

Investigation of Phenolic Defense Properties in *Acer rubrum*

Kaitlin Koch

Ashley Maiuri

Afsheen Motalleb

KC Semrau

Abstract

It is important that plants have the ability to increase levels of chemical defenses to deter herbivores. According to the carbon nutrient balance hypothesis a plant can favor allocation of energy toward growth or toward carbon based defenses depending on the metabolic needs of the plant. Because of this, levels of chemical defenses may fluctuate during a plants lifetime. Phenolic levels in plants are found at a constitutive level until after an attack, at which point the plant can induce the production of phenolics to increase the concentration. Chemical activity within a plant relies largely on the rate of photosynthesis, so accordingly we ask whether a resource such as sunlight could have an effect on the production rate of phenolics. Our experiment focuses on investigating the pre- and post-attack levels of phenolic compounds on *Acer rubrum* leaves receiving different levels of sunlight. We found that leaves from the north and south side both experienced induction, but that the amount of sunlight available to a leaf did not significantly affect the constitutive or induced phenolic concentrations.

Keywords: *Acer rubrum* – phenolics – sunlight availability – constitutive – inducibility – Carbon-Nutrient Balance Hypothesis – herbivory

Introduction

All plants are fed upon by multiple herbivores, yet the majority of land on Earth is still green. Plants have evolved defenses that allow them to maintain their fitness despite the prevalence of herbivory. Defenses can be mechanical, chemical or both. Because of their ecological importance, plant chemical defenses have received much attention within the past few decades. Phenolics are secondary chemicals found in most angiosperms. These chemicals are known to deter many herbivores (Rozema and Verkleij, 1991).

The levels of chemical defense compounds can fluctuate within a plant depending on its metabolic needs. The Carbon-Nutrient Balance Hypothesis suggests that carbon and nitrogen are allocated to the production of secondary metabolites only after the requirements of growth are met (Hamilton, 2001). Because production of phenolics is an expensive process for plants, allocation of resources to phenolic production can lead to a reduction in plant growth. In addition, the amount of sunlight available to a tree may affect the levels of phenolics found in the

individual leaves. Leaves directly exposed to sunlight are expected to photosynthesize more, thereby fixing more carbon dioxide, than those that receive less sunlight, which may allow them to invest more carbon into the production of phenolic compounds. Consistent with this hypothesis, leaf phenolics from *Liriodendron tulipifera* (tulip poplar) and *Cornus florida* (dogwood) were found in higher concentrations when sunlight was greater within field and forest habitats (Dudt and Shure, 1994).

One aspect of plant chemical defense that has been focused on is the inducibility of phenolic compounds. Because production of chemical defenses is a costly process for many plants, they have evolved a way to keep their chemical defenses low and induce them only when needed (Agrawal and Karban, 1999). Inducibility is the ability to increase levels of chemical defenses based on environmental cues. This trait is also advantageous because it limits the possibility of autotoxicity in plants (Agrawal and Karban, 1999). For instance, phenolics in *Cornus florida* (dogwood) and *Acer rubrum* (red maple) can be induced by stress due to lack of nutrients and water (Muller, 1987). Aspen (*Populus tremuloides*) is known to induce phenolics following an artificial herbivore attack (Haukioja, 1990). An experiment conducted on *P. deltoides* demonstrated that upon damage, airborne signals are sent to nearby leaves resulting in an induced chemical defense response in the surrounding undamaged leaves (Yu et al., 2006). Although the properties of chemical defenses in plants have been closely studied, not much is known regarding phenolic concentrations in pre- and post-attack leaves and the inducibility of chemical defenses, particularly in *Acer rubrum* (red maple).

