Laboratory: Clover Cyanogenesis

Teachers Manual

Overview:

This lab consists of one investigation that can be divided into several sections. Through the use of a model organism, white clover (*Trifolium repens);* students will qualitatively measure the production of cyanide as a result of tissue damage in different clover specimens. The lab provides connections between classic Mendelian genetics, population genetics, and natural selection. Students will observe clear evidence of gene interactions resulting in different observable phenotypes.

Objectives:

- Qualitatively measure the amount of cyanide produced by different clover specimens
- Relate the inheritance of two unlinked genes underlying phenotypes
- Demonstrate complementary gene action in metabolism
- Calculate allele and genotype frequencies for populations using Hardy-Weinberg equations
- Relate results of lab to the broader concepts of co-evolution and natural selection

Content Standards (9-12):

Unifying Concepts and Processes

- Evidence, models, and explanation
- Change, constancy, and measurement
- Evolution and equilibrium
- Form and function

Science as inquiry

- Abilities necessary to do scientific inquiry
- Understanding about scientific inquiry

Life Science

- The cell
- Molecular basis of heredity
- Biological evolution
- Organisms and environments
- Populations and ecosystems
- Interdependence of organisms
- Diversity and adaptations of organisms

Science in Personal and Social Perspectives Standards

- Science and technology in local, national, and global challenges

Time Requirements:

- Activity A: Clover Adaptation Pre-Lab - 30 min.
- Activity B: Cyanogenesis Assay - 100 min.
- Activity C: Gathering Class Data - 15 min.
- Activity D: Population Genetics Hardy-Weinberg 30 min.
- Optional Activities: Clover Hunt, Herbivory Lab, Creating a Dichotomous Key

Background for teachers:

White clover (*Trifolium repens*) can be used to study the genetic basis of plant diversity, as well as the concepts of coevolution and natural selection. White clover shows natural variation for cyanogenesis, which is the release of hydrogen cyanide (HCN) as a result of tissue damage. In natural populations, both cyanogenic and acyanogenic plants can be found. The cellular and biochemical basis of clover cyanogenesis has been known for nearly a century, and clover's cyanogenesis polymorphism has long been used as a model system for studying the maintenance of genetic variability in natural populations (reviewed by Hughes 1991, Olsen et al. 2007).

The production of poison is a common defense mechanism in plants, and cyanogenesis can be found in diverse plant species, including apples, almonds, lima beans, and flaxseed. In white clover, ecological studies have demonstrated that cyanogenesis is selectively favored as a defense against herbivores; cyanogenic plants are avoided by small herbivores, including slugs, snails, and voles. However, surveys of clover populations around the world have also revealed that the proportion of cyanogenic plants declines in colder climates. Most plants at higher elevations and latitudes are acyanogenic, while those in warmer climates are mostly cyanogenic. Two hypotheses have been proposed to explain why acyanogenic plants are more common in colder climates. First, since frost can result in cell rupture, it is possible that cyanogenic plants suffer from autotoxicity in areas of frequent freezing, caused by cyanide release within the plant tissue. Alternatively, there may be fewer clover herbivores in colder climates; since the production of the compounds required for cyanogenesis is energetically costly (requiring energy that would otherwise go into flowering and seed production), acyanogenic plants may be at a competitive advantage in low-herbivore environments. In either of these cases, both cyanogenic and acyanogenic plants are being selectively maintained within this species.

Two biochemical components must be present for a clover plant to be cyanogenic. The presence or absence of each of these components is controlled by two independently segregating genes. One gene, *Ac*, controls a plant's ability to produce cyanogenic glucosides (lotaustralin and linamarin), which are cyanide-containing sugars that release cyanide when acted on by the appropriate enzyme. The glucosides are stored in the vacuoles of leaf and stem cells. A plant must carry at least one dominant *Ac* allele to produce cyanogenic glucosides; a homozygous recessive plant (*acac* genotype) lacks cyanogenic glucosides. For the purposes of this investigation, we refer to the Ac gene as "C" for Cyanogenic glucosides.

