PLANT DEFENSES: INVESTIGATING THE DIFFERENTIAL ALLOCATION OF SECONDARY COMPOUNDS

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The study of plant-animal interactions is an area of great interest in ecology (e.g. see Vol. 77 of Ecology, 1996), both from an evolutionary perspective and from a practical standpoint (e.g. the search for pharmaceutically useful chemicals from vanishing plant species, the use of biological control methods in efforts to improve agricultural productivity). Because an investigation of plant-herbivore interactions uses knowledge and techniques from several sub-disciplines – ecology, biochemistry, evolution, physiology, plant morphology – it provides a model for the integrated nature of research. This lab will investigate the differential allocation of secondary compounds within individual plants as evidenced by a bioassay.

Objectives

1. To appreciate the ever-increasing importance of integrating "field" work with "lab" work in answering scientific questions.
2. To practice how to state scientific questions and hypotheses and how to evaluate them.
3. To practice techniques such as pipetting, making dilutions, making careful counts of organisms, and running a common bioassay for toxicity.
4. To use the web to find references from scientific journals.
5. To develop teamwork while working with a group on a scientific investigation.
6. To practice using statistics to answer biological questions.
7. To practice writing a scientific paper.

Background

All higher plants contain secondary substances: these are chemicals that are not part of such major organic groupings as carbohydrates, proteins, fats, and nucleic acids. These compounds mostly have no known role in the metabolism of the plants in which they occur. Secondary substances have many different functions; of interest in this exercise is their role as chemical signals. Chemical signals act on different levels in the living world. Substances produced by one tissue that influence another tissue within the same organism are hormones. Those produced by one individual and influencing another individual of the same species are pheromones. Those active between different species are allelochemics. (For the purposes of this investigation, we will assume that the secondary compounds with which we will be working are allelochemic. However, you should be aware that some controversial recent work suggests that many of these compounds may have evolved to serve a signaling function within the plant.)

Allelochemic interactions are a major realm of species adaptations that are normally invisible to us. Tannins, lignins, terpenes, alkaloids (such as caffeine), nicotine, strychnine, curare, and organic cyanides are just a few examples of the chemical arsenal that plants have evolved to defend themselves against animal consumers and pathogenic microbes. One example is the way in which tannins, sequestered in vacuoles in the leaves of oaks and other plants, combine with leaf proteins and digestive enzymes in a herbivore’s gut, thereby inhibiting protein digestion. Thus, tannins considerably slow the growth of caterpillars and other herbivores. Ruffed grouse (Bonasa umbrellus) pay a significant cost to detoxify the coniferyl benzoate found in the flower buds of quaking aspen (Populus tremuloides); in addition, digestive efficiencies of grouse were reduced via dilution of utilizable nutrients by plant secondary metabolites (Guglielmo et al., 1996). Of course, herbivores (vertebrate or invertebrate) may counter such toxic effects through the evolutionary modification of their own physiology and biochemistry (Tallamy, 1986). Herbivore species that evolve detoxification mechanisms may be able to specialize on plant hosts that are poisonous to most other species.

Plants (with a little help from natural selection) have come up with some amazing defensive strategies. For example, there is increasing evidence that plant defenses may be induced by herbivore damage. That is, in response to wounds, toxic compounds may be produced – either in the area of the wound or systematically throughout the entire plant – that reduce subsequent herbivory (Jones et al., 1993). In fact, there is now evidence that plants can detect the chemicals released into the air by neighbors who are being munched by herbivores and respond to this by increasing their own levels of anti-herbivore compounds (Bruin et al., 1995). (Why is this a more evolutionarily likely explanation than saying that plants warn their neighbors when they themselves are munched by an herbivore?)

Of particular interest to us will be those situations in which individual plants allocate chemical defenses differentially within their own "bodies". For example, some plants maintain higher levels of secondary compounds in the high-nitrogen foliage preferred by herbivores (Newman et al., 1997). In the first week of this lab, we will walk around campus and discuss plant animal interactions, both mutualistic (pollination, seed dispersal) and antagonistic (herbivory/defense). After this introduction, you will choose species with which to work and develop hypotheses to test.

In the second week, we will screen our samples using a "benchtop" bioassay, the brine shrimp bioassay (McGlaughlin, 1991). Brine shrimp (Artemia salina) larvae have been used for over 30 years in toxicologic studies. Many researchers are now using brine shrimp as a pre-screen for plant extracts because they provide a quick, inexpensive, and desirable alternative to testing on larger animals. It is known that a positive correlation exists between brine shrimp lethality and 9KB (human nasopharyngeal carcinoma) cytotoxcity; brine shrimp are therefore used in many prescreens for potential anti-tumor activity. In addition, brine shrimp bioassays effectively predict pesticidal activities (a property pertinent to our own study) and respond to a broad range of chemically and pharmocologically diverse compounds (McGlaughlin, 1991).

