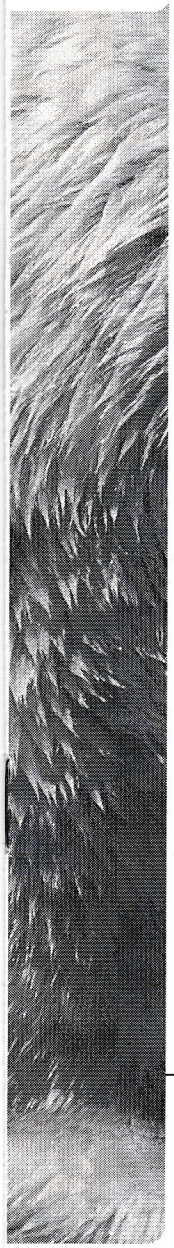

Brine Shrimp Bioassays:

A Useful Technique in Biological Investigations

STANLEY A. RICE IAN B. MANESS

The measurement of toxicity is very useful in biological, especially ecological, investigations (Opler et al., 2002). Some examples include:

1. toxicity of possible sources of pollution, such as runoff and leachate
2. potency and possible danger of new pesticides
3. screening for plant extracts of possible medicinal value
4. determining the anti-herbivore defenses in leaves.

STANLEY A. RICE is in the Department of Biological Sciences at Southeastern Oklahoma State University, Durant, OK 74701-0609; e-mail: srice@sosu.edu. IAN B. MANESS is in the Alvin Independent School District, Alvin, TX 77511; e-mail: IManess@alvin.isd.tenet.edu.

These investigations may overlap; for example, anti-herbivore compounds in leaves and bark often have medicinal value. Vincristine and vinblastine from *Catharanthus roseus,* the Madagascar periwinkle, are now used to treat some forms of leukemia (Wedge & Camper, 2000). There are hundreds of other examples of medicinal extracts from leaves and bark, used both in traditional shamanistic practices and in modern medicine (Maxwell, 1990; Plotkin, 1994). Anti-herbivore compounds may also have anti-fungal effects. Therefore these compounds may be useful as pesticides and fungicides (Karban & Kuć, 1999), and even as herbicides (Rimaldo, Personal Communication).

There are at least three approaches to measuring the potency of leaf compounds against herbivores. The first is to measure the compounds directly. After an extract is made from the leaves, the compound of

interest can be measured by titration, spectrophotometry, or by more advanced techniques such as high-performance liquid chromatography. In some cases, these measurements are fairly simple. For example, tannin concentration in leaf extracts can be measured with a simple spectrophotometer after treatment with saturated sodium carbonate and Folin-Denis reagent (Williams, 1984). It is not necessary to isolate the tannins from the extract. However, this approach is limited in its usefulness to biology instructors; first, because the techniques are often complex, and may require expensive equipment that few students can use. In some cases, the compound must be separated from all the others in order to be measured. Second, you need to know which chemical to measure. The compounds that contribute most to anti-herbivore activity in many leaves are not known. Third, a knowledge of the concentrations of major compounds in leaves will not directly indicate how these compounds interact to produce a toxic effect.

The second approach is to use a bioassay (the approach featured in this article). A bioassay measures the growth or survival of a "naive" organism (e.g., one that has not been exposed during its evolutionary history to the source of the compounds) as a measure of the toxicity of the extract. One of the most commonly used bioassay organisms is the brine shrimp *Artemia salina,* which lives in brackish water. It has not, during its evolutionary history, been exposed to land plants, and shows great sensitivity to many compounds from leaf extracts. It is therefore not necessary to know what the compounds are. High concentrations of an extract will kill all the shrimp (100% mortality); low concentrations will kill none of the shrimp (0% mortality); the concentration of the extract that kills 50% of the shrimp is the LD_{50} (**lethal dose**) or LC_{50} (**lethal concentration**). Dried eggs of brine shrimp are widely available in pet stores and from biological supply companies, and can be easily hatched. Students can count live and dead shrimp in numerous vials under a dissecting microscope, each vial representing a different extract source or different concentration.