The second gene, *Li*, determines the presence or absence of the enzyme linamarase, which is required for the hydrolysis of cyanogenic glucosides to release cyanide. Linamarase is stored in the cell wall, so it is only with tissue damage and cell rupture that cyanide release occurs. A plant that carries two recessive *Li* alleles (*lili* genotype) does not produce linamarase. At the molecular level, the *Ac/ac* and *Li/li* polymorphisms have been determined to arise through two unlinked gene deletion polymorphisms (Olsen *et al*. 2007, 2008). For the purposes of this investigation, we will refer to the linamerase enzyme "li" as "E" for enzyme.

The presence of cyanide release can be detected in clover plants using leaf samples and Feigl-Anger test paper (Feigl and Anger 1966). The production of cyanide will cause a bright blue color change above a test well containing plant tissue that is positive for cyanide. Clover plants found to be acyanogenic can be subjected to further tests to determine if they are positive for the presence of cyanogenic glucosides (*Ac*) or the enzyme linamarase (*Li*), or neither (*acac*, *lili*).

Students will be using a modified Feigl-Anger assay developed by Dr. Kenneth Olsen of Washington University in Saint Louis. By gathering clover plants from their local areas, students will be able to determine the relative percentage of each genotype, compare samples to other locales, and make inferences about the selective forces affecting the clover population in their local climate.

References

Feigl, F., & Anger, V. (1966) Replacement of benzidine by copperethylacetoacetate and tetra base as spot-test reagent for hydrogen cyanide and cyanogen. *Analyst*, 91, 282–284.

Hughes MA (1991) The cyanogenic polymorphism in *Trifolium repens* L. (white clover). *Heredity*, 66, 105–115.

Olsen, K.M., Sutherland, B.L., & Small, L.L. (2007). Molecular evolution of the *Li/li* chemical defence polymorphism in white clover (*Trifolium repens* L.). *Molecular Ecology*, 16, 4180–4193.

Olsen, K.M., Hsu, S.-C., & Small, L.L. (2008). Evidence on the molecular basis of the *Ac/ac* adaptive cyanogenesis polymorphism in white clover (*Trifolium repens* L.). Genetics, 179, 517–526.

Materials

Following is a list of materials needed for one group of students to perform the activities of this lab:

Included in kit:

- 48 well tissue culture plate with lid
- 24 toothpicks or plastic sticks
- 550µl 10mM (11 drops) linamarin (cyanogenic glucoside) solution
- *See appendix A for additional information about this reagent
- 550µl (11 drops) linamarase
- *See appendix A for additional information about this reagent
- 2 microtubes
- 1 sheet of Feigl-Anger test paper (store in the dark and away from moisture)
- 6 large binder clips
- Collection materials for clover samples: 4 1-gallon zip top baggies, large plastic cups, or 3" plastic pots)
- Teachers Manual with Reproducible Student Guide

Required, not included in kit:

Per group

- 4 clover plants (can be shared)
- (You may wish to collect 5 plants if students are doing the herbivory activity)
- 1 20 µl pipettor and 3 tips(or can use standard eye dropper)
- Marker
- Calculator

Per class:

- Incubator (can incubate at room temperature with additional time)
- Freezer
- 1 1000 µl pipettor and tips (or can use standard eye dropper)

Optional:

- Digital camera
- Review activities

Safety:

Ensure that students understand and adhere to safe laboratory practices when performing any activity in the classroom or lab. Demonstrate the protocol for correctly using the instruments and materials necessary to complete the activities, and emphasize the importance of proper usage. Use personal protective equipment such as safety glasses or goggles, gloves, and aprons when appropriate. Feigl-Anger paper is made with caustic chemicals. Be sure and have students wear gloves while handling, or make sure they wash hands thoroughly with soap and water after handling the paper. Model proper laboratory safety practices for your students and require them to adhere to all laboratory safety rules.

Preparation:

Photocopy the black-line master Student Guide for each student.

