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Plant Hormone Signaling Lab Module

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Plant Hormone Signaling Lab Module
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Introduction

The purpose of this project was to create a new lab module for the Principles of Biology II. The lab module would allow students to learn about and experiment with plant hormones in a hands-on environment. Cell communication through hormone signaling is a basic principle in biology and this lab will complement what students are learning in lectures. The modular, repetitive growth patterns of plants make them ideal for studying the effects of hormones on growth dynamics. The project focused on a plant hormone that affects plant growth in a way that can be observed and measured in 10 days' time. For this reason, students would need to be given some time during two consecutive labs to finish the guide and the experiment. The final goal for the laboratory module is to be added to the current laboratory manual.

Arabidopsis thaliana, a genetic plant model species, was used to explore the effect of brassinosteroid hormone signaling, which regulates root growth by inducing cell elongation and division. *Arabidopsis thaliana* has been chosen as the plant to use in the experiments because it has been used as a genetic model species for studying plants for over 70 years (Meinke et al., 1998). As a result, many hormone signaling pathways have been characterized. There are publically available libraries of *Arabidopsis thaliana* mutants and genetic constructs. The last reason *Arabidopsis thaliana* is a great plant to experiment on is that it is budget friendly. A vial of 60 mutant seeds costs around \$10 for academic use, they grow either in soil or on plates, and they do not require any special media or growth chambers (Meinke et al., 1998; TAIR).

Material and methods

Arabidopsis seeds were grown on soil for bulk seed collection. Throughout the project, there were changes made to the seed growing process in order to find the best methods. There were many things modified, including how the seeds were sown, where they were grown, when fertilizer was added, and the kind and amount of light available to the plants. Once seeds were available, preliminary growth assays were done to help determine how students will complete the lab module. The growth assays took place on petri plates containing agar, which consisted of Murashige and Skoog (MS), 2-(N-Morpholino) ethanesulfonic acid (MES), distilled water, and agar.

The lab guide was written while growth assays were being evaluated and included an informational section that students can use to answer questions and create their own hypotheses to test. The goal was to allow students to have some freedom to think of a hypothesis they would actually want to test, instead of having a module and experiment already determined.

There was more than one mutant plant line included in the project so students could have the freedom with the module as mentioned earlier. The brassinosteroid mutant lines that they will choose from include *det2-1*, *bes1-D*, and *bri1_188B5*. These mutant lines were chosen because their roots show different growth dynamics compared to the control wild type lines.

The *det2-1* mutant line was chosen because it is smaller, with darker green leaves and shorter roots than the wildtype plants (TAIR). The roots do not grow as well because *det2-1* is missing a functional enzyme, 5 alpha-reductase, which converts campesterol to campestanol within the brassinosteroid biosynthesis pathway (Noguchi et al., 1999). Thus, although the plants cannot synthesize brassinosteroids, they can still respond to the hormones. If synthetic brassinosteroid hormone is added to the growth media, then the mutant plant should grow like wild type plants. The exact mutation is not known, but the gene is found on chromosome 2 at locus AT2G38050. The *det2-1* mutant is inherited in a recessive manner and the plant lines available to the students are homozygous recessive.

bri1_188B5 is a line that has extremely short roots compared to the wild type *Arabidopsis thaliana* (TAIR). *bri1_188B5* has a G to A nucleotide substitution mutation found on chromosome 4 at locus AT4G39400. This mutant is insensitive to brassinosteroids, meaning it can synthesize the hormone, but it cannot respond to it (Nam & Li, 2002). The insensitivity is caused by a defect in the signaling transduction response pathway. Briefly, when BRI1 binds brassinosteroid it forms a heterodimer with another kinase, BAK1 (Zhu & Wang, 2013). The BRI1-BAK1 heterodimer, inhibits a third kinase, BIN2. Upon inhibition of the BIN2 kinase, the nuclear protein BZR1 and its homolog BZR2/BES1 are dephosphorylated and accumulate in the nucleus. The accumulation of these two proteins activates the BR-induced genes in the nucleus. In the *bri1_188B5* mutant, the BRI1 kinase does not function properly, thus the BIN2 kinase cannot be deactivated and BR-induced genes are not transcribed, even in the presence of brassinosteroids.