The third approach is to expose the leaves or the extract to organisms that would normally encounter it. This has the advantage of providing a more realistic assessment of the ecological effects of the toxicity in the leaves, but does not actually measure the toxicity. For example, an assay using monarch caterpillars would indicate that milkweed leaves are not toxic.

These approaches can be used in combination with one another. For example, some of the suspected toxins of leaves can be separated and measured, then brine shrimp can be exposed to each of these toxins separately. This approach is used in toxicity research (Wedge, Personal Communication) but goes beyond the limitations of most educational biology laboratory activities.

In this article, the techniques for using brine shrimp bioassays as ecology and botany laboratory activities are presented, as well as three examples of hypotheses tested by these bioassays.

Techniques

Each of these steps may be carried out by either students or the instructor, depending on the time allotted for laboratories. If the students perform all the steps, four meetings are required: first, for preparation of extracts; second, for evaporation of subsamples and preparation of shrimp; third, for introducing shrimp to the residues; fourth, for counting. This technique was adapted from Aranson et al. (1991).

Preparation of Extracts

1. **Leaf material.** Obtain fresh leaf material. In testing hypotheses, you will need replicate leaves and different species or treatments. To prevent degradation or enzyme activity, extractions should be carried out right away or on leaves stored at temperatures of -70 °C or lower.

2. **Extraction.** Cut about a half gram of leaf material (of known mass) and place it in a small closable glass vial (about 25 ml capacity works best). Put enough solvent into the vial (of known quantity) to cover the leaf fragments. Close the vial and allow extraction to occur for at least a day. The instructor or students should calculate the effective concentration of the extract: mass of leaf fragments divided by extraction volume, adjusted to mg/ml. The effective concentration is not necessarily the same as the actual concentration of any component compound of the leaf.

   Ethanol (70% v/v in water) is a solvent commonly used for whole extracts, because it extracts both water soluble and water insoluble compounds (though not all of them). This should be sufficient for hypotheses in which comparisons are made, where it is not necessary to extract all compounds (or all of any compound). A universal solvent consisting of acetone, formic acid, methanol, and water can be used, though this is more trouble than it is worth in most educational laboratories. In medical research, citric acid has been found to extract effectively without leaving a toxic residue (Perkins-Veazie, Personal Communication).

3. **Subsamples.** The ethanol is toxic, and the extract described above contains too much of the

leaf compounds for the bioassay. In order to solve these problems, a small subsample (usually 0.1 ml, removed with a 2-ml pipette and pipette-filler) should be saved, and the rest of the extract and leaves set aside for later use. The subsample should be placed in a new, and newly-labeled, vial.

In order to determine LC_{50}, it is necessary to produce a range of effective concentrations to which the shrimp are exposed. There are two components to producing this range of concentrations. The first is to have subsamples of different volumes: some 0.1 ml, some 0.2 ml, etc. The second component (see below) is to place different volumes of shrimp suspension in different vials. The instructor needs to plan ahead (see following steps) before telling the students (or having them figure) how much extract to use for each vial's subsample.

After the subsample is placed in the vial, it should be allowed to evaporate with the lid off, at least overnight and with adequate ventilation. This leaves a leaf extract residue, in which the traces of ethanol that remain are harmless to the shrimp.

It is also possible to obtain several samples from each original extract, and dilute each to a range of concentrations. This has the slight advantage of reducing the number of original leaves and extractions with which you began, but the great disadvantage of reducing the explanatory power of the results. A scatter plot of 60 points starting with only six leaves has no better explanatory power than those six leaves; the 60 points could be considered pseudoreplication (Hurlbert, 1984). Therefore it is better to have each data point used in the analysis to come from a separate leaf.

