December 2016

A comparison of soil pH, leaf caffeine concentration, and disease prevalence in Coffea arabica

Sofia Lochner

A comparison of soil pH, leaf caffeine concentration, and disease prevalence in *Coffea arabica*

Sofia Lochner
Department of Molecular, Cellular, Biology
University of California Berkeley
EAP Tropical Biology and Conservation Program, Fall 2016
16 December 2016

ABSTRACT

This study investigates the relationship between soil pH, caffeine content in leaves, and disease prevalence in *Coffea arabica*. Caffeine is a secondary metabolite that acts as an insecticide and antimicrobial to protect the coffee leaves, shoots, and fruits from fungus and herbivory. I used caffeine water extraction and UV/V spectrophotometry to analyze the caffeine content in leaf samples from a total of forty trees in four different sites at the Life Monteverde farm in Cañitas, Guanacaste. At each tree a soil sample was collected for pH analysis and a disease survey was conducted for *Hemileia vastatrix*, *Mycena citricolor*, and herbivory. Additionally I measured temperature, soil moisture, and light levels at each tree and compared between plots to see if there were significant differences in these factors depending on location. I compared disease prevalence, caffeine content, and soil pH amongst the forty sampled trees and found no significant difference between any of these three factors. The results from this study suggest that leaf caffeine concentration is dependent on other factors.

Una comparación del pH del suelo, la concentración de cafeína en la hoja, y prevalencia de la enfermedad en *Coffea arabica*

RESUMEN

Este estudio investiga la relación entre el pH del suelo, el contenido de cafeína en las hojas y la prevalencia de la enfermedad en *Coffea arabica*. La cafeína es un metabolito secundario que actúa como un insecticida y antimicrobiano para proteger las hojas, brotes y frutos del café de hongos y herbivoría. Utilicé la extracción con agua de la cafeína y el espectrofotómetro UV / V para analizar el contenido de cafeína en muestras de hojas de un total de 40 árboles en cuatro sitios diferentes en la finca Life Monteverde en Cañitas, Guanacaste. En cada árbol, tomé una muestra de suelo para el análisis del pH y realicé un muestreo de presencia de *Hemileia vastatrix*, *Mycena citricolor* y herbivoria. Además, medí la temperatura, la humedad del suelo y los niveles de luz en cada árbol para ver si había diferencias significativas en estos factores dependiendo de la ubicación. No hubo diferencias significativas de prevalencia de la enfermedad, contenido de cafeína y pH del suelo entre los cuarenta árboles muestreados. Estos resultados sugieren que la concentración de cafeína foliar depende de otros factores no estudiados aquí.

The coffee industry is valued at 20 billion dollars and coffee is the main source of income for 25 million small producers worldwide (FAO, 2007). With millions of dollars in losses due to diseases and herbivory, it is important to understand the inherent mechanisms that promote plant

health, in an effort to reduce loss of profits and jobs. This study attempts to understand the relationship between caffeine, disease, and soil pH in *Coffea arabica* plants.

Coffee plants produce caffeine, a secondary metabolite that has well-documented effects on the human central nervous system. With our global population consuming 2.25 billion cups of coffee every day, coffee ranks as the most widely used psychoactive drug in the world (Dicum and Luttinger, 1999). Besides its ability to stimulate the human body, caffeine is shown to be an important insecticide and antimicrobial. High levels of caffeine found in the leaves are toxic to insects that attempt to eat the plant (Freeman, 2008). Caffeine at a dietary concentration of 0.3% kills nearly all larvae of the tobacco hornworm within 24 hours and similar results were obtained with other insects including butterfly larvae, mealworm larvae, milkweed bug nymph, and mosquito larvae. It has been shown that the caffeine toxicity is primarily caused by inhibition of phosphodiesterase activity (Nathanson, 1984). However, caffeine can also be used to attract insects; for example bees get a beneficial energy boost from the low caffeine dose found in the nectar of coffee flowers, enticing them to return and potentially boosting pollination rates (Freeman et al, 2008). Caffeine has also been shown to have antimicrobial and antifungal properties; one study found that caffeine significantly inhibited the growth of *E.Coli* (Ibrahim et al, 2006) and the fungus *Xyleborus fornicates* (Kumar et al, 1995). Caffeine is an energetically costly metabolite for the plant to produce, but helps improve the overall fitness of the coffee plant in many ways (Frischknecht, 1986).

The leaves and seeds of *Coffea arabica* are about 1% caffeine by dry weight and this secondary metabolite is produced through a multistep biosynthetic process (Ashihara et al, 2008). The main pathway for caffeine production is a four-step sequence of three methylation reactions and one nucleosides reaction. Purine nucleotides contain four nitrogen atoms and are the starting material for this pathway; thus for this process to proceed, the plants need an ample source of nitrogen for adequate caffeine production (Ashihara et al, 2008). Since coffee plants attain nitrogen and many other essential compounds from the soil, the quality and nutrient content of soil is very important for overall health of the coffee tree.

