INQUIRY & INVESTIGATION

Studying Plant–Rhizobium Mutualism in the Biology Classroom: Connecting the Big Ideas in Biology through Inquiry

RECOMMENDED FOR AP Biology

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Abstract

We present a guided-inquiry biology lesson, using the plant–rhizobium symbiosis as a model system. This system provides a rich environment for developing connections between the big ideas in biology as outlined in the College Board's new AP Biology Curriculum. Students gain experience with the practice of scientific investigation, from designing and conducting experiments to making claims based on the data they collect. We include one example of a piloted classroom experiment that can easily be modified to test a variety of interesting ecological and evolutionary hypotheses.

Key Words: *Rhizobia; symbiosis; mutualism; parasitism; photosynthesis; nitrogen fixation.*

○ Introduction

The early part of the 21st century has experienced a remarkably synchronized and congruent effort to reform biology education in the United States (e.g., *Next Generation Science Standards*, The College Board's *AP Biology Curriculum Framework*, and AAAS's *Vision and Change*). Targeting both K–12 and undergraduate biology instruction, these efforts focus their goals on helping students develop a conceptual understanding of the "big ideas" of biology while emphasizing that deeper understanding comes through a concurrent development of scientific skills or practices. "The revised AP Biology course addresses this challenge by shifting from a traditional 'content coverage' model of instruction to one that focuses on enduring, conceptual understandings and the content that supports them" (College Board, 2011).

In this environment, the selection and design of the laboratory experiences is critical to help students develop scientific practices and content understanding. "Students who take an AP Biology course designed using this curriculum framework as its foundation will also develop advanced inquiry and reasoning skills, such as designing a plan for collecting data, analyzing data, applying mathematical routines, and connecting concepts in and across domains" (Table 1; College Board, 2011). The AP Investigative Laboratory program provides a potential design model. In these labs, the students are first presented with a laboratory system or topic. Through guided inquiry, they are introduced to the skills and content they will need to apply as they address their own questions (Herron, 1971). Then the students conduct their own independent research in the topic – nonguided inquiry that requires effective scientific argumentation and effective communication of results.

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The rhizobium–legume mutualistic relationship has been a stalwart high school lab topic for the past 60 years (BSCS, 1961; Larson, 1969; Hughes, 1986). Hughes (1986) described how this system provides an "almost ideal lab":

> If all that can be achieved within the constraints of a limited budget, in a 45–50-minute period, with available equipment, in safety and still have a high probability of success in both illustrating the experimental process and a major biological principle, in consonance with the student's developmental level, it would seem almost ideal.

We argue that this claim is even more relevant today. For instance, the AP Biology Curriculum is organized around four big ideas: (1) The process of evolution drives the diversity and unity of life; (2) Biological systems utilize energy and molecular building blocks to grow, reproduce, and maintain homeostasis; (3) Living systems retrieve, transmit, and respond to information essential to life processes; and (4) Biological systems interact, and these interactions possess complex properties (Table 1; College Board, 2011). Likewise, the *Next Generation Science Standards* for high

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Table 1. Learning objectives from the AP Biology Curriculum (College Board, 2012b) that this activity could address.

Learning Objectives	
LO 1.5	The student is able to connect evolutionary changes in a population over time to a change in the environment.
LO 2.2	The student is able to justify a scientific claim that free energy is required for living systems to maintain organization, to grow or to reproduce, but that multiple strategies exist in different living systems.
LO 2.23	The student is able to design a plan for collecting data to show that all biological systems (cells, organisms, populations, communities, and ecosystems) are affected by complex biotic and abiotic interactions.
LO 2.38	The student is able to analyze data to support the claim that responses to information and communication of information affect natural selection.
LO 4.14	The student is able to apply mathematical routines to quantities that describe interactions among living systems and their environment, which result in the movement of matter and energy.
LO 4.16	The student is able to predict the effects of a change of matter or energy availability on communities.

Table 2. *Next Generation Science Standards* for High School Life Science performance expectations that could be addressed with this lab (NGSS Lead States, 2013).