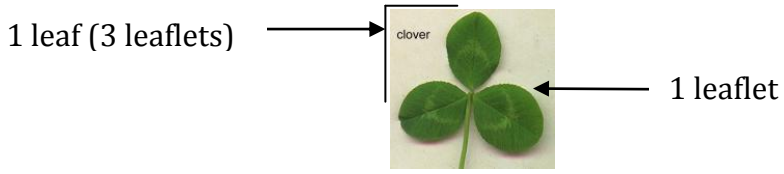
Activity A: Clover Adaptation Pre-Lab

Activity B: Cyanogenesis Assay

Clover Plants

Several days in advance of lab, students need to collect clover samples. Instruct students to dig up (including roots) clover plants from surrounding locales. (See identification activity for accurately identifying white clover.) Patches of clover are commonly found in lawns, and are most easily recognized by their production of white flower heads. Each student should bring in 1-4 plants to ensure sufficient quantities for the class. It may be easier if one class period is used to collect samples from the surrounding school grounds. This would ensure that the correct species of White clover is obtained, and that students have enough samples for the experiment. Samples should be clearly labeled. Students should record the location of their samples and make written observations about the surrounding area, presence of predators, and condition of the clover. If students are collecting on their own, suggest that students collect not only from their yard, but also from school grounds, parks, fields, etc.

It is critical that students understand the difference between leaflets and leaves.



Each test requires a total of 18 **leaves**; therefore, each plant should have a total of 20 – 30 leaves.

Clover plants propagate vegetatively along stolons, and a large clover patch may contain multiple individuals that have grown together. By selecting a sample from a discrete, circular patch of clover (< 3 ft. across), students can be fairly assured of having only one type of clover plant in each of their samples. Having multiple genotypes in one sample can create uninterpretable results in the Feigl-Anger test.

Alternately, clover can be raised indoors well ahead of the time for the lab investigation. Clover can be cultivated easily from seed or propagated from wild stock under normal greenhouse conditions. Growing the clover from seed will not provide students with the opportunity to relate the local genotype to environmental conditions, but is an option if no other clover is available. Experience thus far has shown that while the purchased seed is not a true monoculture, there is very little diversity of cyanogenesis phenotype within a purchased seed sample.

From seed:
Approximately 6 weeks before scheduled lab obtain 4” pots, potting soil, and seed. Moisten soil and place in pots. Scatter clover seeds over the surface and cover lightly with approximately 1/8” of soil. Lightly scarring the seeds with sandpaper (scarification) may increase germination rate. Mist surface, cover loosely with plastic wrap and grow under lights (3” above pot). Plants should germinate within 3-4 days with a high percentage germination rate. As seedlings mature, split the plants out into 2-3 larger pots to provide sufficient samples for each group. Plants grow well with 13 hours of light, moderate water and temperature of 68^{o}F.

From wild stock:
From a sample of clover, select one plant, follow its stolon along to the root, and separate the plant from its clump. The rooted plant can be placed in a 3-4” pot with pre-moistened soil, watered well, and grown under normal greenhouse conditions as above. Keep lights low over pot and keep well moistened. Plants will grow and be ready for transplanting into a larger pot in approximately 6 weeks. This is a good method to preserve samples brought in each year, but is slow to produce the needed quantities of clover for a class.
Large clumps left over from samples brought in by students can be placed in pots and maintained intact as above. Separating out the individual plants is only necessary if it is likely that the original sampled clover patch contained multiple plants.

Pre-lab Preparation:
Day before or day of lab:

For each lab group, aliquot (divide) 550 µl 10mM linamarin solution into a labeled microtube and 550µl 0.2 EU/mL linamarase into another labeled microtube. If you do not have a micropipettor, a standard eye dropper will do. There are 45-50 microliters in a single drop, so you will need about 11 drops of each solution per lab group. Although you should keep the reagents refrigerated while storing, it is not necessary to refrigerate while students are using them.