Bioassays such as this are used for a variety of purposes – in general they allow researchers to screen for plant chemicals that show biological activity – that is, that show a targeted biological effect (e.g. effects on survival, growth, or reproduction) on selected research organisms. For example, botanists and biochemists are currently using bioassays to screen unstudied plants for potentially useful, naturally occurring drugs. In particular, the rapid rates of destruction of tropical rainforests and new research suggesting potential sources of medicinally beneficial chemicals in the tropics have precipitated increased funding for botanical research by the National Institutes of Health and several major pharmaceutical companies. The US National Cancer Institute has also launched an intensive effort in "chemical prospecting", especially in the tropics. This agency alone is screening some 10,000 substances/year for activity against cancer cells, HIV, etc (Wilson, 1989).

While it is unlikely (but not impossible) that we will discover an unidentified cancer-fighting chemical in the plants investigated, it is important for us to keep in mind that the reason most of those chemicals exist in the first place is the result of coevolution between plants and herbivores. The more we know about "who" makes those chemicals, why they are made, how they are made, and the consequences of allelochemics to both parties involved, the more we will know about the evolutionary process and about potential practical uses of such chemical evolutionary products for our own benefit.

Week 2

Protocol for Brine Shrimp Assay -- Day 0

1. Divide your group of 4 students into two pairs. One pair will run the assay for one of your samples (e.g. the ripe fruit = R) and the other pair for the other (e.g. the unripe fruit = U). Each team of 2 will follow all of the procedures below, separately. Use separate data sheets to record results for your 2 samples (e.g. A and B).

Obtain 10 vials and a small amount of your plant specimen (either R or U) for preparing an extract. Label the vials 1-10 and EITHER R or U (or whatever letters you choose to differentiate your samples).

We will run two different kinds of control treatments in this experiment. The water control (vial #1) will allow us to determine if there are significant effects of the plant chemicals as compared with the "normal" survival of brine shrimp over a 24-hr period. Why is this necessary? Do we expect all brine shrimp placed in seawater in small vials to survive? In addition, vial #2 is a methanol control; it will allow us to gauge the effect of one portion of our protocol (the dissolving of plant material in methanol) independently from the effects of plant material. This is necessary because methanol is likely to be toxic to brine shrimp; if not all the methanol is removed during the evaporation procedure, then an additional variable affecting brine shrimp survival has been introduced. Results from which 2 vials will be compared to test whether or not we have introduced a "methanol effect" in brine shrimp survival? Why then are both control treatments required?

2. Mark vial #1 as the water control, vial #2 as the methanol control, vial 3-6 with 100 micrograms/ml and vials 7-10 with 1000 micrograms/ml. These are the two final concentrations of the plant extract you will test.

3. Carefully dry a "mortar" and "pestle" (well plate and test tube). Remove all traces of animal material from your plant specimen and place the plant specimen in the bottom of the well plate. Grind it thoroughly for no less than 5 minutes. Weigh out 40 milligrams (not micrograms or grams) of the plant material on an electronic balance and then transfer the 40 mg sample to a small beaker or vial. Take the sample to the fume hood. The methanol is in the fume hood because it is highly flammable and toxic. It is important to avoid contact with your skin and eyes. Remind yourself of the location of the eyewash stations, just in case. Methanol is being used as a general solvent for the chemicals present in the plant tissue. Carefully add 4 ml of 100% methanol to the plant material. What is the concentration of your extract at this point? (40 mg/4 ml = 10 mg/ml) Allow the material to dissolve in the methanol for 5 minutes.
Ready several airlines for evaporating the methanol from samples you will prepare later. You will want a gentle stream of air coming out of each of the glass pipettes attached to the airlines. Test for "gentleness" by trying the air stream in a beaker of tap water FIRST.

4. Obtain 2 micropipettes for measuring 50 microliters and 500 microliters, respectively. Familiarize yourself with the use of the micropipettors by practicing with small aliquots of water.

5. When the 10 mg/ml plant extract has dissolved in the methanol for 5 minutes, add 50 microliters of the mixture to EACH of the 4 vials labeled 3, 4, 5 and 6, and add 500 microliters to EACH of the vials labeled 7, 8, 9, and 10. Do not add any extract to water control tube #1. To methanol control tube #2, add 50 microliters of plain methanol (not the plant extract mixture).

