GERMINATION MODULE

Time to completion: 15 days
Difficulty level: Easy

GOAL

Test how variation in plant genotype influences the germination response to different environmental stimuli in Arabidopsis. By comparing the dependence of the germination rate of different natural variants on the light and cold treatment, you will gain an understanding of natural variation, adaptation, seed dormancy, seed germination and how to overcome seed dormancy and promote seed germination.

OBJECTIVES

- Plant seeds of 5 genotypes of Arabidopsis: 4 natural variants (ecotypes) and a laboratory strain used as control.
- Germinate seeds in water under light and darkness, and with or without stratification (cold period treatment).
- Observe germination and collect data.
- Analyze the differences in responses between different genotypes and discuss the collected data.

INTRODUCTION

Large genetic variation is present among the many natural variants (ecotypes) of *Arabidopsis thaliana* (Arabidopsis). These variants are adapted to particular environments and exhibit great genetic variation in light and temperature requirements for seed germination. This variation leads to large differences in seed dormancy behavior. Genetic variation for germination-related traits can be detected when genotypes are compared in identical conditions.

Germination is a process by which a seed begins to sprout and grow into a seedling under favorable growth conditions. Dormancy is a temporary inability of an intact, viable seed to germinate despite the presence of favorable growth conditions.

Many Arabidopsis natural variants undergo a dormancy phase, and generally will not germinate until this dormancy is broken. Seed dormancy in Arabidopsis can be broken by germination-promoting factors such as dry storage (after-ripening), light, low temperature (stratification), as well as by applying certain

chemicals such as gibberellins or nitrogen-containing compounds. The requirement for external germination-promoting factors can differ greatly depending on the genotype.

Stratification is a process of subjecting seeds to both a short-term cold (2°C - 5°C) and moist conditions to simulate natural winter that a seed commonly endures before germination in non-tropical climates. The required time to stratify Arabidopsis seeds depends on the genotype and temperature, but 3 to 7 days is usually enough.

Arabidopsis seed germination and dormancy are controlled by both environmental and genetic factors. Light and temperature are the key external factors in control of these processes. Plants must correctly perceive and respond to these stimuli to ensure that seedlings emerge and grow in the most favorable time for mature plant establishment. Photoreceptors called phytochromes are mainly responsible for sensing and responding to light during seed germination. A number of phytohormones act as internal regulators to determine whether a seed will germinate or remain dormant. Germination in Arabidopsis is stimulated by stratification, resulting in increased hormone biosynthesis and phytochrome action.

Seed dormancy plays a significant role in the adaptation of numerous plant species to their habitats. Plants have developed effective dormancy mechanisms to survive in disadvantageous environmental conditions, delaying the germination of mature seeds until favorable conditions return. Seed dormancy is crucial for grain production of some species grown in humid areas (corn, wheat, rice and canola) because it prevents seed germination before harvesting (vivipary), thus averting considerable grain damage and financial loss. However, long seed dormancy can be a problem in forestry and horticulture where germination of mature seeds may need to be induced with chemical treatments. Hence, the optimal level of seed dormancy for the specific growth environment is an important characteristic for any type of crop production.

MATERIALS

1. Arabidopsis seeds:

    - Col-0 (Columbia, CS 70000) is a laboratory strain selected by George Redei from the non-irradiated Laibach Landsberg population. Col-0 genome is completely sequenced and is used as a reference for comparison with the complete genome sequences of other natural variants. With a relatively low level of seed dormancy, which disappears approximately one month after seed ripening, it represents the control for this experiment.
    - Fei-0 (St. Maria d. Feiria, CS76412) is a natural variant collected in St. Maria d. Feiria, Portugal.
    - Kly-4 (Kolyvan, CS76384) is a natural variant collected in Kolyvan, Russia.
    - Lag2-2 (Lagodechi, CS76390) is a natural variant collected in Lagodechi, Republic of Georgia.
    - Xan-1 (Xanbulan, CS76387) is a natural variant collected in Xanbulan, Azerbaijan.