Acer rubrum is a deciduous tree species common throughout eastern North America. As mentioned above, they are known to produce phenolic compounds in response to stressful circumstances involving low nutrient and water availability (Muller, 1987). Our study will

investigate whether the concentration of phenolic defense compounds in *Acer rubrum* varies under other specific circumstances including damage and sunlight availability. We will measure the phenolic concentration in leaves that receive different amounts of sunlight. In contrast to similar experiments performed in the past which focus on chemical defense variation between trees, our study will focus on variation of phenolic levels within an individual tree, in response to sunlight availability and damage. This will allow us to disregard genetic variation between trees. In particular we will focus on the leaves that are on the north and south side of each tree. Because the sun is in the southern part of the sky, we expect that the leaves on the south side of each tree will fix more carbon dioxide than the leaves on the north side of each tree, allowing for the potential to increase phenolic production. We will measure the phenolic concentrations in damaged and undamaged leaves to see whether phenolics are inducible in *A. rubrum* in response to attack. Accordingly, in this study, we ask:

- 1) Does the availability of sunlight increase the concentration of phenolic compounds in the constitutive leaves of *Acer rubrum*?
- 2) Does the concentration of phenolic compounds increase after damage in *A. rubrum*?
- 3) Does the availability of sunlight influence inducibility of phenolics in *A. rubrum*?

Materials and Methods

*Effect of sunlight availability on constitutive phenolic concentration in *Acer rubrum*.*

To determine whether amount of light has an effect on the concentration of phenolics in red maple leaves, we first chose 25 trees that were along the north edge of Riggsville road, in Pellston Michigan, to maximize exposure of the south side of the tree to the sun. The trees were located approximately 60 feet from the road and within 10 feet of the edge of the forest. We assumed that the effect, if any, of the road was equivalent among all leaves per tree in our study.

The trees we chose had distinctive north (shaded) and south (sunny) leaves, and were relatively similar in size (3-8 cm DBH).

We chose a sunny time period in the early afternoon to collect half of each leaf on the north and south sides of each tree. We determined constitutive phenolic levels by cutting the leaf with scissors along the midrib. We then placed the half leaf into a glassine envelope and immediately stored them in an ice-filled cooler. Upon returning to the lab, we put all the samples in a -80°C freezer for 30 minutes, then lyophilized them for approximately 48 hours.

A paired t-test was used to compare the percent phenolics by dry mass of the north constitutive and south constitutive leaves on the same tree.

Effect of damage on the concentration of phenolics in Acer rubrum.

To determine whether damage affects levels of phenolics in *A. rubrum*, we used a garlic press to damage the other half of the leaf that was used to measure constitutive levels and that remained attached to the tree. We damaged two additional leaves found on the same vascular system because, as previously cited, damaged leaves are known to communicate with nearby leaves resulting in an induced phenolic response (Yu et al., 2006). 48 hours later, we collected the damaged half leaf using the same method described above.

A paired t-test was used to compare the percent phenolics by dry mass of the damaged half leaf to the percent phenolics by dry mass of the undamaged half of the same leaf. Since the two halves have the same exposure to sunlight, differences should reflect only the effect of the damage.

Effect of sunlight on the inducibility of phenolics in Acer rubrum.

To determine whether the amount of sunlight affects the inducibility of phenolics in *A. rubrum*, we used a paired t-test to compare the percent phenolics by dry mass present in the north

damaged leaves with the percent phenolics by dry mass present in the south damaged leaves. In addition, we used a paired t-test to compare the percent change in phenolic concentration between leaf pairs on the north and south sides of each tree.

Folin-Denis Phenolic Analysis

We used the Folin-Denis method to determine relative concentrations of total phenolics in our samples.

