Induction of phenolics in black cherry in response to herbivory: Does it occur and, if so, does it matter?

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June 14, 2000
Ecology 381, Spring Term

Abstract: All plants contain secondary compounds that can be used for chemical defense. Some of these compounds, such as phenolics, can induce in response to damage while others remain at constitutive levels in the plant. Phenolic content in leaves of *Prunus serotina,* black cherry, was assayed using the Folin-Denis technique to determine if induction occurs in response to mechanical damage both above and below the damaged leaf. Leaves from the next closest and a distant shoot were also assayed to examine if induction was localized or systemic. We found no evidence for induction in *P. serotina. Malacosoma americanum*, eastern tent caterpillar, the specialist feeder on *P. serotina*, was used to study the interaction of a host plant and its primary herbivore. *M. americanum* were fed leaves from trees of different damage levels to study the effects of damage level on larval performance. The feeding trial showed that *P. serotina* tree damage had no effect on *M. americanum* larval performance. Induction may be favored against a generalist feeder over a specialist herbivore because of the intricate relationship between a specialist feeder and its host plant.

Introduction

Plants contain secondary compounds that presumably have evolved as a defense mechanism to protect plants from herbivory (Stuhlfauth 1987). In all plants, chemical defenses exist at constitutive (baseline) levels, but many are known to increase following injury (Karban and Baldwin 1997). An increase in the level of a chemical defense in response to plant damage is called induction. For instance, wind damage has been shown to induce lignin defense chemicals in the common bean (Cipolli, D.F. 1997), while parsnip webworm herbivory induced a furanocoumarin in wild parsnip (Berenbaum et al., 1989).

Induction has likely evolved in plants to raise defense against injury due to herbivores (Karban and Baldwin 1997). Resource allocation may not allow plants to maintain high levels of chemical defense at all times (Sagers and Coley 1995). Therefore, individual plants may encounter a trade-off in allocation of limited resources to growth vs. defense. Rather than maintaining high constitutive levels of defense at the

cost of growth and reproduction, it is proposed that induction of chemical defenses allow plants to invest in defense primarily when those defenses are most necessary.

Phenolics, a class of carbon based secondary metabolites, are one of the most widespread defense compounds (Whittaker and Feeny, 1971, Porter et al., 1985). Phenolics are known to occur universally in leaf tissue throughout deciduous trees. Phenolic compounds contain an alcohol group attached to a benzene ring. Several studies have demonstrated that phenolic levels are inducible in some species. (Karban and Baldwin 1997). For example, poplar trees have been shown to induce phenolics in response to leaf damage (Baldwin and Schultz, 1983).

A logical assumption is that induction is favored as a defense primarily against generalist feeders, since specialists have presumably adapted to the chemical defenses of their host plant, and therefore would not be adversely affected by elevated levels of those defenses. However, few studies have demonstrated the effect of induction on specialized feeders. The eastern tent caterpillar, *Malacosoma americanum*, is one such specialist feeder. In Northern Michigan, *M. americanum* is the primary herbivore on the black cherry tree, *Prunus serotina,* while *P. serotina* is the primary host plant for *M. americanum*. This specific host/herbivore system between *P. serotina*, and *M. americanum*, provides an opportunity to study the effect of induced responses on a specialist feeder.

In this study we addressed the following questions:

1) Does induction of phenolics occur in *P. serotina* in response to damage?

2) If so, is induction systemic or localized?

3) What affect does response of *P. serotina* to damage have on larval performance *M. americanum*?

Materials and Methods

Plant Selection

We selected 23 *P. serotina* trees for analysis from the Pellston Plain near the University of Michigan Biological Station in Pellston, Michigan. To avoid using plants in which induction may have already occurred, we selected only those trees with minimal observable damage for study.

Leaf Selection and Mechanical Damage Regime

To determine if induction occurs in response to mechanical damage we obtained two samples of the same leaf, one before mechanical damage to determine baseline levels of phenolics in each leaf, and one 24 hours later to measure change in total phenolic content of the leaf in response to damage. Each sample was placed in a glassine envelope and kept on ice during collection. We then transferred all samples to a -80°C freezer at UMBS to maintain levels of leaf phenolic content at time of removal. (We assumed that phenolic levels are consistent throughout the leaf, that induction hadn't already occurred in the leaf, and that by removing half of the leaf we didn't cause induction.)