2. Filter Paper
3. Ultrafine point sharpie or pen

4. Ruler
5. Petri dishes, 9 cm diameter
6. Tap water
7. Parafilm
8. Wax paper
9. Wood toothpicks
10. Aluminum foil
11. Refrigerator
12. Bright fluorescent lights, preferably in a light rack
13. Thermometer
14. Magnifying glass or dissecting microscope

EXPERIMENTAL PROTOCOL

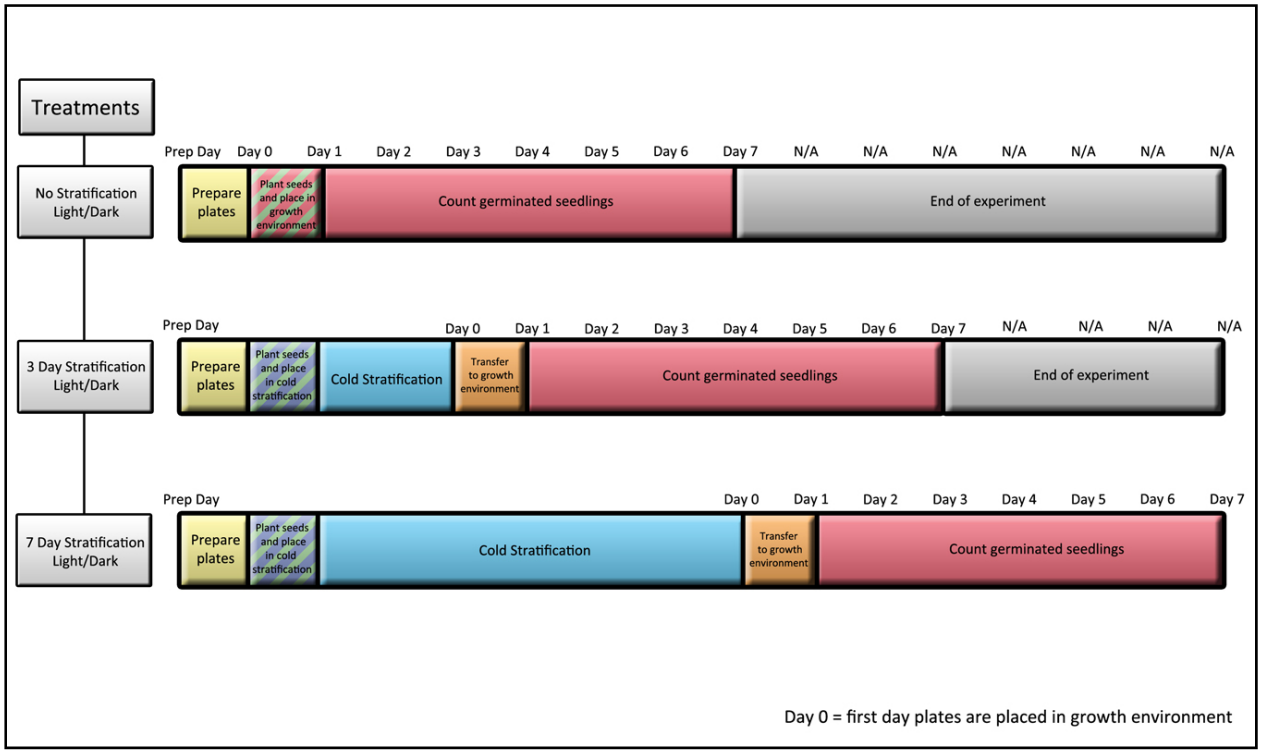
Treatments and Experimental Design

The objective of this experiment is to determine the effects of stratification and light treatments on the germination rates of 5 Arabidopsis genotypes. Seeds will be subjected to the following treatments: a) two stratification periods at 4°C, plus a no stratification control; b) two light treatments including no light (darkness) and continuous light with an intensity of 120-150 µmol/m^2 sec. There will be 6 treatments (3 stratification x 2 light treatments) in the experiment (Table 1). A class needs to be divided in 6 groups of 5-6 students. Each group should perform one treatment in triplicate. Every group will need three plates for the treatment they perform. 18 plates will be required for the whole class. Having replicates for each treatment will allow the students to perform a statistical analysis of the results. A class will receive at least 180 seeds of each genotype (10 seeds of each genotype should be planted on each plate).

