A Methanol-Free Method of Extracting Secondary Chemicals

Jordan P. Sarver[1], Lawrence S. Blumer[1], and Marius K. Denton[2]

[1]Department of Biology
Morehouse College
830 Westview Dr, SW
Atlanta, GA 30314
lblumer@morehouse.edu

[2]Department of Biology
Georgia State University
24 Peachtree Center Ave. NE
Atlanta, GA 30303
mjkcd1@aol.com

Abstract: This experiment examined the inducible responses of tobacco. Leaf toxicity was assessed by a brine shrimp bioassay. A new method of extraction was used in this bioassay. Previously, secondary chemical were extracted from tobacco leaves using methanol. Our new method used a boiling 1% NaCl solution for secondary chemical extraction. This experiment was a test of this new methanol-free extraction method. Three concentrations of extracted secondary chemicals were evaluated by using volumes of boiling 1% NaCl extracts ranging from 1ml to 3ml. This new method is both safer and quicker than methanol extraction, and yielded very robust bioassay results.

Introduction

Plants manifest defenses in a number of forms, including chemical, physical, and temporal defenses. Chemical defenses consist of secondary chemicals (not required for normal metabolism) that may be either, constitutive, induced, or both. Constitutive defenses are those that are always produced by the plant. Induced defenses are those that become activated or mobilized in the event that the plant is injured. This experiment tested the hypothesis that tobacco plants (*Nicotiana*) produce chemical defenses and that such defenses are inducible. This protocol was presented at ABLE 2006 (Blumer, Denton, and Brooks, 2007). A novel approach to chemical extraction was

tested in this experiment. Using a boiling NaCl solution instead of methanol, we evaluated plant extracts that were immediately ready for brine shrimp bioassays (Winnett-Murray, Hertel, and Murray, 1997). This new method provides a safe and quick way to perform secondary chemical extractions for bioassays.

Student Outline

Induction of Secondary Chemical Defenses

Objectives

1. Evaluate the presence of toxins in the leaves of tobacco plants (*Nicotiana*).
2. Perform an experiment to address the question: Does leaf damage, such as that caused by herbivores, induce an increase in secondary compound toxin concentration in tobacco?
3. Address the question: Is there a difference in the response of tobacco plants to physical damage alone and actual herbivore damage?

Introduction

At first glance, plant-herbivore interactions seem to be a highly unequal interaction between a mobile and responsive predator attacking an immobile and helpless prey plant. Yet, first impressions can be deceiving. Plants, in fact, are not helpless prey. Although they are sessil, most plants produce two types of defenses, physical and chemical. The timing of life history events, such as the production of flowers and fruits, can also be considered a form of defenses (for example, seed masting). Physical defenses include increased tissue toughness by means of cellulose and the production of defensive structures such as hairs, spines and thorns. Chemical defenses are part of an extremely diverse collection of compounds that are not part of the metabolic processes that plant require for their growth and maintenance in the absence of herbivores. Given the accessory nature of these chemicals, which include non-photosynthetic pigments and defensive chemicals, these compounds are termed secondary chemicals and secondary chemical defenses.

Thousands of secondary chemicals have been identified in plants and many have clearly demonstrated defensive functions (anti-herbivore, anti-microbial or anti-fungal activity) (Feeney, 1992, Harborne, 1993, Whittaker and Feeney, 1971). These chemicals include nitrogen compounds, terpenoids, and phenolics, and include chemicals that are important in human affairs. Compounds traditionally used as spices are often anti-microbials (Billings and Sherman, 1998). The pleasure we get from consuming the plant products coffee, tea and chocolate is provided by a nitrogen compound, caffeine (an alkaloid), which is produced by plants to poison their herbivores. Cocaine, morphine and nicotine are in this same class of secondary chemicals.

The complexity of many secondary chemical defense compounds and the use of limiting nutrients (particularly nitrogen) in many of these compounds has long suggested that chemical defenses are costly for plants to produce and maintain (Karban and Baldwin, 1997). Such defense expenses could be minimized if plants could produce expensive chemical defenses only when they were needed (Baldwin, 1998). Experimental evidence for rapidly inducible chemical defenses, producing or increasing chemical defenses in response to an initial herbivore attack, is very clear in a

wide variety of plants (Karban and Baldwin, 1997). The interactions between herbivores and plants occur in both ecological (the life span of a given organism) and evolutionary time. The production of an effective chemical defense may be overcome by a specialist herbivore that selectively feeds on the least defended parts of a plant, or evolves detoxification mechanisms that permit it to overcome a particular defensive chemical. The occurrence this kind of plant-herbivore arms race and the resulting co-evolution of chemical defenses and herbivore specializations has produced some of the most unusual and interesting plant-animal interactions (Futuyma and Keese, 1992). Some specialist herbivores even use the toxins of their host plant for their own protection (Harborne, 1993, Sotka, Wares, and Hay, 2003).