On each individual tree, a shoot with at least six fully expanded leaves was identified (figure 1). Where possible, we chose shoots on south facing branches since they presumably receive the most sunlight (therefore photosynthesize more readily) and would be more likely to induce in response to damage by grazing caterpillars. On each selected shoot, a proximal quarter of the second lateral leaf was removed with a razor

blade. The terminal half of this leaf was damaged using a garlic press to mimic herbivore damage and to standardize damage throughout all damaged leaves. To ensure that we would observe induction of phenolics if it did occur, we damaged two leaves above and three leaves below the second lateral leaf because the signal to induce phenolics may only travel in one direction. We returned 24 hours later to collect the opposite proximal quarter of the same damaged leaf. To test whether induction was localized or systemic we removed half of a terminal leaf on a nearby new shoot and half of a terminal leaf on a distant new shoot before damage, and the remaining portion of each test leaf was collected 24 hours after damage.

Leaf Phenolic Content

Phenolic content of leaves was established based on methods described by A. Spickard (unpublished). We lyophilized each test leaf for 48 hours to remove as much water as possible. Individual leaves were then ground to a fine powder using a Wig-L-Bug amalgamator (model 3110B; Crescent Dental Mfg. Co., Lyons, IL.) We then weighed out each sample as close to 10 milligrams as possible; a few samples were smaller than 10 milligrams, yet we were still able to run assays of them. To extract the phenolic compounds from the test leaves, each sample of leaf powder was washed with 0.4 ml of 70% acetone solution to separate the phenolic compounds in the sample from other leaf contents. Following the wash, we sonicated the mixture for 10 minutes in deionized water to break up remaining cell tissue. The sample was then centrifuged at 10,000 rpm for 3 minutes to isolate and collect the supernatant. The extraction process was repeated two more times, for a total volume of 1.2 ml. The leaf extracts were set in a freezer at UMBS until phenolic assays were performed.

The Folin-Denis assay was used to determine total phenolic content of each test leaf. Dried leaf extracts were centrifuged once more for three minutes at 10,000 rpm to ensure isolated phenolic compounds in assay. The extracts were diluted (1:25, 1:10, and 1:50 depending on sample concentration) in 0.5 ml deionized H_20 for each reaction. Following dilution, 0.5 ml Folin-Denis reagent was added to each reaction tube. Immediately following this addition we added 1 ml of a solution of 1 M sodium carbonate and .07 M L-(+)-tartaric acid for a total volume of 2 ml. However, due to apparent cloudiness of our sample solution which interfered with analysis, we ran some samples without L-(+)-tartaric acid to correct for the problem. Samples were incubated for 30 minutes at room temperature. Sample absorbency was determined at 725 nm against a blank of deionized water and a standard curve of four concentrations of .5% tannic acid solution (50 ug/ml, 30 ug/ml, 10 ug/ml and 0 ug/ml.) Total phenolic content was therefore expressed in ug/mg dry leaf material. All samples were run in duplicate.

To determine whether localized induction occurred in the damaged leaf we compared total phenolic contents of the quarter leaf harvested prior to damage and the quarter of the same leaf harvested 24 hours after damage via a paired t-test. To establish whether induction occurred locally or systemically throughout the damaged tree we applied a paired t-test to compare the phenolic contents of the half leaf obtained prior to mechanical damage with the remaining half leaf obtained 24 hours after damage of the terminal leaf on the nearby shoot to the damaged leaf and the terminal leaf on the distant shoot.

Feeding Trials

We ran feeding trials to determine whether extent of tree damage in *P. serotina*

influences the overall performance of *M. americanum*. We obtained a range of leaf phenolic content by selecting forty undamaged leaves, ten from each tree damage category, from a sample size of 22 trees. The four categories of tree damage type are as follows: undamaged (no visible caterpillar damage), mechanically damaged (no visible caterpillar damage with injury via garlic press), caterpillar damaged (visible caterpillar damage on nearby leaves), and a combination of caterpillar and mechanically damaged (visible caterpillar damage on nearby leaves as well as injury via garlic press). We mechanically damaged nearby leaves using a garlic press 24 hours before collection of trial leaves. A proximal quarter of each leaf was removed and we measured the wet weight and final dry weight to use as a control for later calculations. The remaining portion of each leaf was weighed to establish initial wet weight and placed on ice until the start of the feeding trial.