NGSS For Life Science Performance Expectations	
HS-LS1-6	Construct and revise an explanation based on evidence for how carbon, hydrogen, and oxygen from sugar molecules may combine with other elements to form amino acids and/or other large carbon-based molecules.
HS-LS2-1	Use mathematical and/or computational representations to support explanations of factors that affect carrying capacity of ecosystems at different scales.
HS-LS2-2	Use mathematical representations to support and revise explanations based on evidence about factors affecting biodiversity and populations in ecosystems of different scales.
HS-LS2-3	Construct and revise an explanation based on evidence for the cycling of matter and flow of energy in aerobic and anaerobic conditions.
HS-LS2-4	Use mathematical representations to support claims for the cycling of matter and flow of energy among organisms in an ecosystem.
HS-LS2-6	Evaluate the claims, evidence, and reasoning that the complex interactions in ecosystems maintain relatively consistent numbers and types of organisms in stable conditions, but changing conditions may result in a new ecosystem.
HS-LS2-7	Design, evaluate, and refine a solution for reducing the impacts of human activities on the environment and biodiversity.
HS-LS3-3	Apply concepts of statistics and probability to explain the variation and distribution of expressed traits in a population.
HS-LS4-2	Construct an explanation based on evidence that the process of evolution primarily results from four factors: (1) the potential for a species to increase in number, (2) the heritable genetic variation of individuals in a species due to mutation and sexual reproduction, (3) competition for limited resources, and (4) the proliferation of those organisms that are better able to survive and reproduce in the environment.
HS-LS4-3	Apply concepts of statistics and probability to support explanations of why organisms with an advantageous heritable trait tend to increase in proportion to organisms that lack the trait.
HS-LS4-4	Construct an explanation based on evidence for how natural selection leads to adaptation of populations.
HS-LS4-5	Evaluate the evidence supporting claims that changes in environmental conditions may result in (1) increases in the number of individuals of some species, (2) the emergence of new species over time, and (3) the extinction of other species.
HS-LS4-6	Create or revise a simulation to test a solution to mitigate adverse impacts of human activity on biodiversity.

school are organized around similar big ideas (Table 2; NGSS Lead States, 2013).

Interactions between leguminous plants (e.g., clover and soybeans) and a kind of bacteria called "rhizobia" are classic examples of mutualism. Rhizobia infect plant roots and form root bumps, called "nodules" (Figure 1). Inside the nodules, they convert atmospheric nitrogen (N_2) to ammonium (NH_4^+), making it available to their host plants. In return, plants can provide rhizobia with sugar (i.e., carbon)

Figure 1. Nodules on legume roots. Rhizobia grow inside the nodules and convert atmospheric nitrogen (N_2) to ammonium (NH_4^+). (Photo credit: T Suwa.)

produced through photosynthesis. With the rhizobium–legume system, students can address and readily connect multiple big ideas with their questions and investigations while developing their science practice skills.

○ Guided Inquiry

Review of the Scientific Processes

Most high school and college students are familiar with scientific methods. However, they tend to think of the method as linear because they rarely have an opportunity to walk through the entire process. Explain to the students that we are going to follow the entire scientific method – literature search, formulating a question(s) and hypotheses, designing and conducting an experiment, analyzing data and supporting claims based on their evidence – and highlight that the process is circular, not linear (Understanding Science, 2014c).

Background of the Study System: Plants & Rhizobia

Introduce students to the plant–rhizobium system (see online Supplementary Materials). Note that many students from rural areas are familiar with the soybean as a crop. In addition to the life history of soybeans, highlight the importance of soybeans as a crop in the United States. For example, "soybeans rank second, after corn, among the most-planted field crops" in the United States, which is the largest producer and exporter of soybeans in the world (with >279,110 farms producing soybeans; EPA, 2013). Furthermore, rhizobia can provide up to 40–70% of the soybean nitrogen requirement and can increase soybean yields by up to 25% (Hume & Blair, 1992; Egamberdiyeva et al., 2004). On a landscape scale, nitrogen fixation can equal or exceed the amount of synthetic nitrogen fertilizer that is applied to some cropping systems (Klubek et al., 1988). Rhizobia increase plant yields and decrease dependence on synthetic nitrogen fertilizer. Therefore, from an economic and environmental perspective, the legume–rhizobium symbiosis is an important and desirable component of agricultural systems worldwide.