Table 1. Treatments and Experimental Design

Factor	**Treatment**					
Stratification	None		3 Days		7 Days	
Light	Light	Dark	Light	Dark	Light	Dark
Genotype	Col-0	Col-0	Col-0	Col-0	Col-0	Col-0
	Fei-0	Fei-0	Fei-0	Fei-0	Fei-0	Fei-0
	Kly-4	Kly-4	Kly-4	Kly-4	Kly-4	Kly-4
	Lag2-2	Lag2-2	Lag2-2	Lag2-2	Lag2-2	Lag2-2
	Xan-1	Xan-1	Xan-1	Xan-1	Xan-1	Xan-1

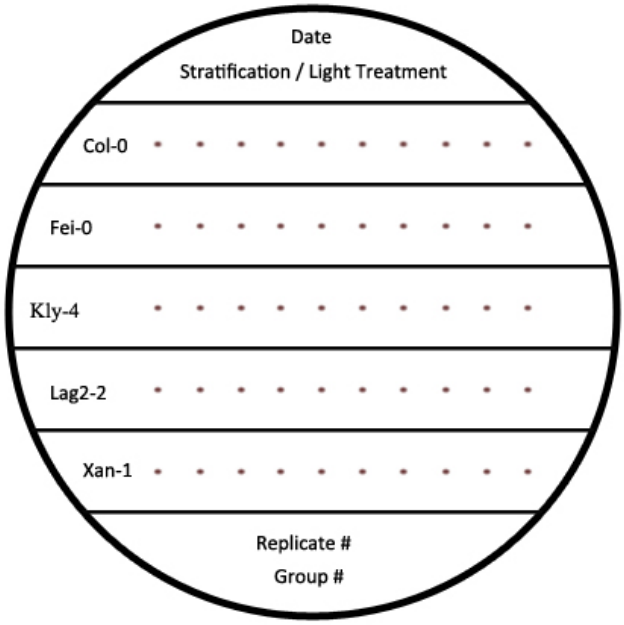
Figure 1. Sample Timeline



Preparation of Petri Dishes

- Cut round pieces of filter paper so that they fit perfectly inside each Petri dish. You will need 4 pieces of filter paper per dish.
- Label 3 x 6 filter papers using a ruler and pen. Draw six horizontal lines on a piece of filter paper to create spaces for planting the 5 types of seed (as shown in the Figure 1 below). Also, include the following information: treatment (3 day stratification, 7 day stratification, no stratification, light, and dark), replicate number, group number, and actual planting date.

Figure 2. Petri dish layout



- Place 3 blank pieces of filter paper into each dish. Push each piece flat against the bottom of the plate using your clean fingers to ensure a close fit. Then place the labeled piece of filter paper on top. Each Petri dish should have 4 layers of filter paper: three unlabeled pieces and the fourth piece with labeling on top. Four sheets help ensure sufficient moisture for germination.
- Add enough water to each dish to soak the paper without leaving any standing water. Soaking typically requires about 8 - 10 ml of water/dish, depending on the type of filter paper. Excess water will cause the different types of seeds to wash out of place and may cause the failure of the experiment or mold contamination. If you have excess water, tilt the plate and drain the water into a container or a trash can.

Seed Planting

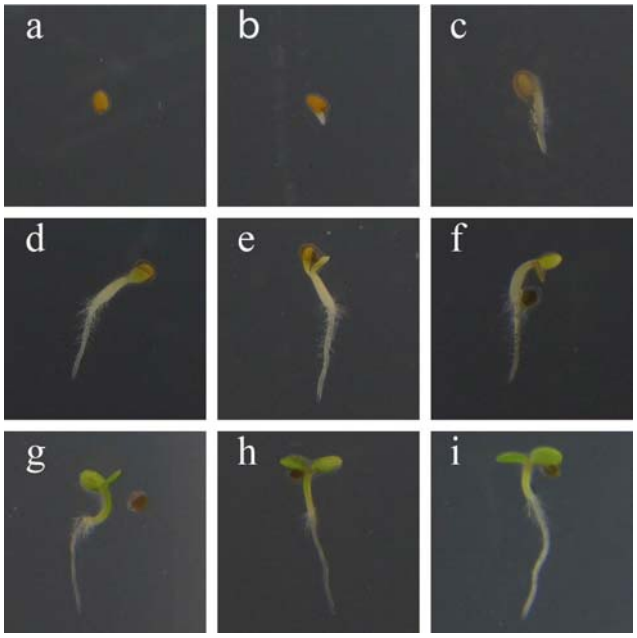
- Each group should carefully and slowly sprinkle about 30 seeds of one genotype on a small piece of wax paper (10 x 10 cm). Soak a toothpick by immersing the tip in water and use the moistened tip to pick up one seed at a time from the wax paper and place it on the corresponding space on the plate (Figure 2); continue until you have placed 10 seeds in the appropriate space. Repeat this procedure for each dish in three replicates until all the genotypes are planted.
- During and after planting the plates should be handled very carefully; always try to hold the plates horizontally in order to avoid mixing seeds of different genotypes.