In this study, we will use a bioassay to evaluate the toxicity of leaves from tobacco plants (*Nicotiana alata* or *N. tabacum*) and determine whether herbivore and herbivore-like damage will induce an increase in toxic secondary compounds. A specialist herbivore on tobacco, larval tobacco hornworms, *Manduca sexta*, will be used as natural herbivores in your experiment (Villanueva, 1998). We will employ a Brine Shrimp Bioassay (Winnett-Murray, Hertel, and Murray, 1997) to evaluate the toxicity of leaf extracts. Brine shrimp (*Artemia salina*) larvae, or nauplii, are commonly used in toxicological studies as a humane and inexpensive proxy for vertebrate animals. The 24-hour brine shrimp nauplii bioassay will provide a fairly rapid measure of leaf toxicity that would not be possible using the natural herbivores of tobacco. However, tobacco hornworms could be used in a more natural bioassay. How might tobacco hornworms be used to evaluate the toxicity of the tobacco plants in this study? Design a protocol for a tobacco hornworm bioassay.

We will address three questions in this study:
1. Do tobacco plants produce a toxic compound in their leaves?
2. Does herbivore damage to tobacco leaves induce the increased concentration of toxic compounds in other leaves on that plant?
3. Does physical damage cause the same responses by tobacco plants as herbivore damage?

Methods and Materials

Pretreatment

At least one week prior to preparing the leaf extracts, each group will perform pre-treatments

on three tobacco plants. Label one plant "undamaged", a second " insect damaged", and a third “physically damaged”. The undamaged leaf should receive no manipulation. Treat the plants as described in below (Table 1). One week after pre-treatment, harvest one leaf from each plant (but not a leaf that was directly damaged) and prepare as described in Leaf Extract Preparation below.

Table 1. Pre-treatment of Tobacco Plants

Treatment Name	Method
Undamaged	Do not damage any leaves.
Physical Damage	Make fabric wheel tracks on each of four leaves.
Insect Damage	Put one tobacco hornworm larvae on the plant for 24 hours.

Leaf Extract Preparation

Extracts of tobacco leaf material can be made by grinding 250mg leaf sample in a glass mortar with a small pinch of sand and 25ml of boiling 1% NaCl solution. Use only clean and dry mortar and pestles, so you do not contaminate between leaf sources. This method will create extracts that contain 10mg/ml (250mg in 25ml of solvent).

Prior to your class meeting, design an experiment to evaluate the potential toxicity of the leaf extracts. A general protocol for evaluating toxicity with a brine shrimp bioassay is described below. You will design an experiment using the brine shrimp bioassay. Come to class ready to present your experimental design and discuss the experiment that the entire group will conduct. Be sure to address the following issues:

What will serve as a control for the experiment?

How much replication will be performed?

What will be the final concentrations of leaf extract in the bioassay?

Brine Shrimp Survival

You will be using 8ml sample vials to conduct your brine shrimp bioassays. Each and every vial will have a **final volume of 5ml** including any volume of liquid used to transfer brine shrimp nauplii to the vial. Each vial can reasonably hold a total of 10 brine shrimp nauplii for a 24-hour period to evaluate survival.

Past experience has indicated that 1.0ml, 2.0ml, and 4.0ml of tobacco leaf extracts, prepared using boiling 1% NaCl, in final volumes of 5ml are appropriate for evaluating toxicity. Start by pipetting the appropriate volume of leaf extract to each vial (make sure your vial is marked with the extract source and concentration). Keep your vials in a vial rack to keep them from spilling.

Using a dissection microscope, carefully isolate groups of 10 vigorously swimming brine shrimp nauplii in small volumes of 1% NaCl. Use glass Pasteur pipettes to isolate and count the nauplii. To each of your bioassay vials, transfer 10 vigorously swimming brine shrimp nauplii. Do not transfer dead animals or unhatched eggs. After you transfer the animals to a vial, examine the vial to be sure there are 10 nauplii present.