Preparation of Shrimp

1. Prepare a brine solution. Brine shrimp grow well at a range of brine solutions. The best brine solution may depend upon the place of origin of the eggs. We used 1% (1 g salt per 100 ml deionized water). (Tap water may contain chlorine or other materials that may affect the shrimp.) It may be a useful class project to hatch shrimp eggs at different brine concentrations to determine which concentration is best. You will need about 1 ml per bioassay vial. Heating is not necessary. In some instances, we have found shrimp eggs from commercial sources to have low viability: It is therefore best to perform a trial run of shrimp hatching before the laboratory activity.
2. Suspend about 0.1-0.2 g of eggs per 100 ml of brine. High shrimp concentrations may cause toxic waste buildup. We used a glass beaker. Many researchers and hobbyists hatch them in inverted 2-liter soda bottles with air bubbled into the suspension. However, bubbling is not necessary to induce hatching.
3. Stir initially and occasionally. Many shrimp will have hatched by one and a half to two days. It is not necessary for all the shrimp to hatch.
4. Estimate the number of hatched shrimp per ml of stirred brine, using a dissecting microscope (lit from below). The concentration of shrimp will vary depending on room temperature and other factors. If your suspension contains more than about 50 live shrimp per ml, you may dilute the suspension or part of the suspension with brine.
5. Without supplemental food, the shrimp will die after about four days.

Bioassay Setup

1. Calculate a range of concentrations for the bioassay in the following manner. If you have 500 mg of leaf fragments in 5 ml of solvent, the extract concentration is 100 mg/ml. If you save a 0.2 ml subsample, and allow it to evaporate, the residue corresponds to 20 mg of leaf tissue. If you expose 1 ml of shrimp suspension to this residue, the effective concentration is 20 mg/ml. To obtain a lower concentration of 10 mg/ml, you could either evaporate only 0.1 ml as your subsample, or use 2 ml of shrimp suspension. By adjusting subsample and shrimp suspension volumes, you should have a range of effective concentrations ranging from about 2 to about 20 mg/ml. Higher concentrations are likely to cause high mortality and not be very useful in determining the LD_{50} of the extract.

   Not all extracted materials will equally redissolve or resuspend into the brine. Chlorophyll and carotenoids, for example, will remain on the bottom of the vial. However, in these small volumes, the shrimp will come in contact with even the residue that does not redissolve.
2. For those vials that require more than 1 ml of shrimp, place an additional ml of brine into the vials before putting in the shrimp suspension.
3. Add 1 ml of frequently-stirred shrimp suspension to each vial. Plastic pipettes (supplied with shrimp eggs by some suppliers) work well. Note that it is not necessary to count the shrimp at this stage. Do not slosh the suspension, as both extract and shrimp will stick to the sides of the vial and be lost from the analysis.

4. Prepare a few vials with shrimp suspension as controls. The best controls are vials in which 0.1 ml of solvent has been allowed to evaporate.
5. Close the vial to prevent evaporation, which would cause the effective concentration to increase.
6. Wait at least 24 hours.

Counting Shrimp

1. If the vials have slanted sides (e.g., glass bottles), which would interfere with the students looking straight down into the vial, it may be helpful to provide small beakers and extra brine with which students can wash out the vials for observation.
2. The students should record live shrimp and dead shrimp in two data columns for each vial.
   - The live shrimp are easily recognized by their swimming. It may prove difficult to count moving shrimp, but most students manage to do it. They should start at one point (say, near "twelve-o-clock") and work their way around the vial or beaker. If there are 50 shrimp in the beaker, and the student miscounts by one, the resulting error is only 2%; thus this system is robust against small errors in counting.
   - Dead shrimp have their front appendages extended and are motionless. Not all motionless shrimp should be counted as dead; many shrimp may be in the process of hatching, and are recognizable because their appendages are not extended. In suspensions with low toxicity, many more shrimp will have hatched during the 24-hour bioassay period. In suspensions with high toxicity (due to toxic leaves or high concentration or both), many shrimp may have died before completely hatching.
   - Ignore the partially hatched shrimp, the unhatched eggs, and the empty shells.
3. After each vial is counted, the students can discard the suspension in a waste beaker and rinse the vial to make cleanup easier.

Calculations & Graphing

If a computer spreadsheet is available, it can calculate the proportion of surviving shrimp from columns of input data. In the graph, the percentage of surviving shrimp is a function of effective exposure concentration. The data should show a negative slope tendency.

