A compound microscope was utilized to measure pollen germination. Measurements were taken under 100x and 400x (10x or 40x objective lens x 10x ocular lens) in order to capture a clear image of the pollen. To adjust the image, the course and fine focus knobs found on the side of the microscope were utilized. The microscope was calibrated using the eyepiece reticule and stage micrometer before taking measurements in order to calculate the conversion factor:

$$\frac{\#\ of\ lines\ on\ stage\ micrometer\ (um) * \frac{0.01\ mm}{1\ um}}{\#\ of\ lines\ on\ eyepiece\ reticule\ (au)} * \frac{1000\ um}{1\ mm}$$

Pollination Experiment

To prepare for the experiment, 7 cover slips and microscope slides with ethanol and deionized water to ensure that there was no lingering residue. In addition, 7 gaskets were lightly greased with agarose gel and positioned on top of the microscope slides to prevent any movement. Using a P200 micropipette, 45 microliters of basic germination media were transferred onto the cover slips. Then, mature pollen from the flowers was collected with a pipette tip by gently rubbing the dehiscent anther lobes and lightly tapping it onto the germination media. The germination media was composed of 1 mM KCl, 0.1 mM $CaCl_2$, 1.6 mM H_3BO_3, and 10% glucose. After transferring the pollen, the cover slip was inverted so that the hanging drop suspended over the gasket. When the slides were not in use, they were kept in a humidity chamber, made up of moistened filter paper in a petri dish in order to prevent the hanging drop from drying out.

Statistical Analysis

Pollen tube growth was checked every 30 minutes and every subsequent 10 minutes after initial signs of germination appeared—the amount of time that had passed, percent germination, and the lengths of at least 30 pollen tubes were noted. Means ± standard error was calculated for all measurements. With this information, multiple unpaired 2 sample t- tests were conducted in order to determine the significance between the average tube lengths and its respective pistil length.

Results

In Figure 1, the flowers are arranged from shortest to longest pistil length on the x-axis. From *Digitalis purpurea* to *Lonicera sempervirens*, the pistil lengths range from 2.6 to 7.4 centimeters. The objective of this experiment is to determine the relationship between pistil length and pollen tube length. Figure 1 displays a positive trend between the two variables—as the pistil length increases, the average pollen tube length also increases. The shortest flower, *Digitalis purpurea* was used as the negative control in this experiment because its pollen did not germinate in the 3 trials that were conducted (Table 1). On average, the pollen from the other 6 flowers germinated within minutes of being transferred onto the basic germination media at a rate of 2.24 micrometers per minute. A sample size of 30 pollen grains was observed and measured in order to determine the mean pollen tube lengths of each flower. *Tabebuia impetiginosa,* the second shortest flower, had an average pollen tube length of 31.3 micrometers while largest flower had the longest average pollen tube length of 717.15 micrometers. The calculated standard error did not have a particular trend but it tells the reader how accurately the sample represents the population.

To determine the significance of this positive relationship, six unpaired 2-sample t-tests were conducted with each individual flower compared to the flower with the longest pistil, *Lonicera sempervirens*. The null hypothesis was that the mean pollen tube length of each of the flower would be the same as the mean pollen tube length of *Lonicera sempervirens* (H_0: $\mu_{tc,aa,co,pc,ti,dp} = \mu_{ls}$). The alternative hypothesis was that the mean pollen tube length of *Lonicera sempervirens* would be greater than the mean pollen tube length of the remainder of the flowers (H_a : $\mu_{ls} > \mu_{tc,aa,co,pc,ti,dp}$).