Set up each student station as follows:

48 well tissue culture plate with lid
24 toothpicks
550 µl 10mM (11 drops) linamarin solution
550 µl 0.2 EU/mL (11 drops) linamarase
1 sheet of Feigl-Anger test paper (Keep Feigl-Anger paper wrapped in foil until ready to use)
6 large binder clips
4 clover plants (can be shared)

If doing the herbivory lab activity, there should be one extra plant to take leaf samples from.
1 20 µl pipettor and tips (or eyedropper)
1 Marker
Distilled water

TIPS:

- If you are going to have students collect their own clover samples, it might be a good idea to have them work through the *Identification of Clover Activity,* to make sure they are working with the correct species of clover during the lab.
- Students should place samples in a gallon Ziploc baggie with a moist paper towel. Once arrived in class, the teacher should continue to keep the plant moist until use.
- Advise students to use the same amount of tissue in each well; use young healthy green leaves; remind them of the difference between a leaf and a leaflet. Try not to include petiole.
- Have students write their initials on the corner of the test paper. If the reaction proceeds too slowly to have students observe results during the 90 minute period, the teacher can remove the Feigl-Anger paper later (after incubation is complete), wrap it in aluminum foil, and store it in a dry, dark location. Alternately, the test paper results can be photographed.
  - When incubating the well plates, do NOT stack them on top of each other. This may cause condensation to occur on the Feigl-Anger paper, which will prevent the color from developing
  - If an incubator is not available, plates can be placed in a warm, dry area and extend the processing time.
  - Do not incubate more than 6 hours – results will fade.
  - Binder clips prevent bleed-over of HCN between wells; to further safeguard against bleed-over, check after 20 – 30 minutes, and remove leaves from wells with dark response.
  - Remind students to use a new toothpick for each well to prevent contamination. Pipettor tips or solid plastic straws can be used in place of toothpicks to mash the clover tissue in wells.
  - There are several ways to manage the timing of the lab. Pre -lab discussion takes approximately 30 minutes. The discussion can be started on one day and combined with placing the clover leaves in wells for freezing. Minimum time for freezing is 45 minutes, but can be left in freezer until next class. Addition of reagents and the development of the test paper can follow the next day or available period. Alternately, the background questions/reading can be assigned for homework, thereby decreasing the amount of class time used for pre-lab discussion.
  - Additional lab activities have been provided, and would increase the amount of time necessary, but are optional depending on the level of understanding of students.

**See Apendix A for suggestions on what to do with your plants when you are finished.

Activity C: Determining Genotypes. Calculating Class Data

Groups collect data
Class activity recording results
Copy table or reproduce for class discussion

Clover Cyanogenesis Class Data

Group	(#) Cyanide Producers	(#) Producing cyanogenic glucosides Only	(#) Producing Linamarase Only	(#) Producing Neither cyanogenic glucosides or linamarase	
Genotype					Total Number of Plants
Total number of plants per genotype					
Percent of Clover Plants Assayed					

Activity D: Population Genetics: Hardy-Weinberg 30 minutes
Have class results available

Optional worksheets, activities, and background
Copy necessary student materials

A. Clover Identification Activity
B. Calculating Herbivory Activity
C. Creating a Dichotomous Key
D. Mendelian genetics and Natural Selection Wkst.

Cyanogenesis Assay Procedure
Activity B

Materials:

- 4 clover plants
- 48 well tissue culture plate with lid
- 4- 20 μl pipette tips or eye droppers
- 24 toothpicks
- linamarin (cyanogenic glucoside) solution
- linamarase solution
- 1 sheet of Feigl-Anger test paper *Keep Test paper in a cool dry location, wrapped in foil at all times*
- 6 large binder clips
- 20 μl pipettor or eyedropper
- Distilled water

Procedure:

1. Label the clover plants #1-4.

2. Inspect the 48 well culture plate. Find the letters and numbers that identify the rows and columns. Label the 48 well culture plate and lid with your lab group number.
   - Next to Rows A and B: Label this "TO" for tissue only
   - Next to Rows C and D: Label this "E" for Testing for Enzyme
   - Next to Rows E and F: Label this "C" for Testing for Cyanogenic glucosides

3. Label the well plate diagram located on the student data sheet with the identity of each of your 4 clover plants. Include the labeling of Rows A-F as well.