- Cover each dish and seal with 2 layers of parafilm to prevent desiccation. Seeds that dry out will not germinate. Handle your dishes according to the treatments:
    1. No stratification – light: place plates under the fluorescent lights.
    2. No stratification – dark: wrap plates with aluminum foil and place them in the growth area.
    3. 3 day stratification- light: wrap plates with aluminum foil and place them in a refrigerator for 3 days, then take off the aluminum foil and put them under the fluorescent lights.
    4. 3 day stratification- dark: wrap plates with aluminum foil and place them in a refrigerator for 3 days, then place them in the growth area but do **not** take off the aluminum foil.
    5. 7 day stratification- light: wrap plates with aluminum foil and place them in a refrigerator for 7 days, then take off the aluminum foil and put them under the fluorescent lights.
    6. 7 day stratification- dark: wrap plates with aluminum foil and place them in a refrigerator for 7 days, then place them in the growth area but do **not** take off the aluminum foil.
- A temperature of 22-23°C is a suitable growth condition under the continuous fluorescent light.

OBSERVATIONS AND DATA COLLECTION

- Record the date you transfer the dishes to the growth environment for each treatment. This date should be counted as Day 0. See Figure 1 for a sample timeline. Observe the seedlings grown in the light on a daily basis for 7 consecutive days.
- For the dishes containing dark-grown seedlings, remove the aluminum foil only on the last day of counting (not earlier than 7 days after being moved to the growth environment or removed from the refrigerator in case of treatments that include stratification).
- Count the number of the light-grown germinated seeds (per genotype and per treatment) daily, using a magnifying glass or a microscope. If condensation on the cover makes it too difficult to see the seeds, gently remove the cover and shake out the water droplets. Be sure to reseal the plate securely.
- The process of germination starts with the uptake of water by the seed and its completion is marked by the appearance of the radicle through the seed coat.
- The initial germination stages of the small Arabidopsis seed cannot be observed with the naked eye. As time passes and with the help of a magnifying device, you will observe an imbibed seed with an intact seed coat (Figure 3**a**), then the radicle (root tip) becomes visible through a broken seed coat (Figure 3**b**). In later germination stages you will notice the development of root hairs (Figure 3**c**), the hypocotyl (stem of a germinating seedling; Figure 3**d**), and the green cotyledons (seed leaves; Figure 3**e**-**i**) of the new seedling.
- Count a seed as germinated when you observe a broken seed coat and the radicle that has emerged out from the seed (Figure 2**b**).

Figure 3: Arabidopsis stages of seed germination and seedling development



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- Prepare an excel data sheet or use the pre-made excel sheet to record the daily germination events for each of the replicates of the 6 following treatments:
  1) No stratification - light
  2) No stratification - dark
  3) 3 day stratification - light
  4) 3 day stratification - dark
  5) 7 day stratification - light
  6) 7 day stratification - dark