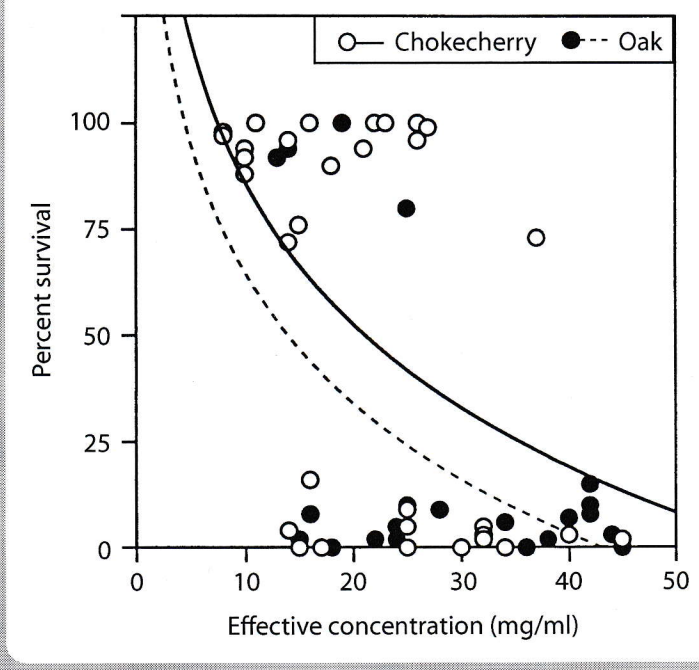

Figure 1.
Bioassay of leaf toxicity of chokecherry (*Prunus virginiana*) and bur oak (*Quercus macrocarpa*). Percent survival of bioassay shrimp is presented as a function of effective concentration (mg leaf material per ml of shrimp).

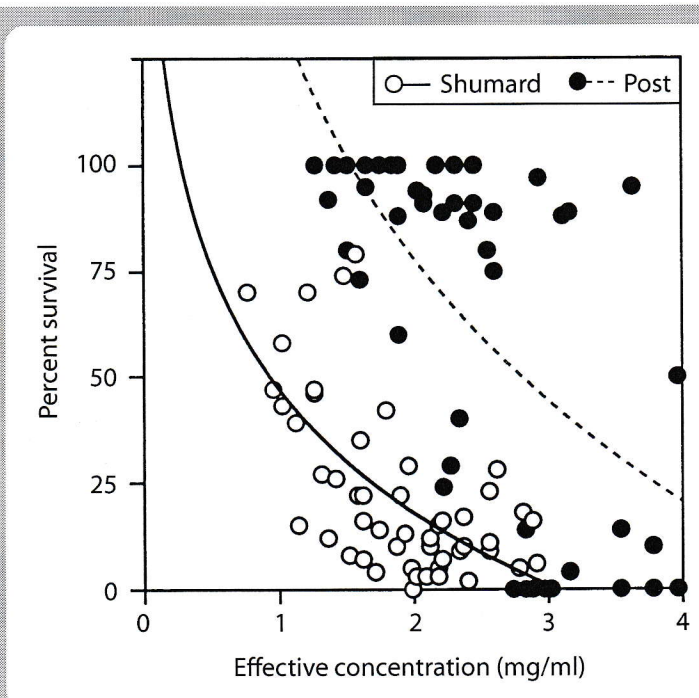

Figure 2.
Bioassay of leaf toxicity of shumard and post oak (*Quercus shumardi* and *Q. stellata*). Percent survival of bioassay shrimp is presented as a function of effective concentration (mg leaf material per ml of shrimp).

Draw a regression line by eye, or have the computer generate a regression line, depending on the mathematical background of the class. LC_{50} can be estimated from the regression line: the x-value at which y = 50%. Higher LC_{50} means lower toxicity.

Because percentages are constrained between 0-100, data distribution should not be expected to be normal. Consequently, parametric regression analysis is not necessarily valid. Probit analysis (Robinson, 1992) is used to avoid this problem. However, the software for probit analysis is not readily available, and the problems caused by the data "topping out" at 100% are not usually great enough to prevent a clear conclusion from being reached.

Examples

In all of these examples of the measurement of toxicity of tree leaves, the assumption is made that the trees experience a trade-off between defense and growth. If they use molecules and energy to produce defensive chemicals, these molecules and energy are not available for growth (Zangerl & Bazzaz, 1992). Because of this trade-off, the trees should produce leaf defensive chemicals only under circumstances in which this would yield a significant avoidance of herbivory. This concept is useful for discussion before and/or after the bioassay project.