The *bes1-D* line has longer roots than wildtype plants and curly leaf petioles (TAIR). This mutant line's exact mutation is not known, but the gene affected is on chromosome 1 at locus AT1G19350. This constitutive signaling mutant causes the plant to always exhibit a brassinosteroid response, even if no hormone is present (Yin et al., 2002). The line is able to do this by suppressing/deactivating the BIN2 kinase mentioned earlier. This in-turn causes brassinosteroid response genes to be expressed, even while brassinosteroid is not present (Zhu & Wang, 2013). *bes1-D* is an incomplete dominant allele.

Results

Due to time restraints, there was not the possibility to run the lab with any students. The biggest challenge was getting seeds for students to use in their experimentation. It took multiple trials and errors to find the best conditions for growing *Arabidopsis thaliana* and the nature of some of the mutants caused them to grow at a very slow rate. The mutant's different rates of growth can be seen in Fig 1 and Fig 2 below. A laboratory guide was still written and is in Appendix A for reference. This guide can be used once enough seeds are collected and more growth assays are completed.

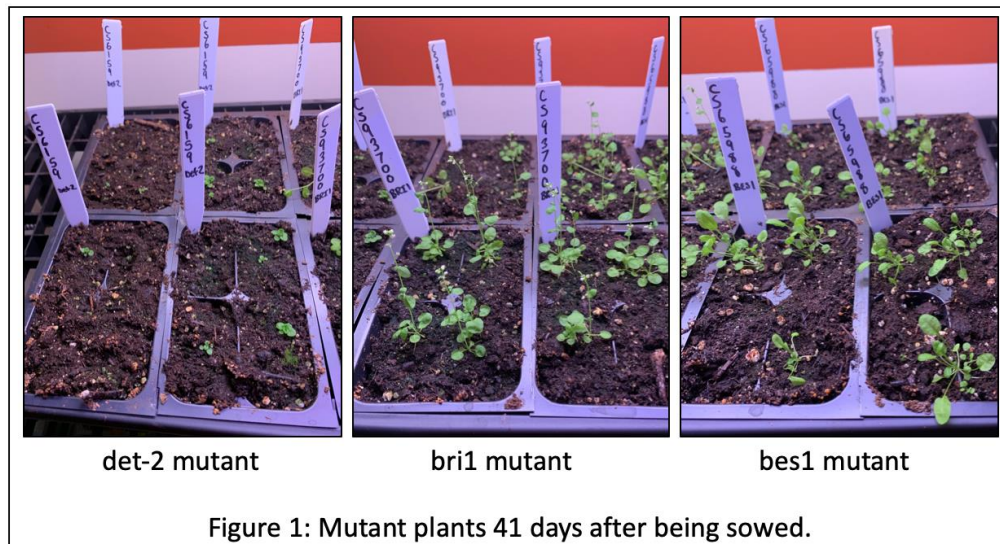
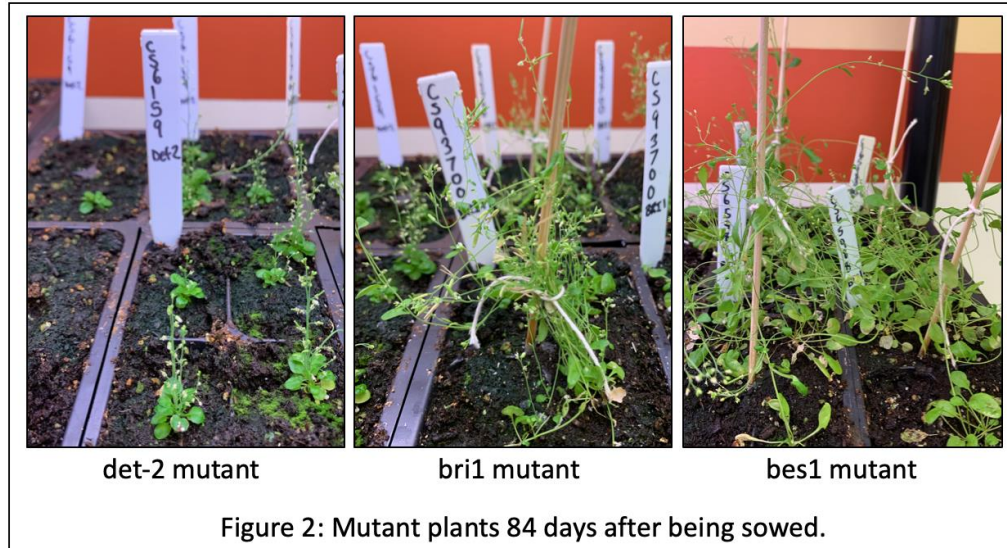
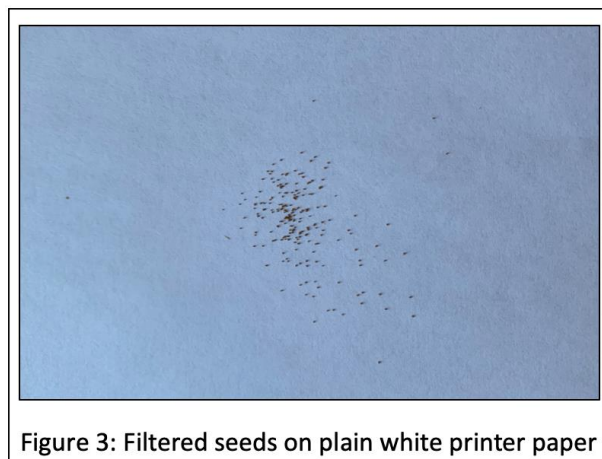