We collected 50 *M. americanum* in their third instar from the Pellston Plain near UMBS a few days prior to the start of the feeding trial. 40 larvae were used in the feeding trials and 10 were kept as controls to estimate initial dry weights. Two hours before the feeding trial, caterpillars were starved to remove gut contents. They were then weighed for initial wet weight and placed in petri dishes with a test leaf. Petri dishes were placed in an environmental chamber for 72 hours with 16 hour days at 18°C and eight hour nights at 14°C. After the trials, caterpillars were starved for one hour and weighed again to obtain their final wet weight. They were then frozen and dried to obtain their final dry weight. Caterpillar frass was also dried and weighed to obtain final dry weight. We also determined the final dry weight of the amount of leaf material uneaten. All weights were expressed in dry weight in order to minimize variation due to water

retention.

We established four factors that we considered to be determinants of overall larva performance: relative growth rate, relative consumption rate, digestive efficiency, and conversion efficiency. To determine the relative growth rate of the caterpillar, we first estimated its initial dry weight using a conversion factor—the dry to wet weight ratio of the ten control caterpillars:

$$\frac{\text{Dry weight control caterpillar}}{\text{Wet weight control caterpillar}} \times \frac{\text{Estimated initial dry weight of trial caterpillar}}{\text{Initial wet weight of trial caterpillar.}}$$

The relative growth rate, or the amount of caterpillar growth per day, could therefore be determined using the following formula:

$$\frac{\text{final dry weight of caterpillar} - \text{initial dry weight of caterpillar}}{\text{\# of trial days*(average larval dry weight throughout trial)}}$$

Total amount of leaf material consumed is needed to calculate the relative consumption rate. To determine the amount of leaf material consumed, we first estimated the initial dry weight of the leaf material fed to caterpillar using the following formula:

$$\frac{\text{Control dry weight}}{\text{Control wet weight}} \times \frac{\text{Estimated Initial dry weight of leaf material fed to caterpillar}}{\text{Initial wet weight of leaf material fed to caterpillar}}$$

and subtracted the uneaten dry leaf matter from the estimated starting dry weight. The relative consumption rate, or the amount of leaf material each caterpillar consumed per day, was then determined using the following formula:

$$\frac{\text{total leaf material consumed}}{\text{\# of trial days*(average larval dry weight throughout trial)}}$$

The digestive efficiency, or percent of consumed leaf material digested (total leaf material consumed - dry weight of frass) was determined as follows:

$$100 * \frac{\text{total leaf material digested}}{\text{total leaf material consumed}}$$

The conversion efficiency, or percent of digested material converted into body tissue, was determined using the following equation:

$$100 * \frac{\text{total growth of caterpillar}}{\text{total leaf material digested}}$$

Using these factors we were able to perform an analysis of variance to determine whether larval performance differed significantly between the four damage categories.

Results

Leaf Phenolic Content

To establish if induction occurred in *P. serotina* in response to mechanical damage, we looked at the results for the damaged leaves (mean difference = -0.83 ug/mg, paired $t = 0.14$, d.f. = 22, $p = 0.89$; Fig. 2). These results led us to conclude that induction did not occur in the damaged leaf. Our next question investigated if induction was systemic or localized. Since there was no evidence of induction in the damaged leaf, we could not test whether induction was localized or systemic. We instead observed the data for nearby and distant leaves for evidence of induction. The results for nearby leaves (mean difference = 0.54 ug/mg, paired $t = 0.05$, d.f. = 18, $p = 0.96$; Fig. 3) and distant leaves (mean difference = 8.6 ug/mg, paired $t = 0.92$, d.f. = 17, $p = 0.37$; Fig. 4) support the conclusion that induction did not occur in *P. serotina*.

Caterpillar Feeding Trial

To answer the second question, what effect does leaf damage have on *M. americanum*, we analyzed the results of the feeding trial. The relative growth rates ($p = 0.20$; Fig. 5) did not vary between the different leaf damage categories, therefore the rate of growth in all caterpillars was equal. Results for relative consumption rates ($p = 0.47$; Fig. 6) demonstrated that there was not a significant difference and each caterpillar tended to consume roughly the same amount of food. Digestive efficiency ($p = 0.76$; Fig. 7) tended to be the same across all leaf damage categories, with each caterpillar digesting similar amounts of leaf material. The final performance type, consumption efficiency ($p = 0.17$; Fig. 8) demonstrated non-significance among the different leaf damage categories; all caterpillars tended to convert similar amounts of digested material into roughly the same amount of body tissue.