In all these projects, the control shrimp experienced very low mortality (about 1%). Extrapolation of the regression curves to zero concentration indicates the same thing, in each case.

Comparison of Leaf Toxicity in Tree Species Differing in Longevity

The first example comes from a class project for Field Botany at the Wheaton College Science Station in Hisega, South Dakota (at about 1000 m elevation in the Black Hills) in early spring (June) of 2001.

The hypothesis was that longer-lived tree species should produce greater levels of anti-herbivore defense than shorter-lived tree species. Longer-lived species may experience a greater buildup of herbivore populations, while a shorter-lived species may have completed its life cycle before herbivore populations have

built up to high levels (Cates, 1981). Oaks are long-lived trees, and have been used in many studies of herbivore defense starting with Feeny's (1970) classical study. Due to time constraints, only one long-lived and one short-lived species were studied here: the long-lived bur oak *Quercus macrocarpa* and the short-lived chokecherry *Prunus virginiana*. Leaves were obtained from ten individuals of each species.

The results (Figure 1) suggest that the bur oak leaves were more toxic (lower LC_{50}) than the chokecherry leaves, consistent with the hypothesis. While the oak leaves were clearly more toxic than the chokecherry leaves, the data do not permit the calculation of a reliable LC_{50} for either species. This occurred because many of the observations (not shown) were at effective concentrations greater than 40 mg/ml. These results suggest that effective exposure concentrations beyond about 40 mg/ml are not likely to be useful, and that a large number (over 30 per species) of observations in the 0-40 mg/ml range are necessary for good analysis of results.

Comparison of Leaf Toxicity in Trees of the Same Genus Differing in Herbivore Load

The second example comes from the second author's student research project at Southeastern Oklahoma State University in April 2002.

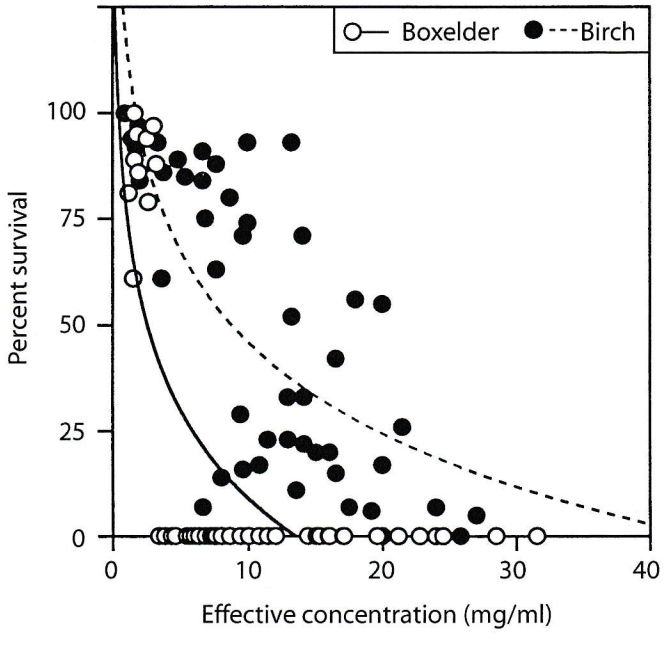

Figure 3.
Bioassay of leaf toxicity of box elder (*Acer negundo*) and mountain birch (*Betula occidentalis*). Percent survival of bioassay shrimp is presented as a function of effective concentration (mg leaf material per ml of shrimp).

The hypotheses were that different oak species produce different levels of leaf toxicity, and that greater leaf toxicity will protect the leaves from herbivory more effectively than lower leaf toxicity. Leaves were obtained from three trees each of the post oak *Quercus stellata* and of the shumard oak *Quercus shumardi* in a cross-timbers forest at Juniper Point on the south shore of Lake Texoma on the Oklahoma-Texas border. Leaves were also collected from three post oak trees from a natural area belonging to the Army Corps of Engineers on the north shore of Lake Texoma near Durant, Oklahoma. Before the leaves were processed, extent of herbivory was estimated from a two-by-ten grid of 20 points each 1 cm apart from the next. The grid points coincided either with fresh leaf, or with a place where herbivores had removed leaf area. Fewer than 20 points were used for smaller leaves. Percent area lost to herbivory could thus be estimated to about the nearest 5%.