Figure 1: Mutant plants 41 days after being sowed.



The guide was made to fit into the manual that students already use, which was written by Ashley Ramer in 2017. For that reason, Appendixes were referenced in the guide that were not actually included in the guide. The proposal worksheet was also taken from the manual to keep consistent with what students are used to doing (Ramer, 2017). Please see Appendix A to see exactly what information students would be given and expected to complete during the two parts of the experiment.



Seeds were collected by first drying the plant out and then picking off dried seed pods. This had to be done carefully, as to not accidentally miss nor drop seeds. Once pods were collected, they would be lightly ground by hand over a piece of paper to release the seeds contained inside. The paper with the seeds on it would then be slightly tilted and tapped to let the seeds roll onto a different piece of paper below. This step filtered the seeds and separated them from other plant material that was not wanted by using static electricity. The seeds roll down onto the paper below before the dead plant material. Fig. 3 above shows seeds after they were sorted from the dead plant material. Seeds were then put into microcentrifuge tube to be held until they are ready to be used. To date, I have collected about 0.2 mLs of seeds from the *bes1* mutant line and approximately 0.05 mLs of seeds from the *bri1* mutants. I am currently

still growing the *det-2* line, but due to their mutation they do not grow nearly as fast as the other mutant plants. I have also collected about 0.02 mLs of wildtype seeds, but have used about a third of them on running experimental Petri plates.

Discussion

One of the biggest parts of this project was to grow plants in order to collect seeds that students could then use for their experimentation. During the summer, seeds were attempted to be grown in the green house on The University of Akron's campus. After a few rounds of watering for a couple of weeks and patiently waiting for sprouts with no success, it was determined that the soil was getting too dry. Seeds were watered daily and fertilizer amounts were changed, but there were never any successful sprouts in the green house.

Flats were then taken into an air-conditioned building and placed underneath a grow light. This method produced sprouts, but the plants were spindly and so an additional light was added. The plants then started to grow a bit faster and healthier, but they were still small. Due to this, we varied the amount and timing of fertilizer addition. Plants that were already growing had the recommended amount of fertilizer supplemented to their water one time. This method seemed to help the plants grow better. Including a slow-release fertilizer in the soil before seeds were sowed resulted in slow growth and low germination rates.

Throughout this processes, it was also discovered that plants grew the best when their seeds were added directly to the soil, as opposed to having them germinate on a Petri plate and then being transplanted to the soil. Some of the plant growth experimentations were done at the same time, but it would still take weeks to complete a test. If the plants were growing, it would take a few of months before they produced pods to be harvested.

Since students are to grow seeds on Petri plates for their experiment, I started trying different experiments with wildtype seeds I grew. From the couple of plates that I was able to test run, I have narrowed down some of the conditions the plated seeds need. The seeds germinated and grew the same under a grow light as they did in regular lighting, both staying on 24 hours. Figures 4 and 5 compares how plated seeds grew under a growth light as compared to ones that grew in regular lighting. I also believe that the plates may need to be poured thicker than I originally made them. I think this because the plates had a tendency to become dry at the edges during the second week the seeds were growing on them. Pouring thicker plates is extra experimentation that can be done once more seeds have been collected.