Introduce students to scientific literature at this point (e.g., Kiers et al., 2002; Denison & Kiers, 2004) and ask them to brainstorm ideas on how synthetic fertilizer may alter plant–rhizobium interactions. To help students think through this big question, teachers can break it into smaller questions. For example, both legume and rhizobium appear to benefit from the symbiotic relationship. Is there any cost associated with the interactions? What would you predict might happen to this relationship if nitrogen were added to the system? How might plants respond, based on your understanding of how they budget their energy? How might rhizobia respond to the plant response? Nitrogen addition can lead to lower nitrogen fixation rates per nodule (Denison & Harter, 1995) and lower production of nodules in legumes (Rubio Arias et al., 1999; Vargas et al., 2000).

Formulating Scientific Questions & Hypotheses

After providing some background information on plant–rhizobium symbiosis, ask students what further questions they have. Students can find many questions to ask in this study system. Here, we present one of the successful classroom experiments we conducted with students. Two example questions that students can test are (1) How do rhizobia affect plant growth? and (2) How does nitrogen fertilization affect plant–rhizobium interaction? A good scientific question will lead to formulation of hypotheses and predictions. A hypothesis is "a tentative answer to a well-framed question – an explanation on trial" (Campbell et al., 2008). In this example, the hypotheses could be that (1) rhizobia increase plant performance and (2) nitrogen fertilization reduces plants' dependency on rhizobia. Predictions are "what we would expect to happen or what we would expect to observe if this idea were accurate" (Understanding Science, 2014a). In this example, the predictions could be that (1) rhizobial inoculation will increase plant biomass (or inoculated plants will have greater biomass than non-inoculated plants) and (2) fertilized plants will have fewer nodules than nonfertilized plants. Good hypotheses and predictions are important because they will lead to a good experimental design.

Experimental Design 101

There are three key concepts that students need to understand prior to designing and conducting an experiment: control, randomization, and replication. These concepts help minimize the problems introduced by confounding factors (Underwood, 1997; Gotelli & Ellison, 2004). Controls are treatments against which manipulations are compared (Karban & Huntzinger, 2006). A control group is "a group of individuals or cases matched to an experimental group and treated in the same way as that group, but which is not exposed to the experimental treatment or factor that the experimental group is" (Understanding Science, 2014a). Replication is the establishment of independent multiple plots or observations within the same treatment or comparison group (Gotelli & Ellison, 2004). Replication increases confidence in experimental results. Plots and observations should be randomly assigned to the treatment or control group (Gotelli & Ellison, 2004), and this can be accomplished by students flipping a fair coin, rolling fair die, or using a computer algorithm to generate random numbers. For more detailed and accessible information on experimental design and hypothesis testing, we recommend the book *How to Do Ecology* by Karban and Huntzinger (2006).

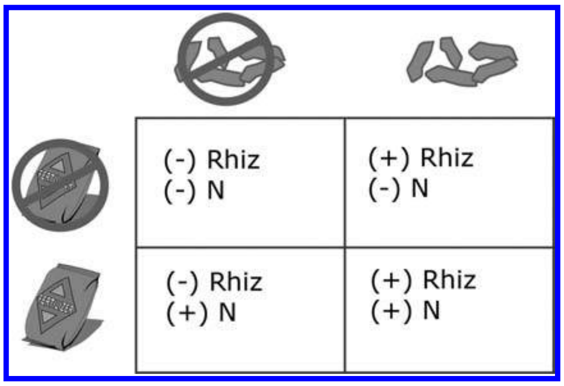

Figure 2. Two-by-two factorial design to test effects of rhizobium inoculation (Rhiz) and fertilization (N) on plant nodulation and growth.

Setting Up the Experiment

Ask students what kind of experiment they would design to answer their questions. In our high school class, we came up with four treatments (Figure 2; 2 × 2 factorial design) to test the two hypotheses described above:

1. Plants with no rhizobia, no fertilizer (control treatment)
2. Plants with rhizobia, no fertilizer
3. Plants with no rhizobia, fertilizer
4. Plants with rhizobia, fertilizer

Review the concepts of control, randomization, and replication in the context of the students' experiment. Divide the class into groups of four. Each group will conduct the experiment described above with a minimum of 3 replicates per treatment combinations (12 samples per group). The total estimated cost of materials is between \$116.25 and \$151.25 (Table 3). Here are the basic procedures to conduct the experiment:

(1) Label each pot with a plot ID number and treatments (e.g., no. 1 –N +Rhiz).