6. Evaporate the methanol from vials 2-10, starting with the vials containing the most methanol. To do this, insert the tip of the glass pipette/airline into the bottom of the vial and gently bubble air through the sample until all liquid vanishes. This should take no longer than 20 minutes for the vials containing the greatest volume. Be ready with the next vial each time an airline is freed up. If you bubble too vigorously and lose some of your extract, you will need to prepare a new vial to replace it -- why?

Table 1. Calculating the final concentration of plant material in ug/ml for each sample vial.

Vial Number	Vol. of 10 mg/ml extract used per vial	ug of tissue in vial	Vol. of seawater	Final [tissue] in ug/ml
1	0	0	5 ml	0
2	0 (50 ul methanol)	0	5 ml	0
3,4,5,6	50 ul	500	5 ml	100 ug/ml
7,8,9,10	500 ul		5 ml	ug/ml

7. While the first few samples are evaporating, do the math below to see how the values in Table 1 were calculated. Fill in the blanks!

Sample calculation for final concentration for vial #3:

50 ul x (10 mg plant tissue/ml) = 0.05 ml x (10 mg/ml) = 0.5 mg

0.5 mg of plant material = 0.5 mg x (1000 ug/mg) = 500 ug of plant material

8. When a sample vial has been evaporated, add 1 ml of plain sea water (2% sea salts) to it.

9. Then add 10 healthy, live brine shrimp to each of the 10 vials (I will demonstrate how to catch and count them).

a. Be sure to add 1-2 ml of plain sea water with them.

b. Do not contaminate your vials with dead brine shrimp or eggs, because the assessment of toxicity is based upon survival as the dependent variable.

c. When you think you are finished with each vial, double-check by holding the vial under the dissecting scope to make sure that it contains no eggs, no dead shrimp, and 10 live shrimp.

d. Leave the screw cap off OR placed on the vial loosely -- brine shrimp need oxygen!

e. Find an extra vial and mark it at the 5 ml level. Using this vial as a "ruler", carefully add plain sea water to each of the 10 vials so that each contains a final volume of 5 ml. Make sure that all vials have exactly the same final volume; if you go over the mark, you will need to do that vial over again because the concentration will not be accurate.

10. Record Day 0 information and all of the headings on Table 2. Label the vial rack belonging to your group with your group name, the date, and your lab section (day). I'll tell you where to store your rack.

Protocol for Brine Shrimp Survival Counts -- Day 1

Return to the lab 24 hours after beginning the cultures to count the shrimp in all 10 vials. Bring this lab handout with you.

1. Count each vial one at a time, as follows: Pour out your shrimp into watch glasses. Count the numbers of live and dead shrimp. If your numbers don't add up to 10, recount (don't erase your first numbers). Dead shrimp fall apart quickly -- don't mistake two halves of a body as two dead shrimp. At least make sure you have an accurate count of live shrimp.

2. Fill in Table 2 completely.

3. Pour out your shrimp vials into the sink, rinse each vial thoroughly, and place the vials and racks in the designated area. If another lab group is using the room, make sure you don't interfere with their work.

Table 2. Brine shrimp bioassay data sheet.

Group Members ____

Lab Day ____________________________

Tissue type (ripe, unripe, etc) ____________________

Vial #	Final [ ] (ug/ml)	# Live Shrimp Day 0	# Live Shrimp Day 1	# Dead Shrimp Day 1	% Survival
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					

Week 3: Data Analysis and Presentation

The simplest way to analyze our data is by using JMP to do a Fisher Exact test. The Fisher Exact test is a 2 x 2 contingency table. The concept is pretty simple: Each of your columns has only two possibilities: shrimp are either alive or dead, the extract they were in were either tissue A (e.g., leaf) or tissue B (e.g., fruit). That means 4 possibilities (alive in fruit, dead in fruit, alive in leaf, dead in leaf). You can build a nice little 2 x 2 table with A&D on the top and F&L on the sides. The trick to this analysis is that you can't just plug in your results. Bummer. You actually have to have a row for every single shrimp! But by pasting, it goes really quickly. To make things go even faster, split your group up. Two of you do the following using the LOW concentration data (see example below); the other two do it using the HIGH concentration data. Whoever gets done first can start the control analyses (to increase your statistical power, pool the data from your high and low concentration controls). Remember to save your data sets!