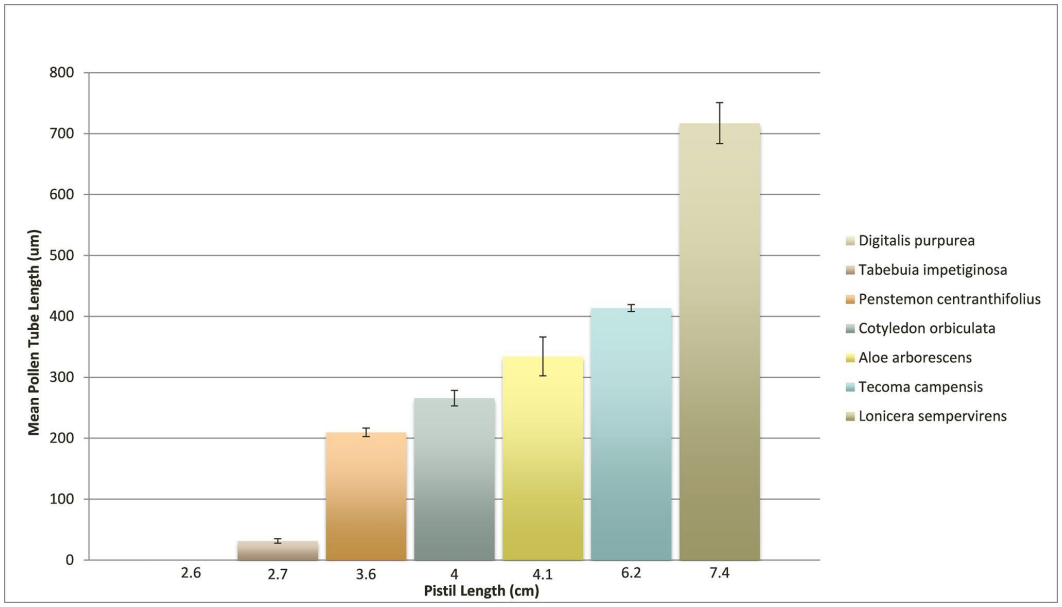


Figure 1. The flowers are arranged from shortest to longest pistil length (2.6-7.4 cm). The bar graph displays the mean pollen tube lengths for each flower in relation to the pistil size. Standard error bars are used to display the variance.

The expectation was to find the longest pollen tube lengths for *Lonicera sempervirens* because it has the longest pistil. Three trials were conducted over the span of two weeks; the results are charted in Table 1. From these three data sets, the set that most accurately represented the data was chosen and statistically analyzed (Table 2). After conducting the 2- sample t-tests, a set of p- values was calculated to determine the significance of the relationship between pistil length and its pollen tube lengths (Table 3).

Table 1. The average pollen tube lengths (um) are depicted for each trial.

	Trial 1	Trial 2	Trial 3
Lonicera sempervirens	30	129.33	717.15
Tecoma campensis	16.8	0	413.7
Aloe arborescens	334.45	162.75	322.35
Cotyledon orbiculata	265.65	98.385	353.85
Penstemon centranthifoliu	126	209.649	430.5
Tabebuia impetiginosa	n/a	n/a	31.325
Digitalis purpurea	0	0	0

Table 2. The table displays the statistical data for each flower.

	Pistil Length (cm)	Mean Pollen Tube Length (um)	Standard Error
L. sempervirens	7.4	717.15	33.65
T. campensis	6.2	413.7	5.753
A. arborescens	4.1	334.425	31.85
C. orbiculata	4	265.65	12.73
P. centranthifolius	3.6	209.649	7.04
T. impetiginosa	2.7	31.325	3.52
D. purpurea	2.6	0	0

Table 3. The table displays the difference in length between each flower and *L. sempervirens.* The p- values from the 2- sample t-test are listed.

	Pistil Length Diff. (cm)	P-value
Tecoma campensis	1.2	2.70E-10
Aloe arborescens	3.3	6.55E-12
Cotyledon orbiculata	3.4	3.15E-15
Penstemon centranthifolius	3.8	5.18E-16
Tabebuia impetiginosa	4.7	3.17E-19
Digitalis purpurea	4.8	1.55E-34

If a significance level of $\alpha < 0.05$ is used, the null hypothesis can be rejected for all the flowers. The p-values ranged from 2.70E-10 to 1.55E-34, therefore, we can conclude that there is statistical significance between the mean pollen tube lengths of each flower and *Lonicera sempervirens*. The data supports our hypothesis because it confirms that the length of the pollen tubes vary depending on the flower's pistil length. In Table 3, we can see that as the difference in pistil lengths increases, the p- values decrease. This means that as the pistil lengths differ by a great amount, the probability that their pollen tube lengths are equal decreases.