Assay

1. Grind each lyophilized leaf with a coffee grinder
2. Using a top-loading balance, weigh 20mg of each leaf and place in separate 1.5ml conical microcentrifuge tubes. This will allow us to estimate the percent of phenolics per 20 mg of each leaf. The vascular tissue of the leaf should not be included in the ground up sample.
3. Dilute 70 ml of acetone with 30 ml of deionized water. To this, add 0.1769 g of ascorbic acid. This will be referred to as the extraction solvent.
4. Add 0.5 ml of the extraction solvent to each tube and vortex for 5 seconds. The ascorbic acid is used to extract the phenolic compounds in the leaf sample.
5. Sonicate for 20 minutes. This will help to disrupt the cell walls and release phenolic compounds into the solvent.
6. Centrifuge for 5 minutes at 6,000 rpm.
7. Using a separate Pasteur pipette for each sample, decant the supernatant into a separate 1.5 ml microcentrifuge tube. Place the collection tubes in the freezer.
8. Repeat steps 4, 6, 7 twice more to obtain a volume of approximately 1.4 ml. The concentration of phenolics should decrease substantially during each extraction, so that by the third extraction only a tiny fraction of phenolics will remain in the ground leaf sample.
9. Add enough of the extraction solvent to each supernatant microcentrifuge tube to bring the total volume in each tube to 1.5 ml and store the sample in the freezer to prevent the phenolic compounds from decaying.
10. Prepare 2 N Na_2CO_3 (sodium carbonate) solution by dissolving 10.6 g of sodium carbonate in 100 ml of deionized water.
11. Prepare dilution series of tannic acid, using concentrations of 2, 10, 30, 50, and 80 ppm.
12. Allow the Folin-Dennis reagent to warm to room temperature. The reagents should all be at the same temperature to standardize the relative rates of the reaction.
13. Dilute each extract with deionized water. We used different dilution factors for the supernatant samples and accounted for that in our data analysis.
14. In a test tube, add 0.5 ml of the diluted phenolic extract (or the tannic acid dilutions to determine the standard curve), then 0.5 ml of the Folin-Dennis reagent and lastly 2.0 ml of the 2N sodium carbonate solution. Allow the solution to react at room temperature for

40 minutes.

15. Run the samples in the spectrophotometer at an absorbance of 700 nm. The concentration of phenolics in each leaf sample can be determined by the absorbance of the slope of the standard curve.
16. Calculate the percent phenol in each 20 mg sample using the formula:

$$[(\text{PPM})(\text{dilution factor})(0.0015)(100)]/\text{sample weight in mg} = \% \text{ dry weight phenolics}$$