Discussion

In our study, there is no evidence that the level of damage in *P. serotina* leaves affects the performance of eastern tent caterpillar larvae. Even with a non-significant value from our ANOVA test on caterpillar growth, there were two interesting values identified by the LSD post-hoc test. One point of interest involved growth of caterpillars fed mechanically damaged leaves (the mechanical group) demonstrating a nearly significantly different ($p = 0.057$) rate of growth than the caterpillars fed leaves damaged both mechanically and by caterpillars (the caterpillar/mechanical group). The other point

suggested that growth of the mechanical group has a nearly significantly different ($p = 0.097$) rate of growth than caterpillars fed undamaged leaves (the undamaged group). We compared the relative growth with the three other performance types to attempt to explain these almost significant results.

The caterpillar/mechanical group not only tended to grow less, but also had a tendency to consume less leaf material than the mechanical group. One possible explanation is that leaves were simply less palatable to the caterpillar/mechanical group. Also, it is possible that mechanical damage in surrounding leaves lowered a feeding deterrent or increased a feeding stimulus of the leaf used in the trial.

Again comparing the two groups of caterpillars, those in the caterpillar/ mechanical category tended to have lower conversion efficiency than the mechanical group of caterpillars. Digestive efficiency also tended to be lower in the caterpillar/ mechanical group than in the caterpillars from the mechanical group. Leaf composition could be a possible explanation for this decreased conversion and digestive efficiency. However, we did not test the leaf compositions to see if there was a difference.

The other point of interest involved the mechanical/caterpillar group tending to grow more than the undamaged caterpillar category. Caterpillars in the mechanical category tended to consume more than the caterpillars from the undamaged category. Conversion efficiency also tended to be higher in the mechanical group of caterpillars than caterpillars from the undamaged category. The same reasons as those above apply for these differences. However, digestive efficiency actually tended to be higher in caterpillars from the undamaged category than those in the mechanical group. Even with

an increased digestive efficiency, caterpillars in the undamaged group may have simply grown less because of their lower consumption rate and/or conversion efficiency.

In response to our first question, does induction occur, we found that induction of phenolics in *P. serotina* in response to simulated herbivore damage over a 24-hour period did not occur. If *P. serotina* doesn't induce, two possible explanations are: the ability to induce responses may never have evolved in *P. serotina*, and the ability to induce response did evolve, but has been selected against.

P. serotina may not contain the genetic variability necessary to induce phenolics. Rather, phenolic levels could simply be constitutive throughout the plant, and lack the pathway for induction to occur. However, induced response may have evolved in *P. serotina*, but is now selected against because it reduces fitness of the tree. *M. americanum* and *P. serotina* have evolved a highly specialized interaction, and the high success of *M. americanum* on *P. serotina* indicates that *M. americanum* has counter-adapted to any chemical defenses its primary food item may have. *M. americanum* may be able to pass phenolics through their gut without any detrimental effects. Phenolics may even be beneficial to *M. americanum*. Phenolics could act as feeding stimulants, assist with host location, and/or serve as defense chemicals for the caterpillar against its predators and parasites.

However, induction may occur in *P. serotina*, but not in response to simulated herbivory. Our damage regime may not have had the necessary stimuli to cause phenolic induction. *M. americanum* saliva may contain an essential catalyst that causes induction. From an evolutionary standpoint, it seems logical to assume that *P. serotina* can distinguish between mechanical and herbivore damage. Defense chemicals can

potentially deter herbivore damage. Mechanical damage, however, is not something that a defense chemical can deter. A tree inducing chemical defenses against mechanical damage would then be wasting resources by investing in a defense that is not beneficial to the tree. Natural selection would supposedly favor trees that had the ability to distinguish between mechanical and herbivore damage, possibly by linking the chemical induction pathway to cues received from herbivory.

It is possible that induction did occur in our study, but was not apparent in our results. We made two crucial assumptions that if violated, could hide evidence of induction. First, we assumed that phenolic levels were equally distributed throughout the leaf at 0 hour when baseline leaf samples were taken. If phenolic levels varied throughout the leaf, then our results would not reflect changes due solely to the mechanical damage inflicted on *P. serotina* leaves. Also, we assumed that our assay techniques did not introduce variation in phenolic level results. If our variation in assay technique changed phenolic level results, then again induction could have been disguised in our results.