Bring the volume of each vial up to 5ml with 1% NaCl. Use a vial containing a measured volume of 5ml as a measurement standard. Screw the cap **loosely** on each vial. The brine shrimp nauplii require oxygen like any other aerobic organism.

After 24 hours, count all brine shrimp nauplii in each vial. Record the number alive and the number dead. Be sure to account for all 10 individuals who went into each vial, animals may get stuck to the vial when it is emptied.

Data Analysis

Start by calculating the mean number of brine shrimp alive and dead (at each concentration) that were from the same individual plant source. This will be the mean of your vial replicates for a given plant source and concentration. Different plants are the independent cases in this experiment.

Now, prepare an Excel spreadsheet file with your mean values for brine shrimp survival. This spreadsheet data file should have the following column headings: Your Name, Extract Source (Plant Treatment), Plant Replicate Number, Extract Concentration, Number Alive, and Number Dead. You will enter the mean number of alive and dead brine shrimp from each category of plant source and concentration. Using the spreadsheet equation function, calculate the percentage of brine shrimp nauplii surviving in each vial for 24-hours.

Data will be analyzed using an analysis of variance (ANOVA) test to compare the brine shrimp survival at different concentrations of extract (for a given plant treatment) and to compare brine shrimp survival between plant treatments (at a given extract concentration). After everyone has entered their data, you will be provided with a complete data file (Induction Raw Data) containing the means (that you entered) of brine shrimp that were alive and dead after 24 hours exposure to each treatment. The output of the ANOVA test also will be provided to you.

Keep in mind that the null hypothesis for this test is no relationship between the concentrations or treatments being compared and the number of brine shrimp alive after 24 hours. A **p** value of less than 0.05 means you can reject the null hypothesis of no difference between the treatments being compared.

Literature Cited

Baldwin, I.T. 1998. Jasmonate-induced responses are costly but benefit plants under attack in native populations. Proceedings of the National Academy of Sciences USA 95:8113-8118.

Billings, J., and P.W. Sherman. 1998. Antimicrobial functions of spices: why some like it hot. Quarterly Review of Biology 73:3-49.

Feeney, P. 1992. The evolution of chemical ecology: Contributions from the study of herbivorous insects. Pages 1-44, *in* Herbivores: Their Interactions with Secondary Plant Metabolites. Volume 2, Ecological and Evolutionary Processes. 2nd Edition. (Rosenthal, G.A., and M.R. Berenbaum eds.). Academic Press, New York, NY, 493 pages.

Futuyma, D.J., and M.C. Keese. 1992. Evolution and coevolution of plants and phytophagous arthropods. Pages 439-475, *in* Herbivores: Their Interactions with Secondary Plant Metabolites. Volume 2, Ecological and Evolutionary Processes. 2nd Edition. (Rosenthal, G.A., and M.R. Berenbaum eds.). Academic Press, New York, NY, 493 pages.

Harborne, J.B. Introduction to Ecological Biochemistry. 4th Edition. Academic Press, New York, NY, 318 pages.

Karban, R., and I.T. Baldwin. 1997. Induced Responses to Herbivory. University of Chicago Press, Chicago, 319 pages.

Sotka, E.E., J.P. Wares, and M.E. Hay. 2003. Geographic and genetic variation in feeding preference for chemically defended seaweeds. Evolution 57:2262-2276.

Villanueva, R. 1998. Tobacco hornworm, *Manduca sexta* (Hawworth), and tomato hornworm, *Manduca quinquemaculata* (Lineaeus). University of Florida Cooperative Extension Service, Publication EENY-031, 4 pages (http://creatures.ifas.ufl.edu/field/hornworm.htm).

Whittaker, R.H., and P.P. Feeney. 1971. Allelochemics: Chemical interactions between species. Science 171:757-770.

Winnett-Murray, K., L. Hertel, and K.G. Murray. 1997. Herbivory and anti-herbivory: Investigating the relationship between the toxicity of plant chemical extracts and insect damage to the leaves. Pages 249-271, *in* Tested studies for laboratory teaching, Volume 18 (J.C. Glase, ed.). Proceedings of the 18th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 320 pages. (Available at www.ableweb.org).