PPM = concentration obtained from the standard curve, in tannic acid equivalents

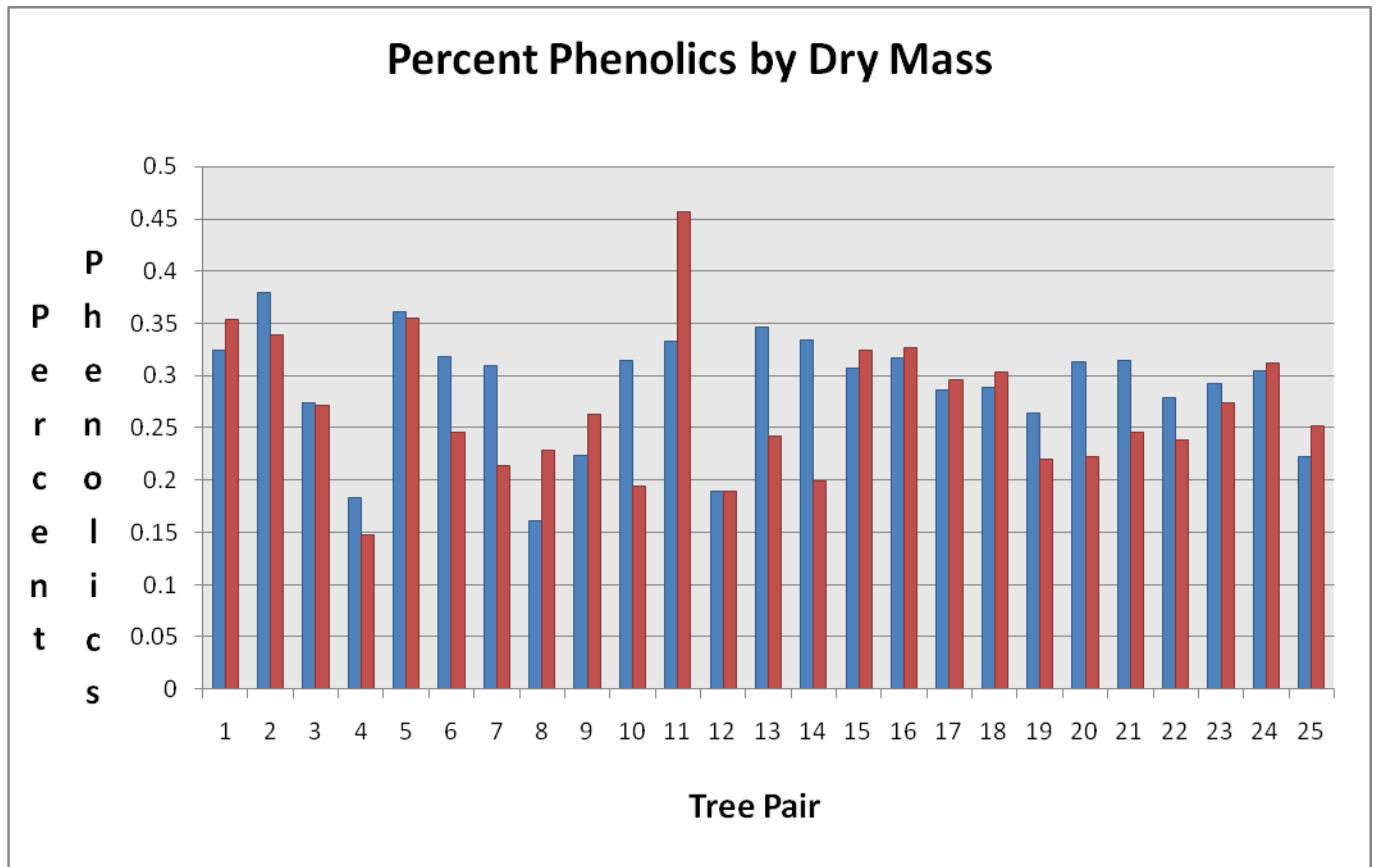
0.0015 = total extraction volume in liters

100 = conversion to percent

We used paired t-tests to check the significance of each hypothesis

Results

Figure 1



The results of the first paired t-test comparing levels of phenolics in north and south constitutive leaves was nonsignificant. We found no evidence that leaves on the north and south

sides of our trees contained different levels of constitutive phenolics (paired $t = 1.486$, $df = 21$, $p = .15$; Figure 1). Constitutive phenolic levels varied from .16 to .38 percent of dry weight and in south leaves, and from .14 to .46 percent in north leaves. In twelve pairs of trees, constitutive levels were higher in the south leaves.