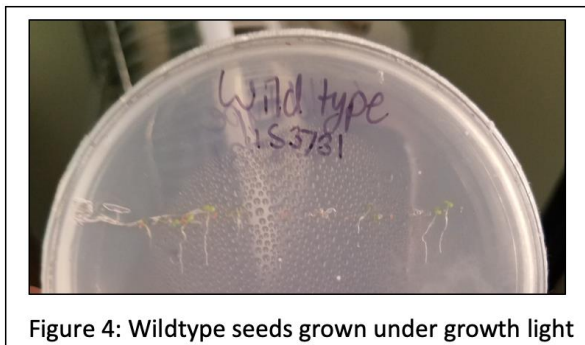


Figure 4: Wildtype seeds grown under growth light



Figure 5: Wildtype seeds grown under regular light

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Plant Hormone Signaling

Lab Outline

Part One

- Take the Prelab quiz over pages 3-6
- Design experiment to test effect of hormone signaling pathways
- Set up Petri plates with seeds on them

What will be turned in after part one

- Experimental proposal

Part Two

- Analyze results from Petri plates and write a summary report
- Research hormone signaling pathways

What will be turned in after part two

- Experiment summary

Points

Prior to Part One

- Prelab Quiz 20 points

Part One

- Proposal worksheet 10 points

Part Two

- Experiment summary 10 points

Plant Hormone Signaling

Background

Hormones

Hormones are signaling molecules produced by multicellular organisms to regulate cell and or tissue functions. Each hormone can be sensed by different parts of the organism and signal for different functions. *Arabidopsis thaliana* is a genetic plant model species, meaning that it has been studied extensively to understand biological phenomena. One of the hormones used by *Arabidopsis thaliana* is **brassinosteroid**. **Brassinosteroids** regulate root growth in plants by inducing cell elongation and division. This factor is important in the length and architecture of the roots, which varies based on a plant's age and environment. Hormone functions are vital to both plant's and animal's survival and act through similar cellular mechanisms.

Brassinosteroid signaling response pathway and biosynthesis pathway

The hormone signaling pathway of brassinosteroids is quite complex. The image below will help you visualize how this hormone works. When Brassinosteroids are present, the BRI1 and BAK1 kinases form a heterodimer. This heterodimer starts a signal transduction pathway that eventually brings the BIN2 protein out of the nucleus and destroys it. Once the BIN2 is inactivated, the BES1 and BZR1 transcription factors are dephosphorylated (have phosphate groups removed) and can move into the nucleus, where they will “turn on” or “induce” BR-inducible genes by attaching their promoters (part of the gene that dictates whether it will be transcribed).