The results (Figure 2) indicate that the leaves of shumard oak were much more toxic than the leaves of post oak. The LC_{50} of shumard oak leaves was about 1 mg/ml, while the LC_{50} of post oak leaves was about 3 mg/ml. However, it was the shumard oaks that sustained greater levels of herbivore damage (53%) than the post oaks (20%). The 95% confidence intervals for percent herbivory did not overlap. The herbivores that ate shumard oak leaves were therefore not inhibited by the greater level of toxins found in them. We did not determine whether the guilds of herbivores differed on the two species. Therefore, some other factors (such as leaf tenderness, or the population dynamics of the herbivores) determined herbivore success, and the second hypothesis was contradicted.

We have also noticed that these young oak leaves have greater toxicity than leaves produced later in the season (unpublished data).

Comparison of Leaf Toxicity in Trees Differing in Induction of Herbivore Defense

The third example comes from a class project for Field Botany at the Wheaton College Science Station (see Example 1) in June 2002.

The hypothesis was that tree species with constitutive defense will have more toxic leaves than those with induced defense in early spring before insect populations are large. Constitutive defenses are produced in leaves on a set developmental pattern, while induced defenses are not produced until stimulated by herbivory, in a pathway involving

jasmonic acid (Creelman & Mullet, 1995). We assumed that the mountain birch *Betula occidentalis* had induced defense, since such defense has been found in other birches (Baldwin & Schultz, 1984). We chose the box-elder *Acer negundo* as an example of a tree likely to have constitutive leaf defense. These species could also have constituted a test of the first hypothesis, since mountain birches (in which small new trunks continually resprout from old clumps) may live much longer than the fast-growing, single-trunked boxelders. However, the hypothesis in Example 1 can be tested only in trees in which the potential leaf defense has been induced. Later in the summer, therefore, a comparison of these two species may have constituted a test of the hypothesis in Example 1.

The results (Figure 3) indicate that box elder leaves were more toxic than mountain birch leaves. The LC_{50} of box elder leaves was less than 5 mg/ml, while the LC_{50} of birch leaves was about 10 mg/ml.

Conclusion

As demonstrated above, brine shrimp bioassays allow experience with many aspects of biological experimentation, including experimental design, hypothesis formation, and graphical and statistical inference. They lead to interesting discussions, such as what would be necessary to really test the three hypothesis above: a comparison of two species may be consistent with or contradict the hypothesis, but only a massive comparison of many species (or a meta-analysis of many studies, Christensen, 2001) could really address these hypotheses. Laboratory writeup scores from class bioassays in the Wheaton College botany classes, and in summer ecology classes at Southeastern Oklahoma State University, were all very high. A final examination essay question, administered to ecology students in summer 2002, who had conducted a brine shrimp bioassay (Rice, unpublished), indicated that 63% of them could define a bioassay, 75% of them understood what a bioassay is used for, and 81% knew how to perform one.

Bioassays are useful in classes populated by students who have career plans outside of ecology and botany. In particular, those who are interested in medical studies will understand that bioassays can be a useful tool in screening plants for possible medicinal potency (Massele & Nshimo, 1995; Meyer et al., 1982; Nick, Rali & Sticher, 1995). For example, we found bark extracts of the seaside alder *Alnus maritima* (an endangered tree species in Oklahoma) to kill more shrimp than either the hazel alder *Alnus serrulata* or the black willow *Salix nigra* (Rice et al., unpublished). Because willow bark is famous in herbal medicine, the seaside alder may prove to have great medicinal value. A brine shrimp bioassay was the first step in a new line of research to investigate this possibility.

Acknowledgments

The author acknowledges the assistance of members of the Field Botany classes of 2001 and 2002 at the Wheaton College Science Station. Caroll Richards assisted with one of the bioassays. Judy Williams helped with figure production, and Erica Corbett with statistical analysis.