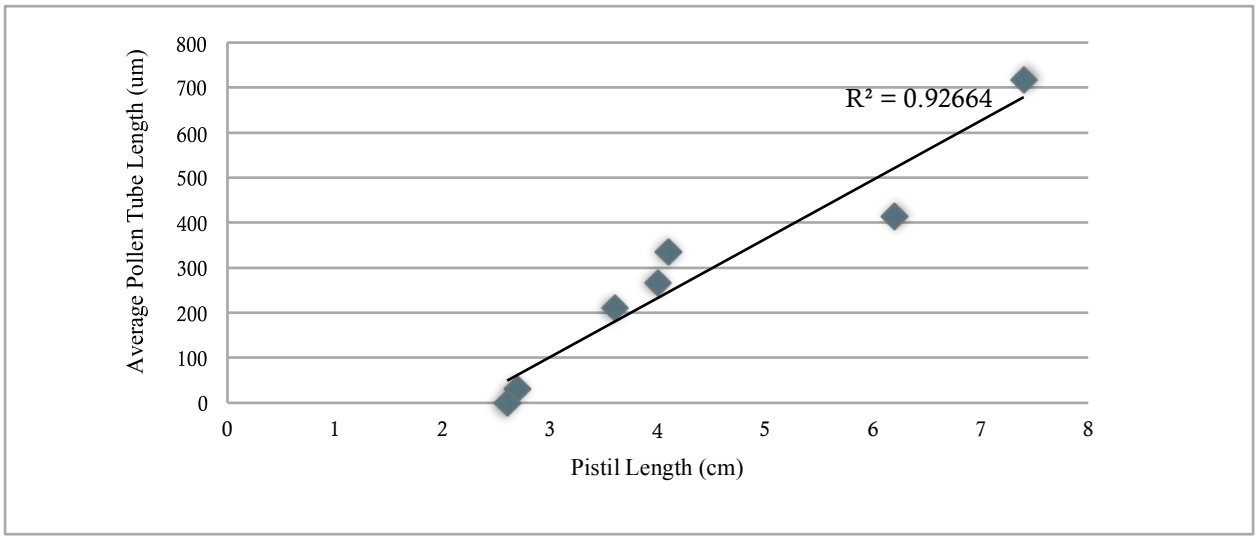


Figure 2. The positive correlation between the pollen tube lengths and pistil length is very strong.

Figure 2 depicts an x-y graph displaying the relationship between pistil length and pollen tube length. The R^2 value is 0.93, indicating that there is a strong positive correlation between the two variables. In other words, there is a clear increase in pollen tube length as the pistil length increases. The large R^2 value also indicates that this model accounts for 93% of the variability along the regression line; the data fits the statistical model very well.

Discussion

Pollen collected from the different tubular flowers elongated readily in the basic germination media. Apart from the negative control, the remainder of the 6 flowers began germinating within 35 minutes of being transferred onto the media. The results of the conducted experiment support the initial hypothesis that flowers with long pistils produce longer pollen tube lengths. The strong, positive relationship between these two variables was confirmed by the R^2 value and the conducted t-test. The R^2 value of 0.93 proved that the size of the pistil of the flower determined the length to which its pollen tubes would grow. In addition, the resulting p-values, ranging from 2.70E-10 to 1.55E-34, indicate that there is a significant difference in pollen tube lengths between different flowers. As the pistil length difference between