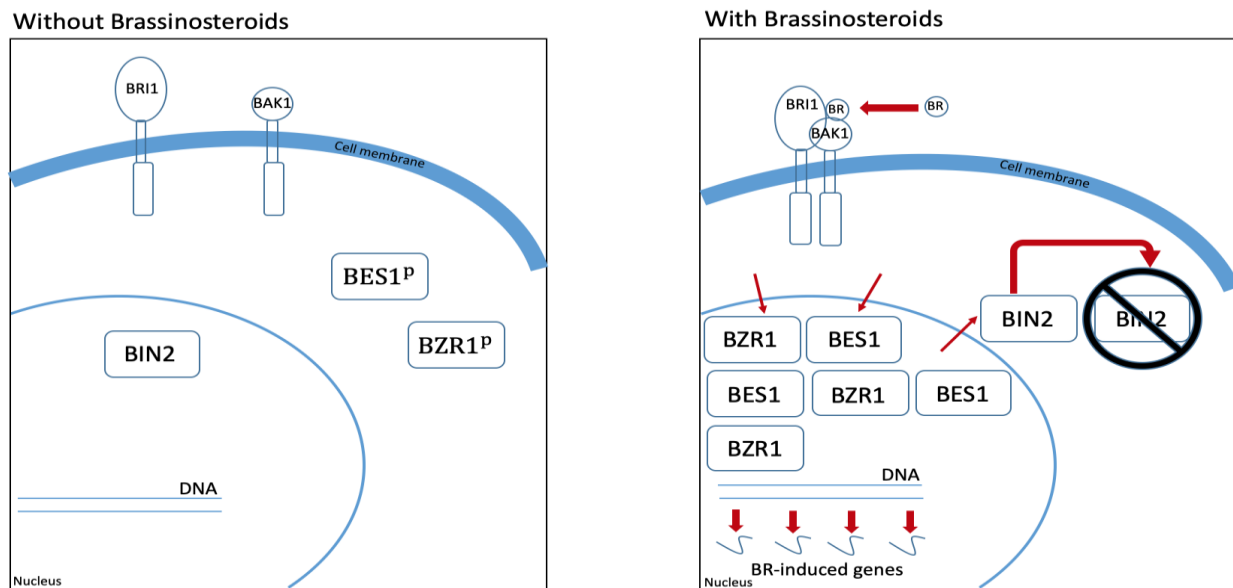


Figure 1: The brassinosteroid signaling pathway

The pathway above is used in cells to *respond* to brassinosteroids. There is a completely independent pathway for synthesizing brassinosteroid hormones. The biosynthesis pathway for brassinosteroids involves many steps. One important step in the biosynthesis pathway is when campesterol is converted to campestanol. This is catalyzed by the 5 alpha-reductase enzyme (5 α -R), which is encoded by the gene DET2 (figure 4).

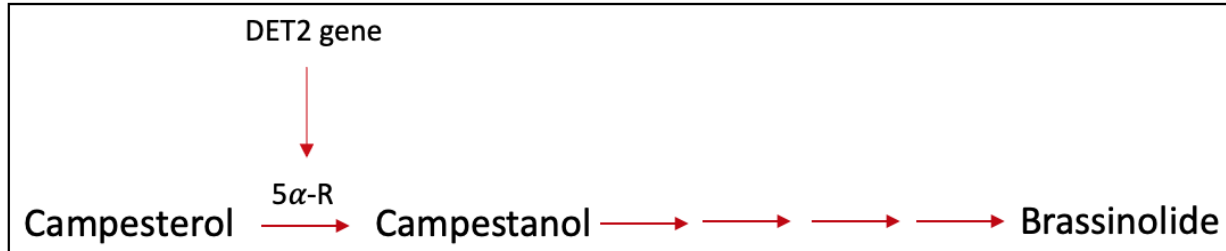


Figure 2: The Brassinolide biosynthesis pathway

Arabidopsis thaliana mutant seed lines

During this laboratory experiment, students will have access to three brassinosteroid mutant lines of *Arabidopsis thaliana* along with a wildtype line. The three mutants are *det2-1*, *bri1_188B5*, and *bes1-D*. *det2-1* has extremely short roots compared to the wild type because it is missing the functional enzyme 5 alpha-reductase (fig 2). This means that the *det2-1* mutant cannot synthesis brassinosteroids, but is still sensitive to them and responds to the hormone when it is present.

The next mutant that is available in this lab is the *bri1_188B5* (*bri1*) line. Compared to the wild type, this mutant has shorter roots, but they are longer than the *det2-1*'s. This is due to the fact that *bri1* is insensitive to brassinosteroids, meaning it can make the hormone, but cannot respond to it. The insensitivity is due to the BRI1 kinase (an enzyme that adds phosphates on to proteins) not functioning properly, which in turns means the BIN2 kinase cannot be deactivated, even in the presence of brassinosteroids.

The final seed type that could be used is the *bes1-D* mutant line. This line has longer roots than the wildtype plant. The mutant has longer roots because the plant is a constitutive signaling mutant. This means that the plant is always exhibiting the brassinosteroid response, even if none of the hormone is present. *bes1-D* does this by suppressing/deactivating the BIN2 kinase constantly.

Mutant	Root phenotype compared to wildtype
det2-1	Smaller roots
bri1_188B5	Smaller roots (longer than the det2-1 mutant)
bes1-D	Longer roots

Figure 3: Mutants' root lengths compared to the wildtype's root length

Objective for today:**Learn about the effects of mutations on plant growth and form an experiment**

Due to the nature of brassinosteroids, how will mutations associated with it affect the root growth of *Arabidopsis thaliana*? How would a homozygous recessive mutant have altered root growth? What about an insensitive mutant that cannot process brassinosteroids? Or an incomplete dominant allele? What might happen if brassinosteroid hormone were placed onto the plates with the seeds? How might different mutants compare to one another or to the wildtype?