Open JMP to a blank spreadsheet. You will use this for your comparison in the LOW concentration treatment. To name the column and set up the parameters, double click on the empty area just above the words "column 1" and between the 2 little boxes. Name the column and set it for character data. Once this is done, go to "add rows" and add 80. Let's assume you were comparing leaf vs green fruit. So that's 40 shrimp in the 4 leaf vials and 40 in the 4 fruit vials (as you can see, we're lumping the data from the 4 replicate vials. There are more complex statistical tests in which you keep them separate and can thus test for the effects of the specific vials, but we're not going to worry about that).

Go to the first row of the Tissue column. Type in "Leaf". Then select that cell and copy. Then select cells 2-40. Hit paste and they should all say Leaf. Now do the same for rows 41-80, except put in the word "Fruit". Now you're ready for column 2. Make the data type character and call it "Fate". Here's the tricky part. If you had a total of 30 of your 40 Leaf shrimp (in the low concentration experiment, of course) alive the next day, type in the word “Alive” in the first cell and paste it in down to row 30. Put the word “Dead” in the cells in rows 31-40. Now do the same alive/dead thing with your data in the Fruit portion of your Fate column. Now go to the Analyze menu, choose fit y by x, assign tissue to “x” and fate to “y” and poof, it will give you your results, both in a figure and as stats.

In the LAP lab, your research hypothesis for the genotype frequency comparison was (for most of you) “population X is different from population Y.” The null hypothesis was “population X is NOT different from population Y.”

For this lab, you weren’t just predicting that tissue A had a DIFFERENT level of toxicity than tissue B; you had a reason to think that one tissue type might be MORE toxic than the other (and here the null hypothesis is “A is not more than B”). The probability that something is “different” is different than the probability that something is “more.” So your P values for a two-tailed test (looking at both “tails” of the bell-shaped frequency distribution) are different from the P values of a one-tailed test (looking at only one end of the frequency distribution). Moreover, the P value also depends on which you predict is greater. We’ll talk about this more in lab, but the rule of thumb is that if your research hypothesis was supported (look at the JMP graph), you’ll go with the lower of the one-tailed P values (for now, don’t worry about “right” and “left”. If the results were the opposite of what you predicted, you’ll go with the greater of the one-tailed P values. Note that it is easier to get a significant result with a 2-tailed test (a test of difference) than it is with a one-tailed test (a test of directionality). In your lab report, you will report your sample size for each analysis along with the Fisher Exact p-value.

You will end up doing 5 different analyses. For example:

1. [Low] Leaf vs. [Low] Fruit
2. High Leaf vs. High Fruit
3. Low Leaf vs. High Leaf
4. Low Fruit vs. High Fruit
5. Water Control vs. Methanol Control

The first two represent your initial research hypothesis (you’re separate analyses of low and high concentrations in case your high concentration is too toxic or your low concentration is not toxic enough). The second two represent an analysis of whether the concentration makes a difference. For these, our hypotheses will be that the higher concentration is more toxic.

For your controls, compare the mortality in your pooled water vs. pooled methanol controls. If the mortality in your methanol controls is significantly greater, that would suggest that some of the mortality you observed in your tissue comparisons was due to the toxicity of methanol, not the toxicity of the plant tissue. While this would be problematic, it would not negate your ability to draw some conclusions from your other comparisons. Why not? Unless you get a dramatic difference between your water and methanol control, don’t worry about making a figure for it. Just report your results in the text of the Results section.

Make two figures in Excel for your lab report. The first should include the results from #1 & 2 above. The second should include 3&4. The Y-axis should represent survivorship or mortality (proportional – that’s decimals). Remember, you’ll want a row labeled as “low” and another as “high” and columns labeled as “leaf” and “fruit”. Fill in the cells with the proportion alive (or dead – but not both – that would be redundant). Your lab group may work together to make the figures and legends, but the rest of your lab report will be your own work. Because the study of secondary compounds is such an active field, I expect your 5 journal citations to be different from the ones I list below.

Each group will make a brief oral presentation in lab NEXT week (the week after data analysis).

Lab reports will be due the following week (19 and 20 October).

Table 3. Summary of results

Comparison	Fisher Exact P	Accept Null?	Conclusion
Water vs. methanol control (pooled)			
100 ug/ml			
1000 ug/ml			

Questions:

1. What is the relationship between the concentration of plant extract and percent shrimp survival (if any) and what is the biological significance of this?

2. Even if the data support your hypothesis, is your hypothesis the ONLY explanation for what you found, or are there still other possibilities? If so, name some.

3. If producing anti-herbivore chemicals is beneficial to plants, then why don't all plants produce lots of them all the time? (In other words, think about what the costs might be to the plants of producing such chemicals).

LITERATURE CITED

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