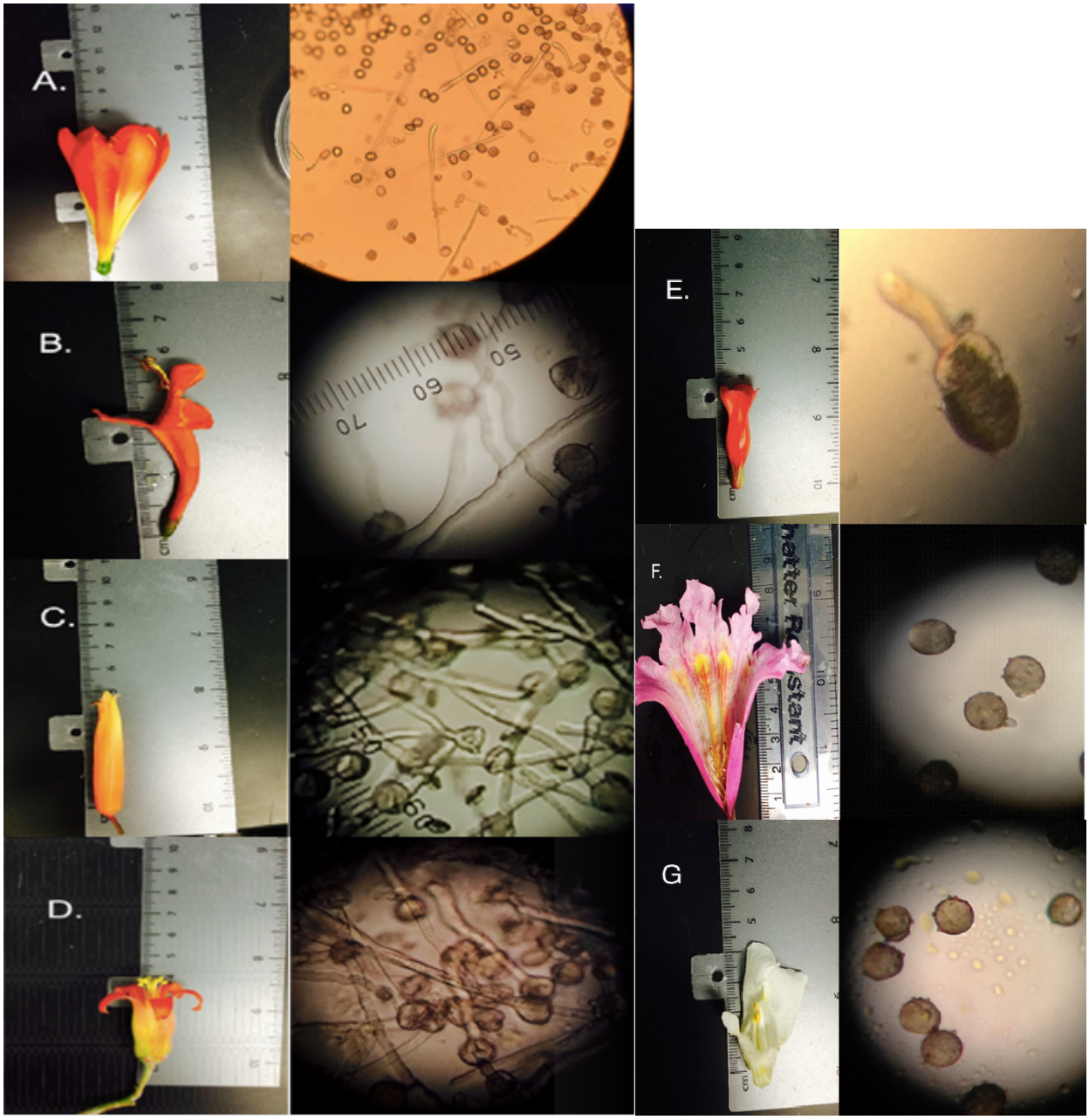


Figure 3. Shown above are images of the flower that was tested and its respective pollen germination.
A. *Lonicera sempervirens* had the longest pistil length and highest average of pollen tube lengths
B. *Tecoma campensis*
C. *Aloe arborescens*
D. *Cotyledon orbiculata*
E. *Penstemon centranthifolius*
F. *Tabebuia impetiginos*
G. *Digitalis purpurea* showed no sign of germination in all 3 trials that were conducted.

the positive control and each flower increased, the probability that the pollen tube lengths of both flowers would be equal decreased. Each p-value was less than 0.05, signifying that the average length of pollen tubes was highly dependent on its pistil length.

The results of the experiment can be attributed to pollen-pistil interactions. The pistil, composed of the stigma, style, and ovary, is essential for pollen tube elongation (Figure 4). According to author Herrero, each component of the pistil supports the elongation of the pollen tubes—the stigma rehydrates the pollen before it enters the style where it is provided nutritive support, such as glycoproteins which guides the tube down towards the ovary (Herrero, 1996). In our experiment, the flower that had the longest pistil length, *Lonicera sempervirens,* produced pollen tube lengths that were nearly 23 times longer than that of the shortest flower, *Tabebuia impetiginosa*. This can be attributed to the larger amount of support and nutrients provided to the pollen tube during its journey through the longer pistils. In addition, flowers that have long pistils tend to have stronger signaling cues and larger chemical gradients, ultimately resulting in longer pollen tubes.

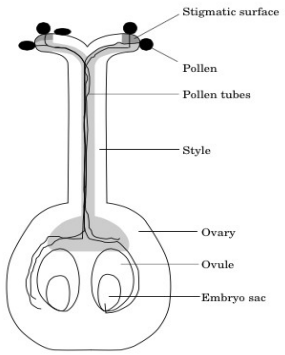


Figure 4. Image displays the anatomy of the pistil.

Furthermore, the outcomes of the conducted experiment were analogous to those of the study mentioned previously by E.G. Williams. In his experiment, flowers with longer pistil lengths produced pollen tubes that penetrated past the embryo sac whereas pollen tubes from shorter flowers could not reach it at all. In our experiment, the relationship between pistil and pollen tube length had a definite, positive trend.

To eliminate confounding factors and achieve accuracy, three trials were conducted in the span of two weeks. However, much of the data from trials 1 and 2 was inaccurate due to the heavy fog and rainfall that was experienced throughout the week. Flowers that bloomed upwards showed the most obvious signs of pollen damage while flowers that droop, such as *Cotyledon orbiculata*, were not impacted by the weather. The rain may have rehydrated the pollen grains prematurely before the flower had fully developed or may have completely shaken off all the pollen off the stigma. Biological replicates were tested—flowers of the same species were picked off of different branches to account for any confounding variables.

This experiment is relevant to the scientific community because when pollinators, such as bees and hummingbirds, feed on nectar from a tubular flower, they are likely to get pollen on themselves. If the pollen falls off onto a stigma of a different flower, it could potentially produce a hybrid flower if the pollen tubes grow long enough to penetrate the ovary at the base of the flower (Mascarenhas, 1993). We now know that this is only possible if the pollen is from a long pistil flower and it falls onto a short flower. However, pollen from a short flower will not grow a pollen tube long enough to reach the base of a long pistil flower. If the pollen tube is successful in penetrating the ovary, this could produce new species of flowers by cross- pollination. In conclusion, the overall purpose of this report was to determine the relationship between pistil size and its average pollen tube lengths. We found that there is a very strong, positive correlation between the two variables, which confirms the initial hypothesis that longer pistils require longer pollen tube lengths in order for successful plant reproduction.

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