To examine and test your hypothesis further, your group will be provided with 2 Murashige and Skoog (MS) plates and a variety of seeds with different genotypes. Seeds will be added to both plates in a straight line in order to analyze root growth in the future. Only one type of seed will be added to each plate, to ensure none of the genotypes get confused. However, each group could have two different types of seeds by having a different line on each of the two plates. Different additives can be placed onto the plates as well.

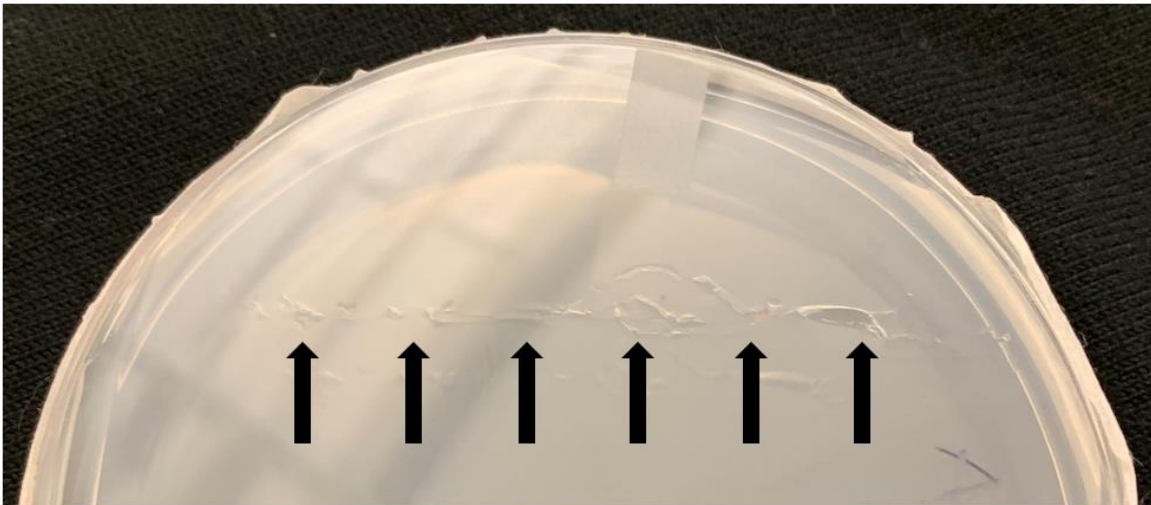


Figure 4: MS Petri plate with wild type seeds adhered

In figure 1, the seeds are not visible due to their small size, however, it shows where the seeds should be placed onto the MS Petri plate. Only wild type seeds were used in figure 4 and 5 and there were no additives on the MS petri plate. Once the seeds have adhered to the plates students will put them in a refrigerator so all of the seeds germinate at the same time. After three days the seeds will be moved to a growth chamber for 10 days. Figure 5 shows the seeds 10 days after they were added to plates and demonstrates how they germinated and grew. Two weeks after part one, students will measure root lengths on their two MS Petri plates and then analyze and compare the results.

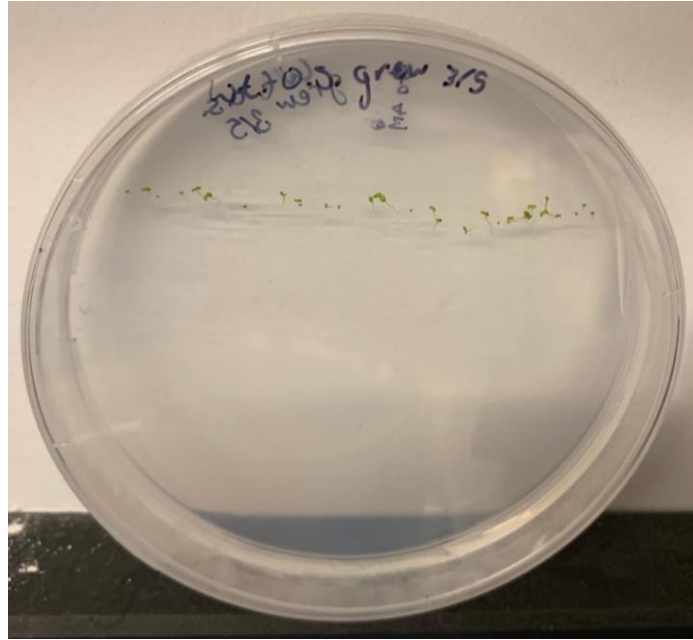


Figure 5: MS Petri plate with germinated wild type seeds 10 days after adhering.