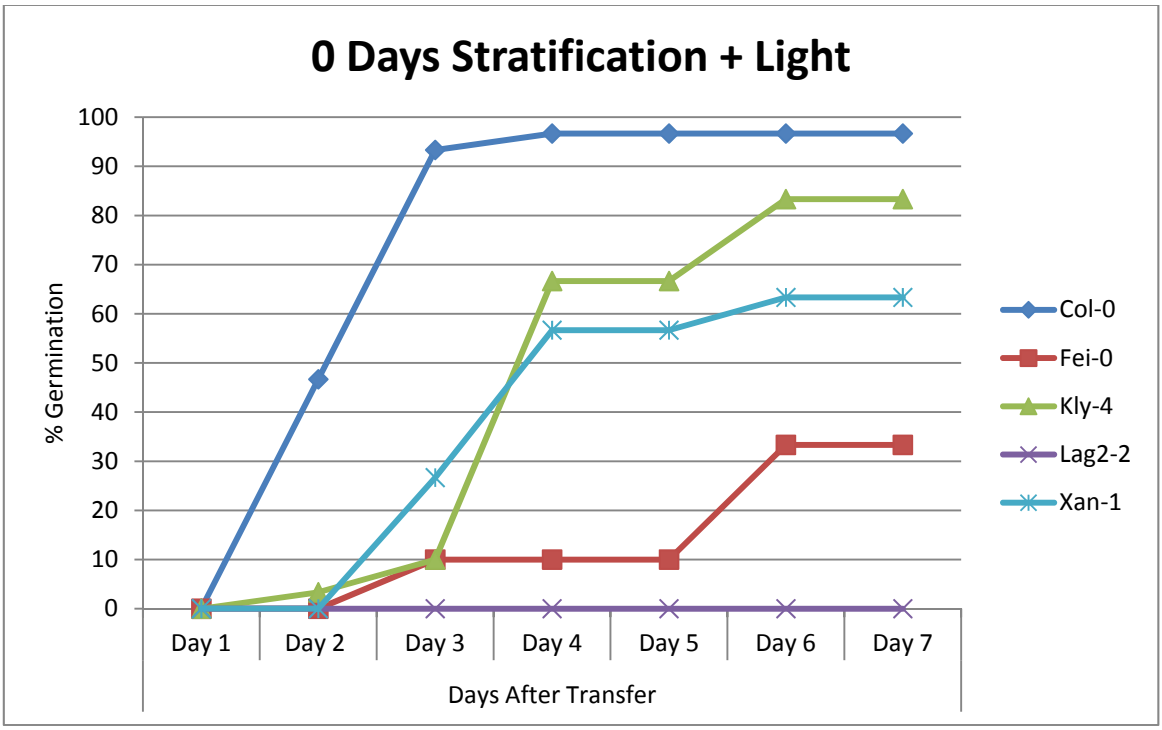
DATA ANALYSIS AND DISCUSSION

- There are various ways to do the analysis of this experiment: a) compare the effects of each treatment on the germination rate of different genotypes (Example 1), b) compare the performance of a single genotype under different conditions (Example 2), c) compare the final rates for all genotypes under each treatment (Example 3), d) compare treatments in general, e.g. stratification vs. no stratification in the light or in the dark for each genotype.
- Within each treatment and for each genotype, calculate the daily percent germination for each replicate. Then calculate the average and standard deviation of the 3 replicates or use a Germination Module Data Analysis Spreadsheet (http://abrcoutreach.osu.edu/greening-classroom) for data entry and calculations. Use the calculated means and standard deviation to generate a scatter plot to compare the germination rate among the 5 genotypes within each treatment. If the Germination Module Data Analysis Spreadsheet is used, the graphs will be

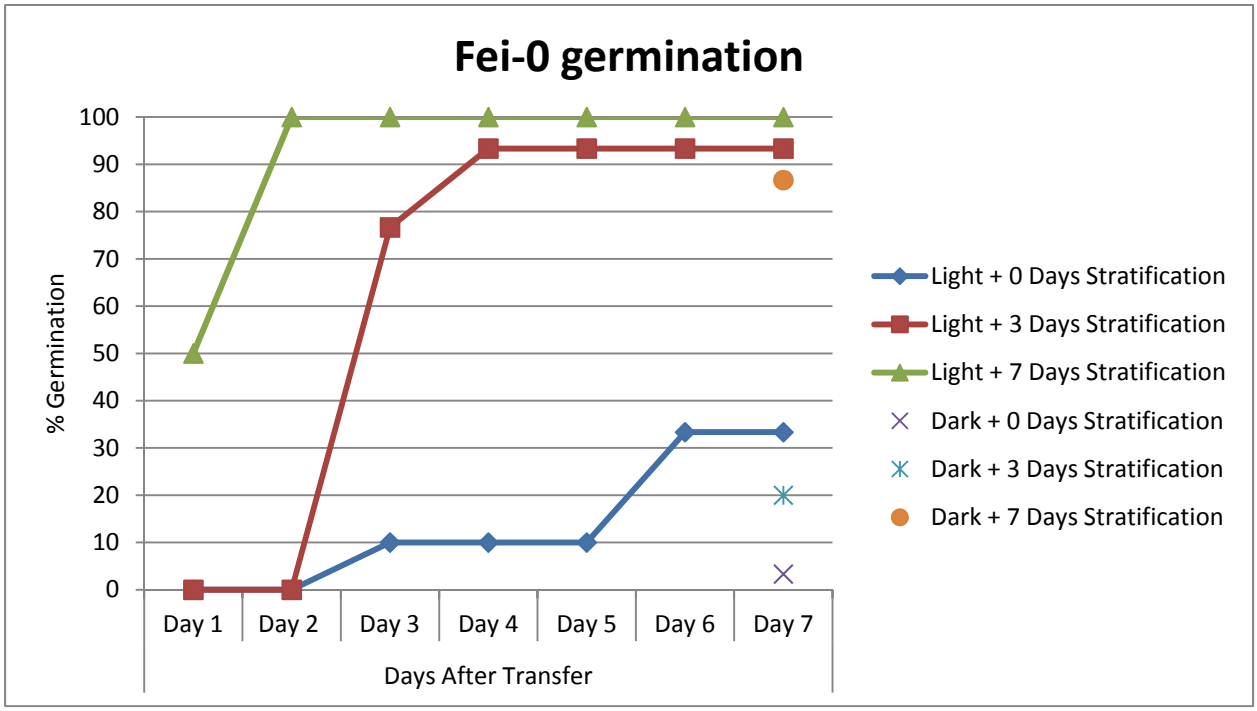
generated automatically. In the plot, the Y axis will represent the germination percentage and the X axis will represent the days of evaluation after the plates are transferred to the growth environment (Example 1). Make this plot for each of the 6 treatments.