To test these two assumptions, we ran a regression comparing phenolic levels in the same leaf in the pre- and post- damages leaves ($p = 0.0072$; Fig. 7). We found a significant positive correlation between the two phenolic levels, supporting our conclusion that induction did not occur. However, the phenolic level of the leaf at 0 hour explains only 12% of the variation in phenolic content of the leaves at 24 hours, suggesting phenolic levels were not equally distributed throughout the leaves, and our assay technique may have introduced variation. Despite the low R-squared value, the

significant positive correlation indicates we did not violate our assumptions to a level that would affect our conclusion.

Therefore, we still have no evidence that induction occurred in *P. serotina*, nor that leaf damage levels has an effect on larval performance of *M. americanum.* Again, induction may never have evolved in *P. serotina.* Just as plausible is that the very specific host/herbivore system between *P. serotina* and *M. americanum* allowed the opportunity for co-evolution. *M. americanum* could have evolved immunity to phenolics, causing natural selection to select against trees that induce, and thus waste resources on a non-effective chemical defense. This is consistent with the scenario whereby plants do not induce defense against their specialist herbivores.

References

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Figure 1:

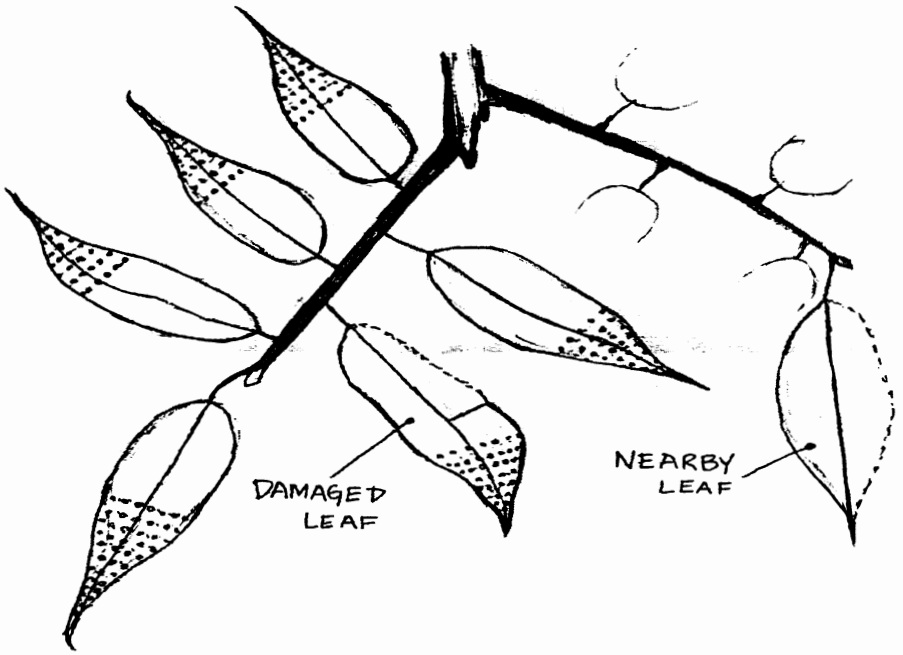

Figure 1: Leaf damage regime

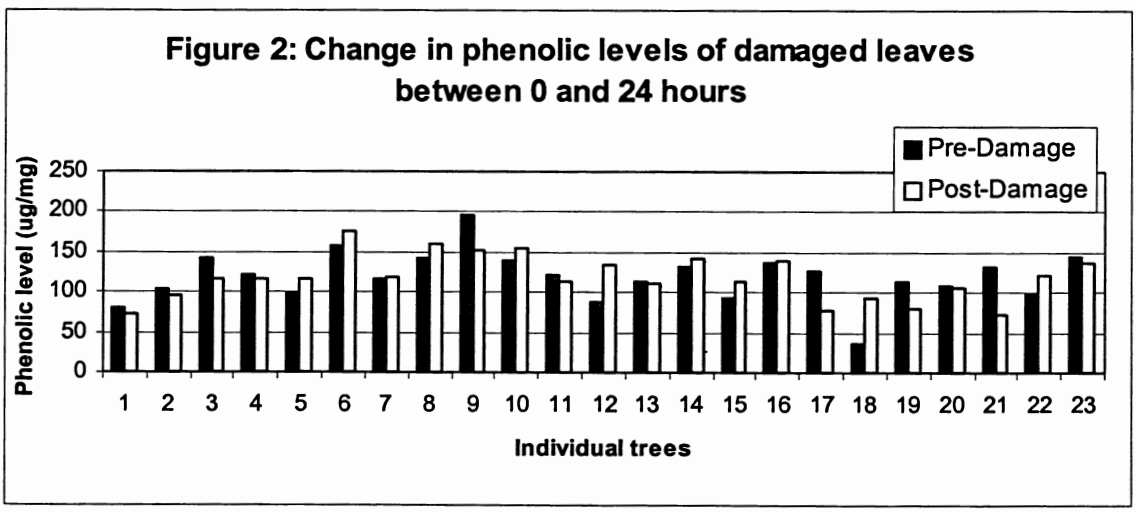

Figure 2: mean difference = -0.83 ug/mg, paired t = 0.14, d.f. = 22, p = 0.89