Figure 2

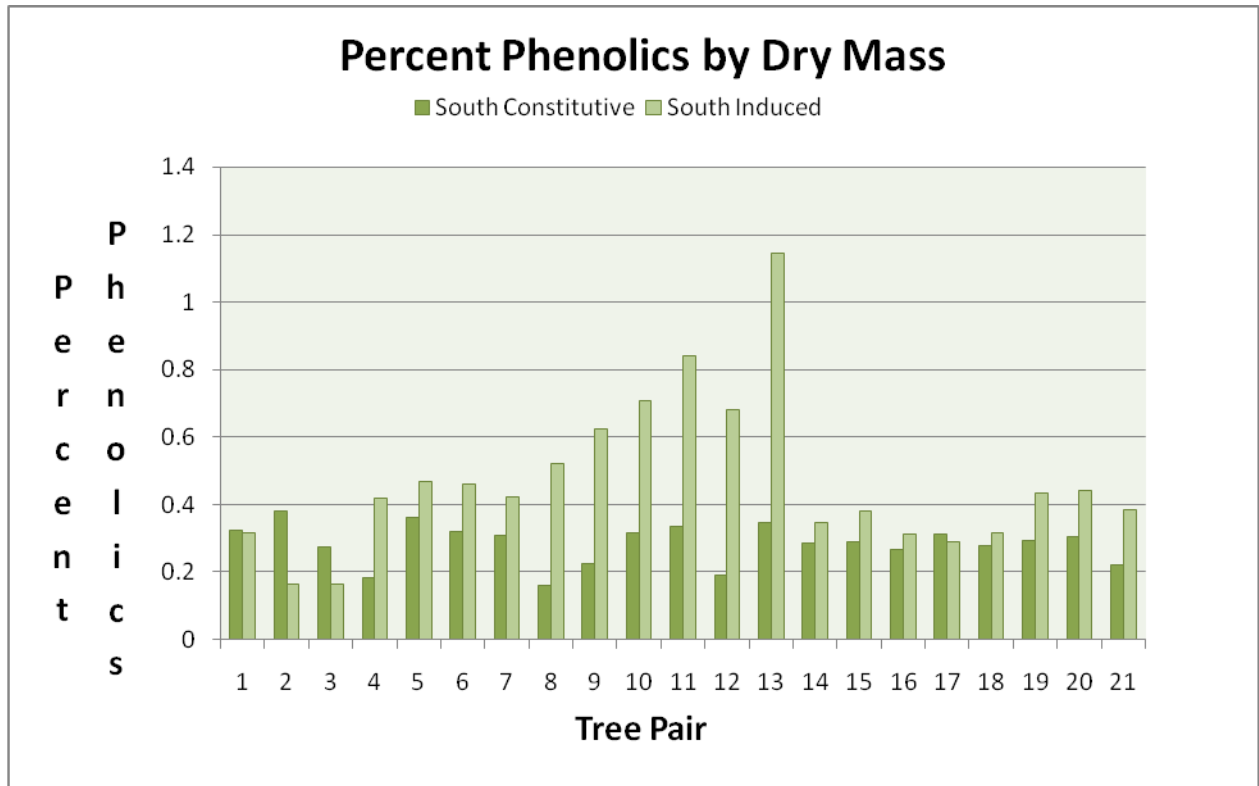