4. For plant 1, place 3 fresh, young, green leaves into each of the wells in column #1, a total of 6 wells. These will be wells A1-F1.
   Skip a column and Place 3 leaves from plant #2 in wells A3-F3 and so on, for all four plants. Label your diagram carefully as to which plant is in each well. There should be a column of empty wells between each sample to help catch any bleed over.

5. Place the lid on top of the plate and place it in a -20^{o}C freezer (normal freezer) for 45 minutes.

*Optional stopping point. Plates can be left in freezer until next class (or for much longer, e.g., several weeks).

6. Remove the plate from the freezer and thaw the samples by placing the plate in a 37°C incubator for 10 minutes. If an incubator is not available, tissue can sit out at room temperature for approximately 20 minutes in a warm, dry location, or the teacher can remove them from the freezer prior to the start of the class period.

7. Remove plate from incubator and add the reagents as follows:
    - In wells A and B: Marked “TO” leaf tissue only + 20 μl or 1 drop distilled water (no reagents added)
    - In wells C and D: Marked “E” leaf tissue + 20 μl or 1 drop **linamarin** solution
    - In wells E and F: Marked “C”leaf tissue + 20 μl or 1 drop **linamarase** solution
    - Repeat for all plants and all wells.

8. Use well F8 as a positive control by placing 20 μl or 1 drop linamarin solution + 20 μl or 1 drop linamarase, no plant tissue. Be sure to mix the contents of the control well.

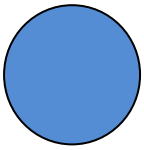
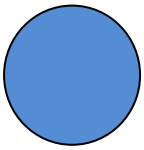
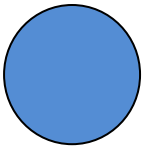
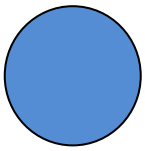
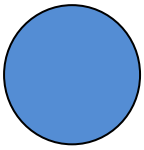
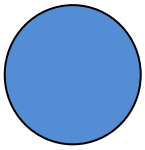
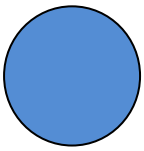
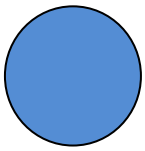
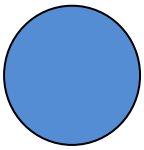
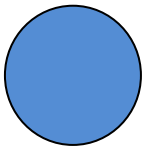
9. Using a new toothpick for each well, mash the tissue and reagents in each well to mix compounds. Mash until a small amount of liquid is visible.
    *Be sure to use a new toothpick for each well. *Mashing can be somewhat time-consuming (approximately 20 min.)*

10. Wipe off the top of the plate to remove any moisture. Place a piece of Feigl-Anger test paper on top of the well-plate. Place the lid over the paper on the plate, and then clamp together using 6 large binder clips (prevents bleed-over of HCN between wells). *Binder clips should all fit around the perimeter of the plate. Use all 6 clips.*

11. Incubate the plate in a 37°C incubator for 30 minutes to 2 hours (or longer, if needed but not longer than 6 hours) then photograph or record results. If incubator is not available, wrap plates in aluminum foil, or a thick envelope, and place plates in a warm, dry, area in the room, and let sit for approximately 90 minutes. Check samples after 20 minutes, and remove any tissue from positive wells to help prevent bleeding. At this point, the teacher can remove the Feigl-Anger test paper, and wrap in foil for analysis the next class period.

For reliable scoring of positive results in weakly cyanogenic plants, a minimum of 90 minutes in an incubator and 3 hours at room temperature. Scoring plants after shorter durations may yield false negative results for plants that produce low levels of cyanogenic compounds.