In order to complete the proposal worksheet, students and their group members need to decide which seed strains they plan on using and if they want to add brassinosteroid hormone onto their Petri plates. The Petri plates will be poured ahead of time so the students will not be completing that step. If elected to add the hormone, it will be placed onto the plates and spread out with glass beads before seeds are added to the plates. The seed lines available are three mutants (*det2-1*, *bri1_188B5*, and *bes1-D*), and a wild type. One example of an additive available is the brassinosteroid hormone.

Example hypotheses

The following are examples of good hypotheses. Groups may use one of the listed hypotheses or create their own.

1. Due to the insensitivity of *bri1* to the brassinosteroid hormone, *bri1*'s roots will be shorter than the roots of an *Arabidopsis thaliana* wild type plant.
2. Adding brassinosteroid hormone to a Petri plate prior to adding *det2-1* mutants will cause the plant's roots to grow longer.
3. Since *bes1-D* is constantly suppressing the BIN2 kinase, its roots will be longer than the roots of an *Arabidopsis thaliana* wild type plant.

Plant Hormone Signaling – Proposal Worksheet

Names: _____

Refer to the rubric in **Appendix A** and the example proposal in **Appendix B**.**Introduction:**

____/0.5 1. Brainstorm what factors might influence (the inquiry challenge topic).
Conduct a literature search to see what is already known about the research topic.
a) List three relevant search strings that include the Boolean operators AND or OR.
i.
ii.
iii.

____/1 2. Locate one relevant primary literature article.
a) How would you reference this article as a full citation?

b) How would you cite it in text?

____/1 3. What is your specific research question?

____/1 4. Why is this a good question?

____/0.5 5. What are the variables you will include in your study?
a) Independent variable:
Is this variable qualitative or quantitative:
b) Dependent variable:
Is this variable qualitative or quantitative:

____/1 6. What is your hypothesis?

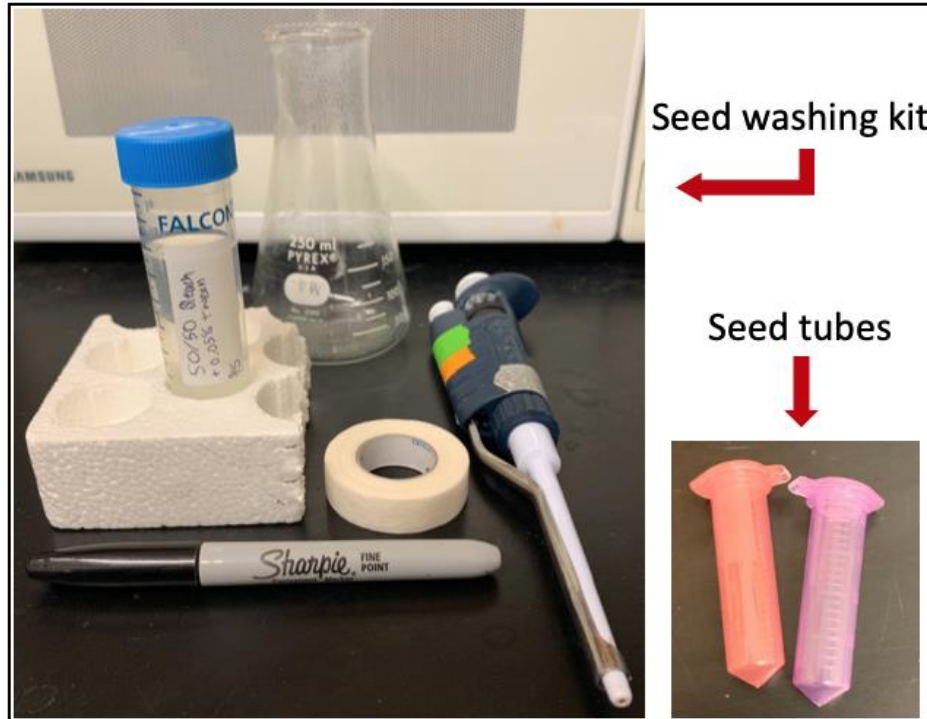
Methods:

____/3.5 1. Describe your groups, sample sizes(s), experimental methods, and control variables.

____/1.5 2. Discuss the statistical test you'll use, why it's appropriate, and how you'll interpret the results.
a) Statistical test
b) It's an appropriate test because
c) How to interpret results:

Steps to perform part one: setting up the Petri plates

1. Once your group has completed the proposal worksheet obtain from your TA: two tubes of seeds, two Petri plates, a seed washing kit (which includes Micropore tape, bleach/tween cleaning solution, 150 μ L pipet, an empty flask, and a marker), a small beaker (~100mL) of distilled water, and brassinosteroid hormone (based on your hypothesis). Seeds are separated into groups of ~10-15, which is the amount needed per plate. You will need to pick up two tubes of seeds total, even if you are experimenting on only one seed type.



2. Start by labeling the bottom of the petri dish (the side with media on it) with the seed type chosen, information if you added the hormone, and your name.
3. If you have chosen to put brassinosteroid onto your Petri plate, you will do that now. Add the recommended amount onto the plate and use ~5 sterile glass beads to spread the additive around evenly onto the medium. You will then need to pour the used glass beads into a container provided by your TA. Place the lid back onto the Petri plate and set aside.
4. **Steps 4-6 will be done for both tubes of seeds at the same time, be sure to keep the seed sets separated.** You will now start washing the seeds. To do this, you will first take 160 μ L (set the 150 μ L pipet to 80 μ L and pipet twice) of the bleach + Tween solution and add it to the microcentrifuge tube the seeds are originally in. You will need to tightly close the lid on the tube flick the bottom of the tube in order to mix the solution and seeds. Let the solution and seeds sit for 20 minutes.
5. The seeds will now need to be rinsed. To do this, you will need to pipe out the solution currently in the microcentrifuge tube, but **be careful not to suck up the seeds!** The solution can be discarded into the flask. Then you will add 160 μ L of distilled water to

the seed tube (with the same technique as before). Flick the bottom of the tube to mix the seeds and water. Then pipe out the water, which again can be discarded into the flask.

6. Add another 160 μ L of distilled water to the seed tube, flick the bottom of the tube to mix, remove and dispose of used water. Repeat this five more times, thoroughly rinsing the seeds with the distilled water.
7. After the last wash, add 80 μ L of distilled water into the microcentrifuge tube.
8. You will now need your labeled Petri plates to add the seeds to them. Take off the lid to the first plate. (only take off the lid to one Petri plate at a time, so there is a lower chance of contamination).
9. You will need to pick up the seeds of one tube using the pipet and place them onto the Petri plate in a straight line. Try to even out the seeds along the line made as much as possible. Pipe up and discard any extra water on the plate so that only the seeds in a line remain.



10. Place the lid back onto the Petri plate and adhere the lid and base together with Micropore tape.
11. Repeat steps 8-10 for the second tube of seeds that you have.
12. Give your labeled Petri plates with seeds adhered to them to your TA.
13. The TA will put the plates into a refrigerator for 3 days, and then they will be transported to a growth chamber for 10 days.

Steps to perform part two: analyzing germinated seeds' root length

1. Record the number of seeds that germinated and the number that did not per plate.
2. Measure the root length on all of the germinated seeds in millimeters (mm) and record your findings. If your plates contain fungus, be careful when opening the lids as to not spread the fungal spores.
3. Once you are finished, put the lids back on the Petri plates and place them on the table in the front of the room.
4. Graph your measurements and analyze the results from your experiment.
5. Create a typed summary using Appendix C: Summary Rubric found at the back of the laboratory manual book.
6. Students can use the following primary literatures and reviews while writing their summaries.

Clouse, S.D., Langford, M., and McMorris, T.C. 2002. A Brassinosteroid-insensitive mutant in *Arabidopsis thaliana* exhibits multiple defects in growth and development'. *Plant Physiology*, 111(3): 671-678.

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Plate 1	Number of germinated seeds	Number of non-germinated seeds	Total seeds
Plate 2	Number of germinated seeds	Number of non-germinated seeds	Total seeds

Plate 1 root lengths	Plate 2 root lengths	Plate 1 root lengths	Plate 2 root lengths
1.	1.	9.	9.
2.	2.	10.	10.
3.	3.	11.	11.
4.	4.	12.	12.
5.	5.	13.	13.
6.	6.	14.	14.
7.	7.	15.	15.
8.	8.	16.	16.