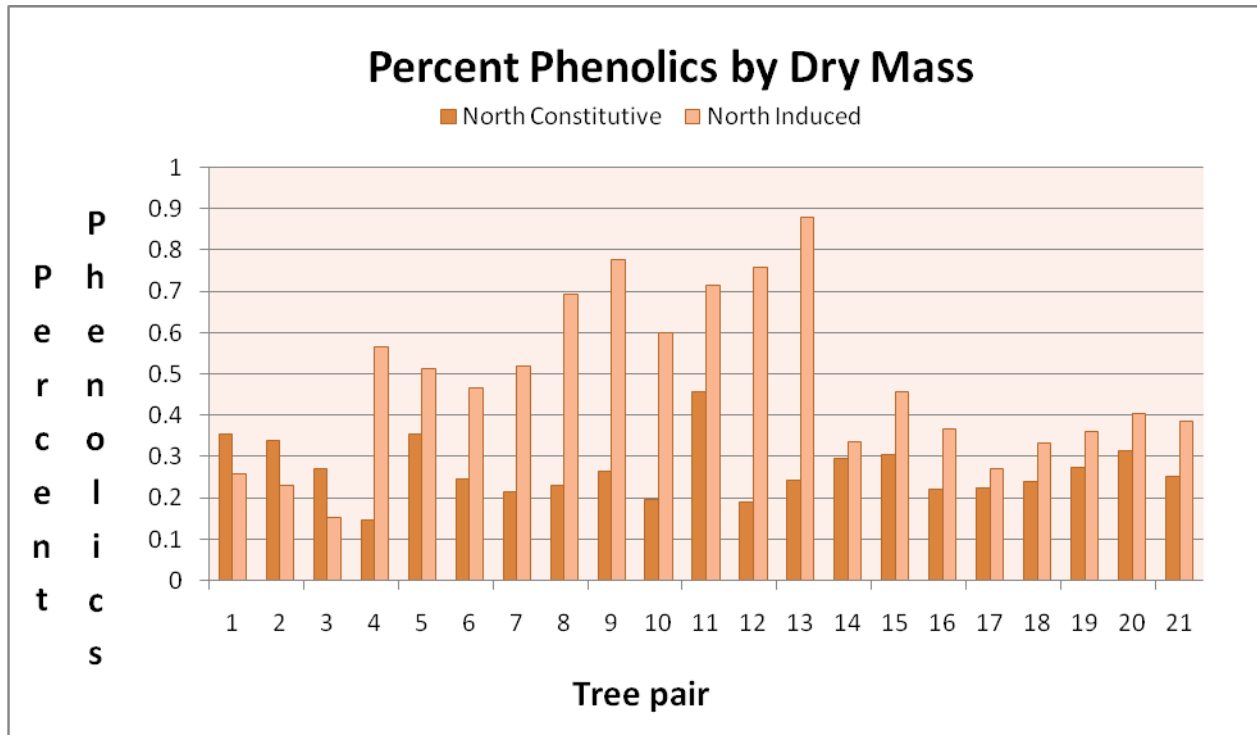
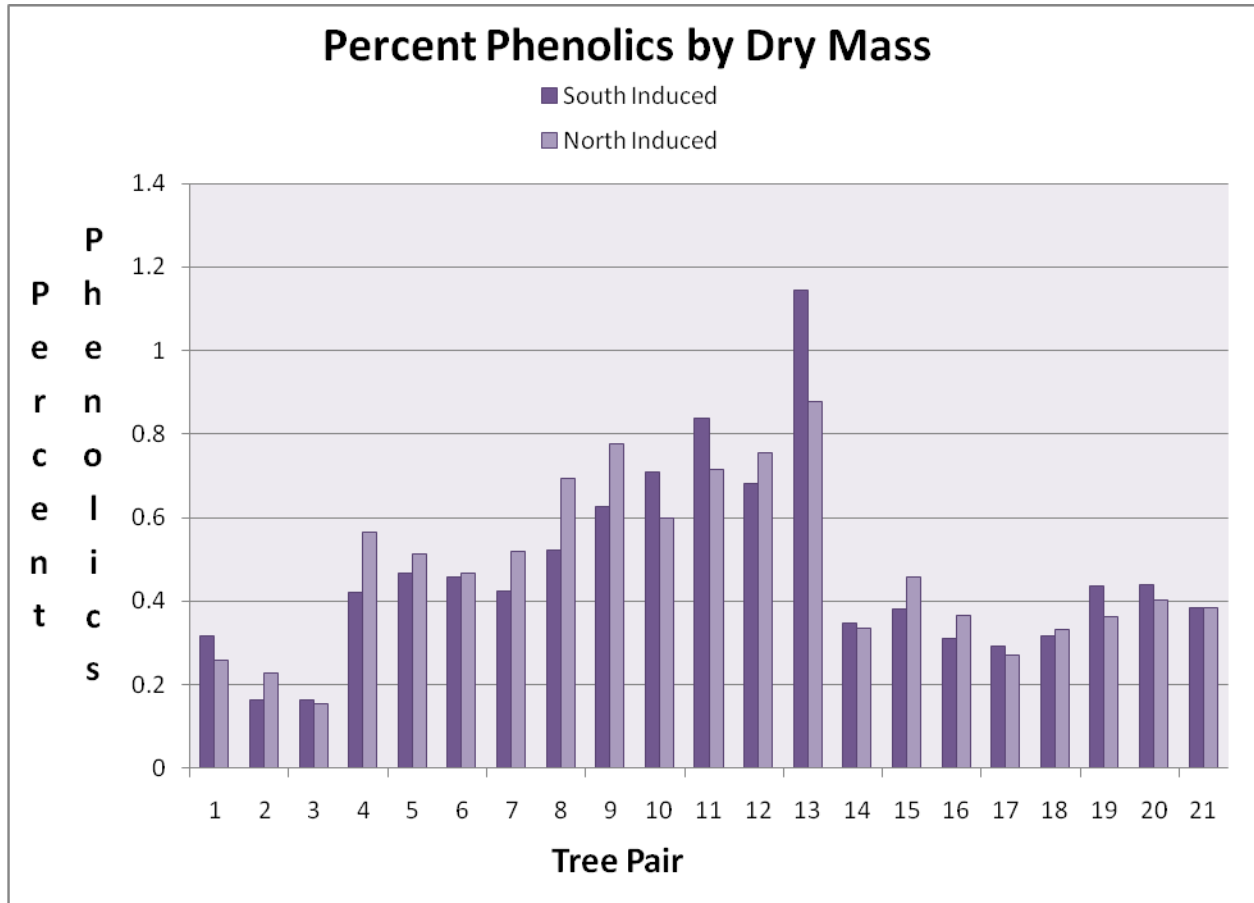