Well Plate Diagram (Sample Data)

	1	2	3	4	5	6	7	8
	Plant1		Plant2		Plant3		Plant 4	
TOA								Distilled Water Only
TOB								Distilled Water Only
+CC								Add Linamarin to activate Enzyme if present
+CD								Add Linamarin to activate Enzyme if present
+EE								Add Linamerase to release cyanide if present
+EF								Add Linamerase to release cyanide if present  Control
GenoType	C_E_		C_ee		ccee		ccE_	

RESULTS

1. If cyanide is produced, a blue color change will appear on the paper. Carefully inspect your paper. Record on your well-plate diagram which wells turned blue and which did not. Inspect the paper from the underside, if results are questionable.

a. If the clover has the ability to produce cyanide, which wells will be blue? all

b. If the clover produces the sugar-cyanide compound, but not the enzyme, which wells will be blue?
E and F

c. If the clover plant produces the enzyme, but not the sugar-cyanide compound, which wells will be blue?
C and D

d. If the clover plant produces neither sugar-cyanide compound nor the enzyme, which wells will be blue? none

e. What is the purpose of well F8? *Control to show that a blue color results when linamarin and linamarase combine*

2. Record the phenotype and the genotype of your plants:

Plant	1	2	3	4
Cyanide producer(y/n)	Y			
Produces Linamarase only (y/n)				Y
Produces Linamarin only (y/n)		Y		
Genotype	C _E _	C _ee	ccee	ccE _

3. Collect class data. Activity C. (Data sheet located in Instructor's Manual)

Based on Class Data:

4. Calculate the percentage of plants that are cyanogenic.
Answers will vary

5. Compare the percentage of plants that are cyanogenic to your predictions and to the percentages from other climates.
Answers will vary

6. Based on climate, do the results for your local area make sense? Explain

Answers will vary

7. Calculate the percentage of the plants that contain the gene for the linamarase ONLY:

Answers will vary

8. Calculate the percentage of the plants that contain the gene for cyanogenic glucosides (sugar-cyanide) ONLY:

Answers will vary

9. Calculate the percentage of the plants that contain NEITHER gene for cyanogenic glucosides or linamarase?

Answers will vary

10. Explain why natural selection would favor plants that produce cyanide in warm climates, but not in cold climates.
The increased metabolic cost of producing cyanide would be offset in warmer climates by a higher survival rate due to less herbivory. In cold climates the increased metabolic cost combined with the self toxicity resulting from ruptured cells would place the cyanide producers at a greater competitive disadvantage.

11. Explain why natural selection would favor plants that produce neither compound in cold climates versus a plant that produces only one of the compounds.
The metabolic cost of producing one gene product without the other would place those organisms at a slight survival disadvantage.

12. Assume more plants express the gene for the production of the sugar-cyanide compound than express the gene for the production of the enzyme. What can you infer about the relative metabolic "cost" of producing the enzyme?
The conclusion would be that the energy cost of producing or maintaining a "stockpile" of the enzyme is greater and would therefore be more strongly selected against.

13. Some plants in the class may have appeared stronger in the levels of cyanogenic compounds produced in the class plants as a whole. What are some possibilities as to why there would be varying levels of cyanogenic compounds in clover plants growing in the same general area?

Answer: Answers may vary- some possibilities:
1) environmental (non-genetic) factors that are affecting the overall health of a plant and its resources available for making the cyanogenesis compounds (e.g., drought, fungus infection, virus infection, too little light, etc. etc.);
2) heterozygosity at the C and E genes — for example, a Cc plant, while producing cyanogenic glucosides, is likely making only about half the amount as you'd find in a CC plant;
3) other 'modifier' genes besides C and E that control *quantitative* rather than *qualitative* variation in cyanogenesis (e.g., a gene that controls the level of expression of the Li gene will be affecting how much linamarase protein is produced in Li_ plants).

Appendix A

Should you find you would like to perform this assay with multiple classes, or teachers in your building, and there is a shortage of linamarin, and linamarase, you may choose this alternative method of performing the assay:

1. Once one class has performed the assay, and achieved results, identify the plants that had the genotypes of C_ee, and ccE_. This means you have plants that are producing only the glucoside “linamarin” and “linamarase”.
2. Label all plants with those genotypes.
3. Place in 6 inch pots with potting soil and place in a well lit area, or under grow lights.
4. Use tissue from these plants in place of the reagents in the well plates of the assay for unknown plants.
5. Should you wish, these plants can be maintained and used indefinitely as a personal source of the reagents.