References

Aranson, J.T., Marles, R.J. & Aucoin, R.R.. (1991). Isolation and biological activity of derived phototoxins. In D.P. Valenzeno, R. H. Pottier, P. Mathis & R. H. Douglas (Eds.), *Photobiological Techniques* (p. 187-196). New York: Plenum.

Baldwin, I.T. & Schultz, J.C. (1984). Damage- and communication-induced changes in yellow birch leaf phenolics. In R. M. Lanner (Ed.), *Proceedings of the Eighth North American Forest Biology Workshop* (p. 25-33). Logan, UT.

Cates, R. G. (1981). Host plant predictability and the feeding patterns of monophagous, oligophagous, and polyphagous insect herbivores. *Oecologia, 48,* 319-326.

Christensen, L.B. (2001). *Experimental Methodology, 8th Ed.* (p. 42). Boston: Allyn and Bacon.

Creelman, R.A. &. Mullet, J. (1995). Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress. *Proceedings of the National Academy of Sciences USA, 92,* 4114-4119.

Feeny, P. (1970). Seasonal changes in oak leaf tannins and nutrients as a cause of spring feeding by winter moth caterpillars. *Ecology, 51,* 565-581.

Hurlbert, S.H. (1984). Pseudoreplication and the design of ecological field experiments. *Ecological Monographs, 54*(2), 187-211.

Karban, R. & Kuć, J. (1999). Induced resistance against pathogens and herbivores: an overview. In A.A. Agrawal, S. Tuzun & E. Bent (Eds.), *Induced Plant Defenses Against Pathogens and Herbivores: Biochemistry, Ecology, and Agriculture* (p. 1-16) . St. Paul MN: APS Press.

Massele, A.Y. & Nshimo, C.M. (1995). Brine shrimp bioassay for biological activity of medicinal plants used in traditional medicines in Tanzania. *East African Medical Journal, 72,* 661-663.

Maxwell, N. (1990). *Witch-Doctor's Apprentice: Hunting for Medicinal Plants in the Amazon.* New York: Citadel.

Meyer, B.N., Ferrigni, N.R., Putnam, J.E., Jacobsen, L.B., Nichols, D.E. & McLaughlin, J.L. (1982). Brine shrimp: a convenient general bioassay for active plant constituents. *Journal of Medical Plant Research, 45,* 31-34.

Nick, A., Rali, T. & Sticher, O. (1995). Biological screening of traditional medicinal plants from Papua New Guinea. *Journal of Ethnopharmacology, 49,* 147-156.

Opler, A., Mizell, R., Robert, A., Cervantes-Cervantes, M., Kincaid, D. & Kennelly, E.J. (2002). Use of a brine shrimp assay to study herbal teas in the classroom. *The American Biology Teacher 64*(8), 596-604.

Perkins-Veazie, P. (2002). Personal Communication. South-Central Agricultural Research Laboratory, USDA.

Plotkin, M.J. (1994). *Tales of a Shaman's Apprentice: An Ethnobotanist Searches for New Medicines in the Amazon Rain Forest.* New York: Penguin USA.

Rimaldo, A. (2002). Personal Communication. Natural Products Utilization Research Unit, USDA.

Robinson, J. L. (1992). *Pesticide Bioassays with Arthropods.* Boca Raton FL: CRC Press.

Wedge, D. (2002). Personal Communication. Natural Products Utilization Research Unit, USDA.

Wedge, D.E. & Camper , N.D. (2000). Connections between agrochemicals and pharmaceuticals.) In S.J. Cutler and H.G. Cutler (Eds.), *Biologically Active Natural Products: Pharmaceuticals* (p. 1-15). Boca Raton FL: CRC Press.

Williams S. (1984). *Official Methods of Analysis of the Association of Official Analytical Chemists,14th Ed.* Arlington VA: Association of Official Analytical Chemists Inc.

Zangerl, A. R. & Bazzaz , F. A. (1992). Theory and Pattern in Plant Defense Allocation. In R.S. Fritz and E.L. Simms (Eds.), *Plant Resistance to Herbivores and Pathogens* (p. 363-391). University of Chicago Press.