Figure 3



The results of the paired t-test comparing the percent phenolics present between constitutive and induced leaves in their respective cardinal directions showed a significant difference, where the induced levels were higher (South pairs $t=-3.97$, $df=20$, $p=.001$, Figure 2; North pairs $t=-3.85$, $df=20$, $p=.001$; Figure 3). This indicates that the induced leaves had higher levels of phenolic compounds present. A paired t-test was also used to compare the difference of phenolic induction in the north and south pairs. The results of this test were non-significant ($p=.23$), which indicates that there was not a noteworthy difference between levels of induction within the north and south constitutive and induced pairs.

Figure 4



The results of the paired t-test comparing the percent phenolics present in the south and north induced leaves was non-significant ($t=.41$, $df=20$, $p=.69$; Figure 4). Induced phenolic levels varied from .14 to .88 percent of dry weight and in south leaves, and from .16 to 1.14 percent in north leaves. In seven pairs of trees, induced levels were higher in the south leaves.

Discussion

We found no evidence that sunlight availability had a significant effect on constitutive phenolic levels in the leaves of *Acer rubrum*. Although we detected significant induction of phenolics in response to mechanical damage, we could not conclude that sunlight availability had an effect on the inducibility of phenolics. There are several possible explanations for these

results. For instance, according to the carbon nutrient balance hypothesis, in the early stages of leaf development, carbon obtained or fixed from external sources such as sunlight, is more favorably allocated towards growth rather than defense. Because we collected our leaves during the spring season, the young leaves may have been experiencing a phase in which any additional carbon was used for growth rather than phenolic production. This suggests the possibility that constitutive levels of phenolics, as well as their inducibility, are phenologically dependent. This means that because leaf development in *A. rubrum* begins in the spring, a large amount of phenolic production may have been selected to occur in the summer when the leaves have matured and growth is not the highest priority. If this is the case, then there would be no significant difference in the constitutive phenolic levels between the north and south sides of each tree during the spring. However, if this experiment were repeated in the summer when the leaves have matured, we may see higher phenolic levels produced in the south leaves compared to the north leaves, assuming that defense is a higher priority than growth in adult leaves. Limiting phenolic production in young leaves may be important from the plant's perspective because not only are the leaves able to invest resources toward growth early in development, but the potential for autotoxicity to occur in young susceptible leaves is also avoided.

It is also possible that our hypothesis was correct, but our assumption that the south side of each tree was receiving more sunlight than the north side of each tree may have been incorrect. For instance, the trees chosen were relatively young with large open spaces between the branches. This may have allowed more sunlight to shine on the north leaves than we had initially expected. If a similar experiment is conducted in the future, it may be helpful to quantify the amount of sunlight available to each leaf using a light meter, to make sure that there is a significant difference in the amount of sunlight available to each leaf pair. It would also be

beneficial to measure the carbon to nitrogen ratio in each leaf to understand where the leaf may be allocating its resources. For example, when the carbon to nitrogen ratio is high, the leaf will allocate more carbon towards defense instead of growth if the plant is following the carbon nutrient balance hypothesis.