Instructor's Notes

Experimental Design

We have conducted this experiment with upper-level ecology students. Although the experimental design of damaged and undamaged plants is a given in this study, it is both possible and reasonable to ask students to design a protocol to address the question: Does leaf damage, such as that caused by herbivores, induce an increase in secondary compound toxin concentration in tobacco? The design of the brine shrimp bioassay is suggested to include three concentration levels of leaf extract, but also should include a control treatment that contains no extract, only 1% NaCl. Replication within each bioassay treatment is always a good idea and a minimum of three replicates in each treatment would use 21ml of the 25ml of extract prepared by each student. More replication with a given extract could be performed if more extract were prepared, but keep the leaf mass to solvent ratio at 10mg/ml.

Materials for a class of 24 students, working in groups of 3

- Dissection microscopes: 24 needed, one per student to isolate and count brine shrimp nauplii.
- Analytical balance to weigh 250mg quantities. Use aluminum foil to make small weigh boats.
- Plants: Each group needs three plants so 24 individually potted flowering tobacco (*Nicotiana alata*) are needed. They can be purchased at a garden shop ($3.00 per potted plant) or grown in advance from seed (*Nicotiana* seeds available from W. Atlee Burpee and Co. for 1.00 - $4.00 US per packet of 50-100 seeds). If started from seed, start several months (two or more months) prior to use date. *Nicotiana* plugs also can be purchased (Park Wholesale 800 845-3366). Plugs are sold in trays of 125 and may need to be ordered 4-months prior to the desired delivery date. Transplant plugs to 3" pots and grow for one-month prior to use in this experiment. We have found that plugs and garden shop plants are most easily purchased in spring and early summer so the lead-time will be much shorter then.
- Tobacco hornworm larvae: Only eight larvae are needed to create the insect damage treatment. Live larvae can be purchased from Carolina Biological (Tobacco hornworm larvae, living, pk 12, #14-3882, $34.75 US + shipping in 2005). See Blumer, Denton, and Brooks (2007) for notes on maintaining larvae until use in the experiment and on conducting the tobacco hornworm damage treatment.
- Brine shrimp nauplii: A total of 2880 newly hatched nauplii will be needed for a class of 24 Brine shrimp "eggs" are available from Carolina Biological (Brine shrimp eggs, 1 oz bottle, 14-2240, $6.45 US). See Blumer, Denton, and Brooks (2007) for techniques on hatching large numbers.
- Fabric marking wheels: one per group or 8 total needed. Serrated tracing wheel Dritz #745 are the correct size and are available at fabric and sewing supply stores (Jo-Ann Fabrics $3.00 each).
- 2-dram screw cap vials: Each group will need 36 vials so a total of 288 vials would be needed for 8 groups. An alternative to using individual vials is a 12-well plastic tissue culture plate. Each well would replace one vial so one plate would be needed for each run a 12-vial bioassay. Each group would need three plates and a total of 24 plates would be needed for 8 groups.
- Vial racks: 24 to hold 2 dram vials
- Glass mortars and pestles: 24 needed, 2 oz size is ideal

- Short disposable glass Pasteur pipettes 48 needed and 24 latex bulbs
- Graduated pipettes (1ml, 5ml, and 25ml) with pipette helpers: Need 24 pipette helpers and 24 graduated pipettes of each size.
- 50ml beakers (24) for boiling 1% NaCl and for transferring 1% NaCl from supply bottle to bench for distribution in bioassay vials
- Hot plate for boiling 1% NaCl
- 25ml or 50ml graduated cylinders (glass) for measuring boiling 1% NaCl (24)
- Small plastic Petri dishes for isolating and counting brine shrimp nauplii: 35 x 10mm (Falcon 351008) 144 should be more than enough if both lids and dishes are used to hold nauplii being counted for the start of the bioassay and are reused after animals are transferred to vials. These same dishes will be needed to count the nauplii at the end of the bioassay. Plastic tissue culture plates may be used instead of individual Petri dishes to temporarily hold the counted nauplii (and to save on supply funds). Twelve-well plates would be appropriate for this task.
- Small scissors and single edge razors 24 needed
- Marking pens to write on glass vials and Petri dishes (24 needed)
- 1% NaCl solution in deionized water: The water used to make this solution must be aquarium quality free of chlorine and metal ions that would be toxic to the brine shrimp nauplii. The volume needed for each group will be 180ml so the entire class of 8 groups would need approximately 1.5L. Prepare 3-4L since a minimum of 2L will be used to hatch the brine shrimp nauplii (see Blumer, Denton, and Brooks, 2007) two days prior to the day the bioassay will be started.