(2) Fill pots with soil (4/5 full). Add 6 g of fertilizer pellets on plots with +N treatment. Place each pot on an individual saucer and water all the pots until the soil is completely saturated and water drips out the drain holes. Saucers are important to minimize cross-contamination.

(3) Make a 1–1.5 cm indentation in the soil in the middle of the pot with your finger. Add 3 seeds and cover loosely with moist soil. Planting of 3 seeds maximizes successful germination in each pot.

(4) One week later (or when seeds have germinated), thin the seedlings to only 1 per pot, removing extra seedlings with forceps while being careful not to disturb the remaining one.

(5) Add 5 mL of rhizobial inoculum using pipette to the center of each pot designated to receive a rhizobial treatment. Inoculum can be made in two ways: (1) make a soil slurry by mixing 10 g of soil from a soybean field with 50 mL of water or (2) purchase rhizobia inoculum from a vender (e.g., Carolina Biological Supply). Add 5 mL of water to all other pots not receiving the rhizobial inoculum for an experimental control.

Table 3. A list of required materials and their estimated costs for the experiment conducted by six groups of four students (24 students total).

Amount	Materials	Cost
1 pack	Soybean seeds	\$3–10
1 bag	Potting soil	\$20–40
72 cups	10–12 oz. plastic cups or pots	\$15–20
72	Small plastic plates as saucers	\$5–10
1 bag	Slow-release fertilizer (e.g., Osmocote slow-release fertilizer, 14N-14P-14K; Scotts-Sierra Horticultural Products, Marysville, OH)	\$8
1 bag	Rhizobial inoculum (available at Carolina Biological Supply)	\$18.5
6	Plastic disposable pipette	\$1.25
1	Masking tape	\$2
6	Dice	\$3
6	Permanent markers	\$4.5
1	Tray or bucket to dispose of soil	\$5
6	Ruler	\$8
1–6	Balance	NA
6	Weigh boats or paper towel	\$0
1 pack	Microcentrifuge tubes	\$12
6	Forceps	\$12
1–6	Stereo microscope (optional, to look at the roots)	NA

Notes: The total estimated cost of materials is between \$116.25 and \$151.25. However, most of the materials (e.g., masking tape, ruler, forceps) are likely already available in the classroom, so the cost of new materials could be less than \$70.

(6) Randomize the location of the pot by rolling a die.

(7) Place all plants in a warm, well-lighted location and water ~100 mL every other day for a month.

Note: A typical experimental error that students may encounter is rhizobial contamination in control plots. To prevent contamination, avoid touching the soil, avoid splashing water between pots when watering the plants, and do not set pots in a windy location. Clean any supplies and materials that contacted rhizobia with bleach or ethanol.

Data Collection & Analysis

Each student group should be responsible for collecting their own data. Examples of response variables that students can measure include plant height, number of leaves, number of nodules, aboveground biomass, and belowground biomass. Before weighing for belowground biomass, clean roots in water to remove soil as much as possible and then dry the plant material by placing it on a hot air vent or in a sunny window for a week to ensure that most of the water has left the plant tissue.

After collecting all the data, pool the class data to plot bar graphs and analyze statistically. First, students calculate the mean and the error bars (standard error of the mean) of a chosen response variable for each treatment. Depending on the students' level, they can statistically analyze the data using a t-test or analysis of variance (ANOVA). Figure 3 shows the data collected by high school students from Kalamazoo Area Mathematics and Science Center. Karban and Huntzinger (2006, pp. 60–76) provide an accessible conceptual explanation of hypothesis testing and data analysis. For technical assistance on the data analysis, we recommend Gotelli and Ellison (2004) and *Advanced Placement Quantitative Skills* (College Board, 2012a).

Evidence-based Claims & Conclusion

Revisit questions and hypotheses discussed earlier and discuss whether students' hypotheses were supported by the data collected. Specifically, ask students to identify which treatments to compare in order to test whether rhizobia affect plant growth. Students should compare plant biomass from –N,–R– vs. –N+R treatments. If rhizobia are mutualistic, plants should increase in biomass when inoculated with rhizobia. We would call rhizobia parasitic if plants had reduced biomass when inoculated with rhizobia.