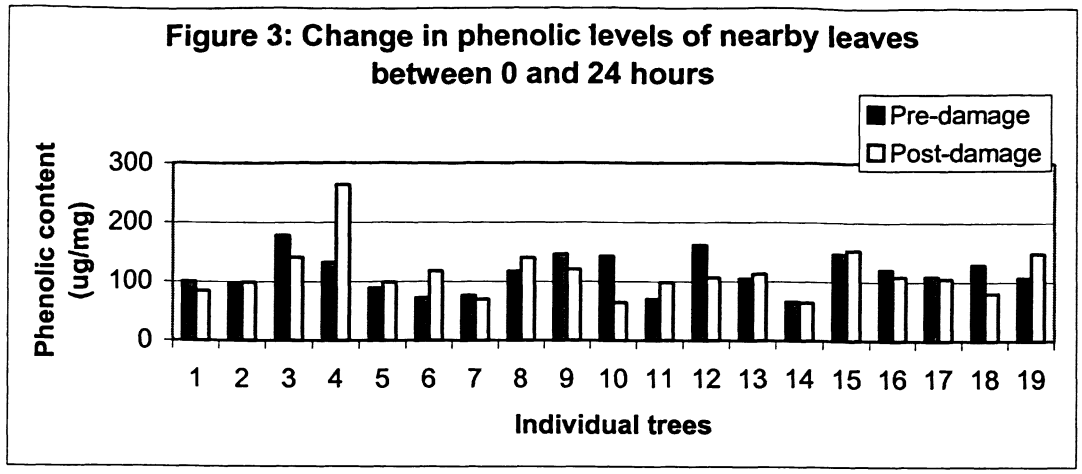

Figure 3: mean difference = 0.54 ug/mg, paired t = 0.05, d.f. = 18, p = 0.96

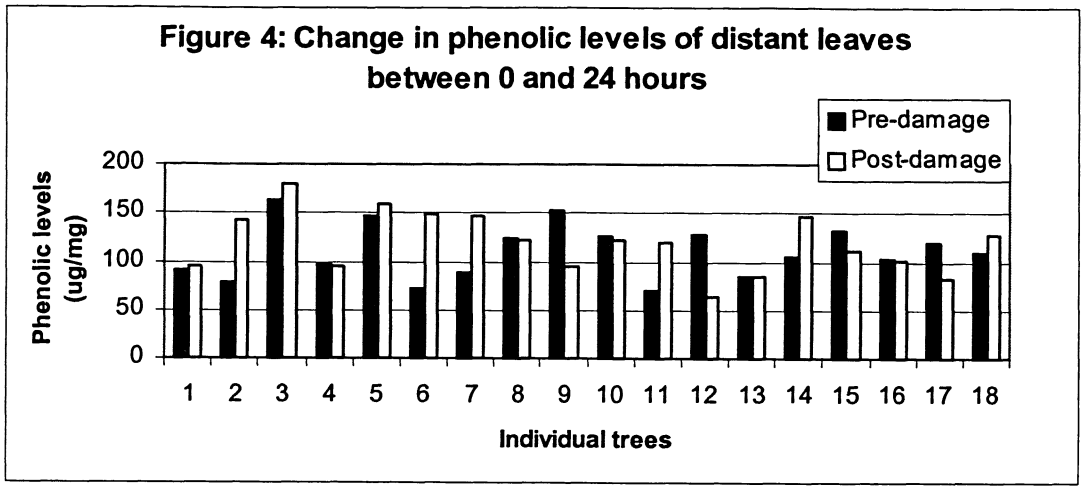

Figure 4: mean difference = 0.86 ug/mg, paired t = 0.092, d.f. = 17, p = 0.37

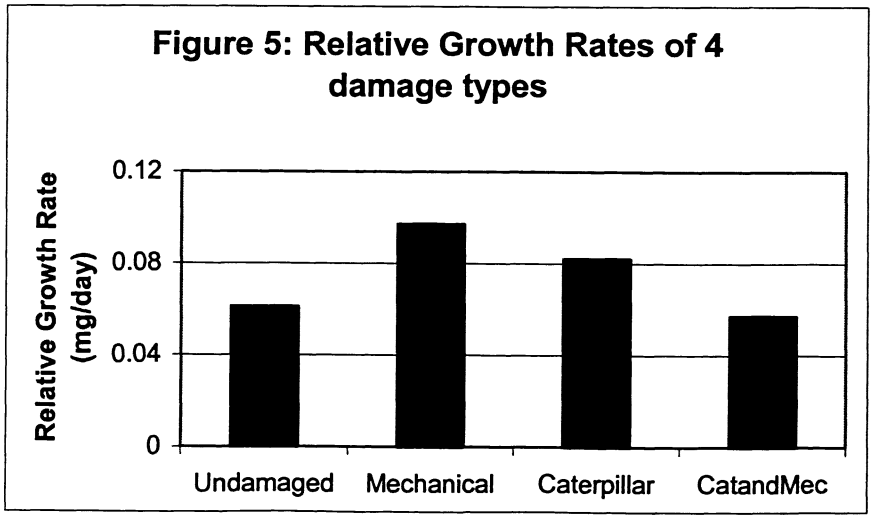

Figure 5: p = 0.20

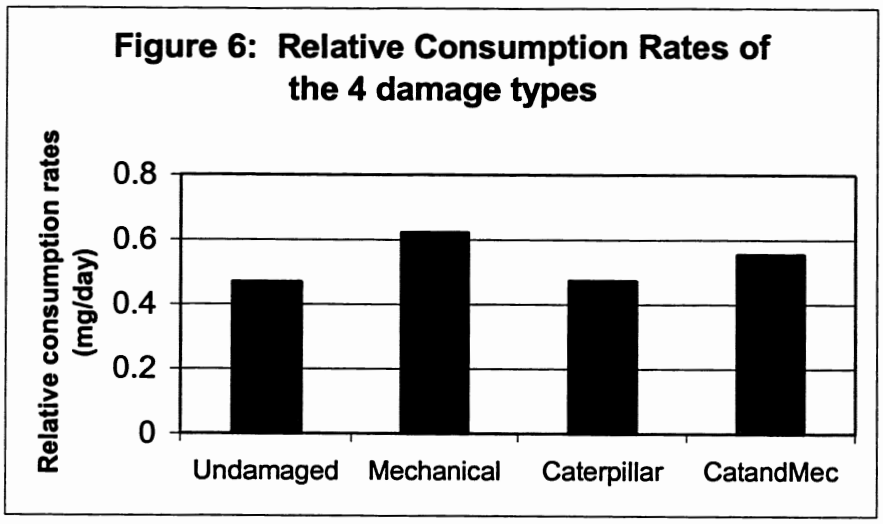

Figure 6: p = 0.47

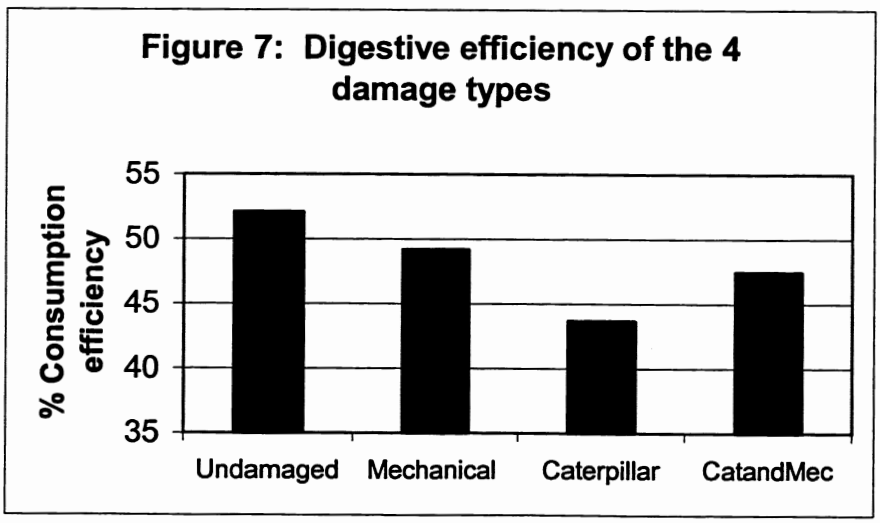

Figure 7: p = 0.76

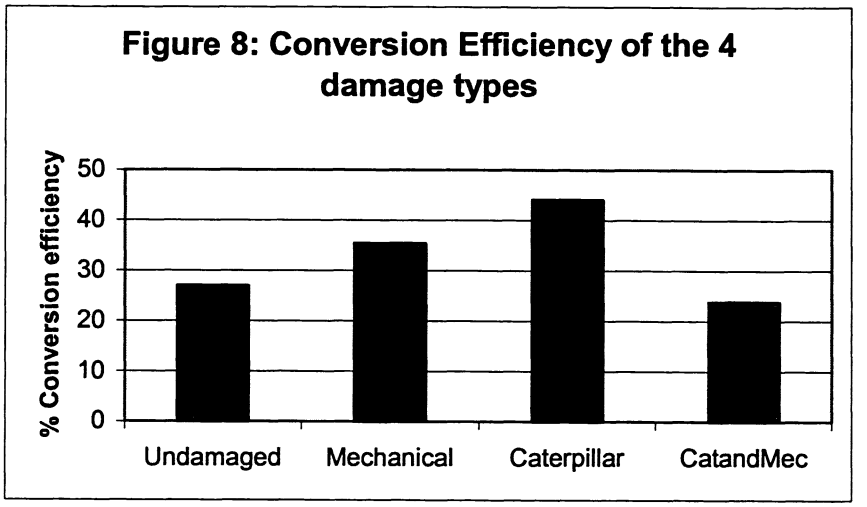

Figure 8: p = 0.17

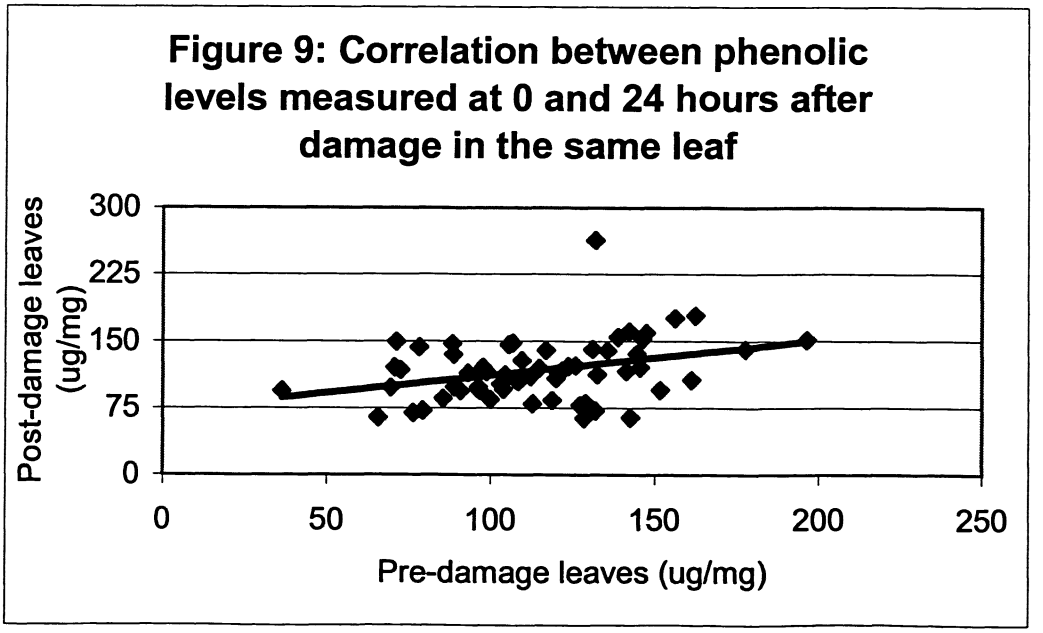

Figure 9: p = 0.007, R-squared = 0.012