Counting and Transferring Brine Shrimp Nauplii

Detailed advice on counting and transferring brine shrimp nauplii are in Blumer, Denton, and Brooks (2007).

Previous Results

Students in the Morehouse College Ecology Laboratory course, BIO 320L, collected these data in the Spring 2007. The bioassay only was conducted on undamaged plants, but the results

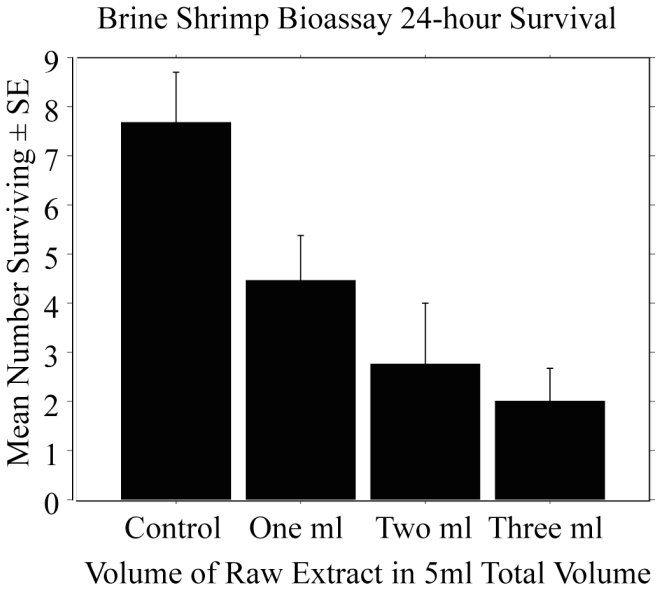

Figure 1. There was a highly significant (ANOVA F=6.198 p=0.0013 df=3) decrease in survival in response to increasing concentrations of leaf extract from undamaged plants (n=8). Controls contained no leaf extract.

(Figure 1) clearly show the negative effect of extract concentration on 24-hour brine shrimp survival.

Acknowledgments

The bioassay part of this study is based on a protocol developed by K. Winnett-Murray, L. Hertel, and K.G. Murray. 1997. Herbivory and anti-herbivory: Investigating the relationship between the toxicity of plant chemical extracts and insect damage to the leaves. Pages 249-271, in Tested studies for laboratory teaching, Volume 18 (J.C. Glase, ed.). Proceedings of the 18th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 320 pages.

This secondary chemical defense induction protocol was developed by L.S. Blumer, M.K. Denton, and L.E. Brooks. 2007. Induction of Secondary Chemical Defenses. Pages 1-16, *in* Tested Studies for Laboratory Teaching, Volume 28 (M.A. O'Donnell, Editor). Proceedings of the 28th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 403 pages. The brine shrimp bioassay was modified in 2007 by J. Sarver to replace methanol extraction with a boiling NaCl extraction.

Literature Cited

Blumer, L.S., M.K. Denton, and L.E. Brooks. 2007. Induction of Secondary Chemical Defenses. Pages 1-16, *in* Tested Studies for Laboratory Teaching, Volume 28 (M.A. O'Donnell, Editor). Proceedings of the 28th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 403 pages.

Winnett-Murray, K., L. Hertel, and K.G. Murray. 1997. Herbivory and anti-herbivory: Investigating the relationship between the toxicity of plant chemical extracts and insect damage to the leaves. Pages 249-271, *in* Tested studies for laboratory teaching, Volume 18 (J.C. Glase, ed.). Proceedings of the 18th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 320 pages. (Available at www.ableweb.org).

About the Authors

Jordan Sarver is a graduating senior Biology major at Morehouse College. He plans to pursue a Master's Degree in Environmental journalism to increase awareness about environmental issues and offer solutions for change.

Larry Blumer earned his Ph.D. from the University of Michigan in 1982 and he is Professor of Biology and Director of Environmental Studies at Morehouse College. He teaches Ecology, Environmental Biology, and Introductory Biology.

Marius Denton earned a BS in Biology from Morehouse College in 2006. A native of West Memphis, AR, he is presently a graduate student at Georgia State University in Atlanta.