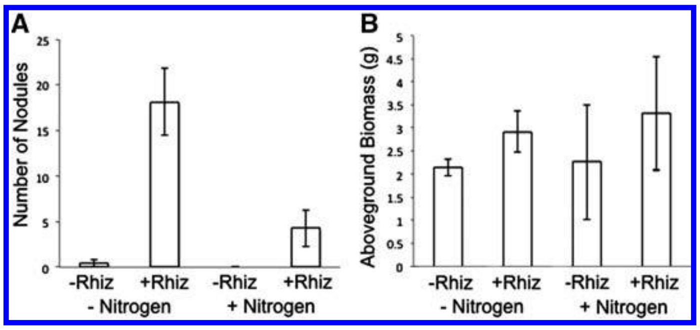

Figure 3. Total number of nodules (A) and aboveground biomass (B) of soybean under four different treatment combinations. "+Rhiz" and "–Rhiz" indicate presence and absence of rhizobial inoculation. "+Nitrogen" and "–Nitrogen" indicate presence and absence of nitrogen fertilization. Error bars indicate standard error of mean. Plants were grown for one month and the data were collected by high school students from Kalamazoo Mathematics Area Science Center in 2012.

To address how nitrogen fertilization affects plant–rhizobium interactions, students need to look at all the treatment combinations. Rhizobia may be mutualistic in the absence of fertilization (–N,–R– vs. –N,+R) but they may not be as beneficial when plants are fertilized (compare +N,–R– vs. +N+R). When plants are fertilized, students may find no difference among inoculated versus non-inoculated plants (+N,–R– vs. +N+R), suggesting that both fertilizers and rhizobia provide the same limiting resource, nitrogen. Another interesting result that students may report is that fertilization reduces nodule numbers (–N,+R– vs. +N,+R). Discuss possible explanations for this pattern.

Communicate Your Findings

Answering questions and generating alternative hypotheses is not the end of the scientific process. Science can advance only when you communicate what you learned to others (Karban & Hutzinger, 2006). To do that, scientists give a poster or oral presentation at conferences and, ultimately, publish articles in journals. For high school and undergraduate students, we recommend creating a space for students to present their findings. For example, organizing a poster session or minisymposium where students give oral presentations would be an excellent way to share their findings with peers and the general public.

○ Advancement

Here, we have introduced an experiment manipulating nitrogen and rhizobia. Students can easily manipulate other environmental variables such as light, soil moisture, and even simulated herbivory to address their own questions. This activity can also be performed using other common legume species such as clovers (*Trifolium* spp.) and medick (*Mediago* spp.). We listed some specific examples to cover "Big Ideas" in Biology (Table 4).

Table 4. Examples of questions and experimental design to address four "big ideas" in biology using the plant–rhizobium system (College Board, 2012a, b).

Big Idea	Question	Potential Lab Activity
Evolution	What is the evolutionary response of rhizobia to long-term N fertilization?	Isolate rhizobia from fertilized and nonfertilized fields (microbiology technique needed). Then inoculate rhizobia from a fertilized and a nonfertilized field to a legume (e.g., clover) to test how rhizobia with different evolutionary history affect plant performance.
Cellular Process: Energy and Communication	How does light (or water) affect plant photosynthesis and interaction with rhizobia?	Manipulate rhizobium inoculation and light (or water) availability.
Genetics and Information Transfer	How common is lateral (horizontal) transfer among rhizobial strains?	Build phylogenetic trees using genes from chromosome and from plasmid (sequence data available at NCBI GenBank) and compare them. If they are the same, there is no evidence for lateral gene transfer; if they're different, there is evidence of lateral gene transfer.
Interactions	How is plant–rhizobium symbiosis influenced by biotic and abiotic factors?	Manipulate rhizobium inoculation and biotic (e.g., herbivores, competitors, pathogen) and abiotic factors (e.g., nitrogen [as in this article], soil moisture, light, pesticide).

Conclusions

Performing inquiry experiments in the classroom can be challenging; however, we hope that students will connect big scientific concepts to the world around them by collaborating with peers in conducting an experiment and by drawing conclusions based on the data they collected. We believe that the legume–rhizobium symbiosis is an excellent study system to teach basic scientific methods and the four "big ideas" that guide each of the recent reforms in biology education.

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Online Supplementary Materials

A presentation (PowerPoint) and worksheet to guide students through the entire procedure are are available at the KBS GK–12 website (http://kbsgk12project.kbs.msu.edu/blog/2012/03/19/mutualism-in-action/).

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