- Use the means and standard deviation calculated above to create another scatter plot to compare the effect of the 6 treatments on germination rate of each genotype. In the plot, the Y axis will represent the germination percentage and the X axis will represent the days of evaluation (Example 2). Make a plot for each of the 5 genotypes.
- Compare the final germination rates of different genotypes under different conditions (Example 3). Do different accessions have different requirements for light and stratification to be able to germinate?

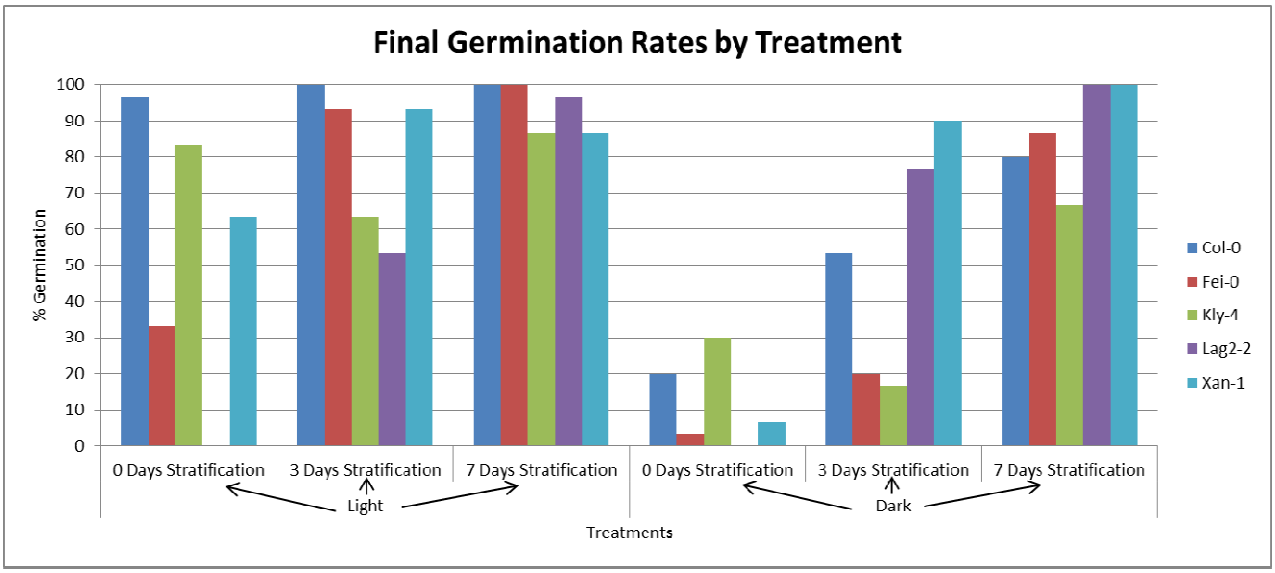
Example 1: Germination: No Stratification - Light



Example 2: Fei-0 germination



Example 3: Final germination rates



- Discuss different requirements for stratification by comparing final rates. Do certain treatments increase the speed of germination? Why is it important for crop seeds to germinate quickly and reach maximum germination in a narrow timeframe?
- Are some genotypes affected more than others by the extended cold treatments? Which genotypes have more dormancy? How many days of stratification are sufficient to break the dormancy and attain near 100% germination for each genotype?
- Were there dark-germinating genotypes?
- Examine and discuss the effects of the possible interactions between genotype, stratification and light on seed germination.
- Based on what you observed, predict which genotypes will be more likely to survive under winter conditions. Why is seed dormancy an important economic trait for agricultural production?
- Discuss your results with the members of your group and then with other groups.