We did detect significant induction of phenolics after mechanical damage. Based on this result, we can say with confidence that *Acer rubrum* appears to have followed the same evolutionary trend as many other plants; the evolution of inducible chemical defenses. This evolutionary adaptation may have been a response to plants producing excessive amounts of chemical defenses during times when they are not needed. As described above when plants, especially those with juvenile leaves, produce excessive amounts of secondary chemical compounds they increase the risk of autotoxicity. This also may have resulted in selection for a trait which allows the leaves to maintain low constitutive levels of phenolics until an attack.

Further implications that we can consider based on our results include how variation in phenolic levels may influence the feeding patterns of herbivores. This variation may be very important in determining where and how much an herbivore feeds on *A. rubrum*. Eventually, as a result of chemical coevolution we can expect that *A. rubrum*, as well as many other plants, will evolve new chemical defenses. Subsequently, herbivores will follow a similar trend by evolving adaptations allowing them to feed on chemically defended plants. While chemical defense strategies do not eliminate herbivory, they control the amount of herbivory that occurs by influencing most herbivores to become specialists.

Acknowledgements

Dave Karowe

Mike Grant

Jesse Lewis

Katie Goodall

We would like to thank the above people for providing us with guidance and support throughout our investigation of phenolics in *Acer rubrum*.

Literature Cited

- Baldwin, I.T. and J.C. Schultz, 1982. Rapid changes in tree leaf chemistry induced by damage: Evidence for communication between plants. *Science* 221: 277-279.
- Baldwin, I.T., J.C. Schultz and D. Ward, 1987. Patterns and sources of leaf tannin variation in yellow birch (*Betula allegheniensis*) and sugar maple (*Acer saccharum*). *Journal of Chemical Ecology* 13: 1069-1078.
- Bryant, J.P. and R. Julkunen-Tiitto, 1995. Ontogenic development of chemical defense by seedling resin birch: Energy cost of defense production. *Journal of Chemical Ecology* 21: 883-896.
- Coley, P. D., 1986. Costs and benefits of defense by tannins in a neotropical tree. *Oecologia* 70: 238-241.
- Dudt, J.F. and D.J. Shure, 1994. The influence of light and nutrients on foliar phenolics and insect herbivory. *Ecology* 75: 86-98.
- Hamilton, J.G., A.R. Zangerl, E.H. DeLucia, and M.R. Berenbaum, 2001. The carbon-nutrient balance hypothesis: its rise and fall. *Ecology Letters* 4: 86-95
- Haukioja, E., 1991. Induction of defenses in trees. *Annual Review of Entomology* 36: 25-42.
- Mole, S., J.A.M. Ross, and P.G. Waterman, 1988. Light-induced variation in phenolic levels in foliage of rain-forest plants. *Journal of Chemical Ecology* 14: 1-21.
- Muller, R.N., P.J. Kalisz, and T.W. Kimmerer, 1987. Intraspecific variation in production of astringent phenolics over a vegetation-resource availability gradient. *Oecologia* 72: 211-215.
- Riipi, M., V. Ossipov, K. Lempa, E. Haukioja, J. Koricheva, S. Ossipova, and K. Pihlaja, 2002. Seasonal changes in birch leaf chemistry: are there trade-offs between leaf growth and accumulation of phenolics? *Oecologia* 130: 380-390.
- Rozema, J, and J.A. Verkleij. 1991. *Ecological Responses to Environmental Stresses*. Kluwer Academic Publishers.
- Tuomi J., P. Miemela, and M. Rousi. Induced accumulation of foliage phenols in mouthain birch: Branch response to defoliation. *The American Naturalist* 132: 602-608.
- Yu, A., S. Ying-bai, W. Li-juan and Z. Zhi-xiang, 2006. A change of phenolic acids content in poplar leaves induced by methyl salicylate and methyl jasmonate. *Journal of Forestry University and Ecological Society of China* 17: 107-110.