Coffea arabica thrives in volcanic, slightly acidic, and fertile soil (Zuchowski, 2007). Among the many factors that affect the health of coffee plants, soil pH is critical; coffee plants can grow in neutral soil, but the optimum pH for best overall health and growth of a tree is between 5.0 and 6.0. However when pH is too low it can cause aluminum toxicity and deficiencies in critical nutrients such as phosphorous, calcium, and magnesium (Department of Agriculture, Forestry, and Fisheries, 2012). Soil pH is hypothesized to be a major component in inorganic nitrogen production in agricultural soils (Kemmitt et al, 2006). Total gaseous emissions of N_2O, NO, and N_2 have repeatedly been shown to be less in acidic soil than in slightly alkaline soils. This may be attributable to smaller amounts of organic carbon and mineral nitrogen available to the denitrifying population under acid condition (Cooper, 2002). A possible factor influencing caffeine concentration could be suboptimal soil pH causing a deficiency in soil nitrogen reserves and thus limiting the caffeine biosynthetic pathway.

Although it is known amongst farmers that soil pH is important for the health of coffee plants, there is very little research about the correlation between soil pH and caffeine content. When soil pH is too low coffee plants are more prone to disease and predation; since caffeine is a defense mechanism (Ceja-Navarro, 2015) it might be that soil pH affects the caffeine synthesis pathway. My central question is: does suboptimal soil pH affect the production of caffeine in *Coffea arabica* leaves? I predicted that if a coffee plant grows in soil with a low pH it will

produce less caffeine, and thus be more prone to disease and predation. Presented here is an analysis of leaf caffeine content, soil pH, and disease prevalence in 40 coffee plants from four different farm sites at the Life Monteverde farm.

METHODS

ONSITE SAMPLE COLLECTION:

The study site was a sustainable coffee farm in Cañitas, Guanacaste called Life Monteverde Farm. The farm plots were dispersed through secondary forest with somewhat variable conditions with respect to elevation, slope of field, integration of shade plants, and wind exposure. I randomly selected ten trees to sample from each of the four selected farm sites. These four sites were selected because previous nutrient and pH data from the Instituto del Café de Costa Rica (ICAFE) existed for these areas.

Each sample consisted of information about the health of one coffee tree and the surrounding soil. To control for other factors that might affect disease and caffeine concentration I recorded soil moisture, light, and temperature. At each tree the soil moisture content was measured with a moisture meter probe, sunlight reaching the tree was measured with the Rapitest 4-way meter, and air temperature was measured with a thermometer (following the manufactures instructions for each instrument). I also measured time of day and weather conditions when the sample was taken. These metrics were not recorded for intensive study of their effect on caffeine concentration and disease, but more to get a general sense of differences between farm sites.

To test for disease incidence I randomly sampled three branches from the bottom, middle, and top of each tree and recorded how many leaves on each branch were affected by the fungi *Hemileia vastatrix* (Roya) and *Mycena citricolor* (ojo de gallo) and general herbivory (Figures 9-11). I recorded all incidences of disease on these nine branches, if more than one affliction occurred on a single leaf, each was recorded separately.

Finally soil and leaf samples were collected. I collected a soil core about 10 cm deep at the base of the tree to later be analyzed for pH in the lab. I also picked five healthy, medium sized leaves from the bottom, middle, and top of the tree. If there were enough healthy, similar-sized leaves I did not pick leaves from the same branch. From the forty trees I collected 600 leaves and checked 5,464 leaves for disease. In total, for each tree I collected a sample of 15 healthy leaves and one soil core and recorded the soil moisture, temperature, light levels, and disease incidence.

TESTING SOIL PH:

First I measured and recorded the pH of the distilled water. I then put 20 g of the soil sample into a beaker and filled the beaker to 40 ml with distilled water. I agitated the mixture for one minute and then filtered the mud with a fine mesh strainer twice. Once the sediments had settled I put the Extech stick in the solution and waited for the pH reading to stabilize for five seconds before recording the value. Since I did not have access to an accurate soil pH meter I used this method with a water pH meter. Thus the pH reading stated here are relative to each other and the water.

DETERMINATION OF CAFFEINE STANDARD CURVE:

The following methods were adapted from Mary Madera's Spring 2014 EAP project. Albino Rodriquez from ICAFE provided the original methods for Madera's experiment. To find the equation relating concentration of caffeine in a solution to absorbance, I measured the absorbance of serial dilutions of a pure caffeine solution. I then applied the y=mx+b equation for this serial dilution regression line to solve for concentration in the coffee leaf solutions.

To make the serial dilution I mixed 10 mg of pure dry caffeine into one liter of distilled water. I put 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 ml of this solution into different graduated cylinders and then filled each one to 10 ml with distilled water (no additional water was added to the 10 ml cylinder) and shook vigorously to combine. The quartz cuvette was cleaned with tap water twice and distilled water once before being filled with the caffeine solution. Before each absorbance recording I blanked the spectrophotometer with distilled water at 274 nm against a cuvette of distilled water. I then measured the absorbance of the dilution twice at 274 nm. I plotted the average absorbance against the known concentration to find the regression line for caffeine absorbance (Figure 1). Using the equation of the line A=0.0828C-0.0016 (where A is absorbance and C is concentration), I solved for concentration (Equation 1). Given the R^2 value was greater than 0.995, I believe that this standard curve provides a reliable equation to determine the concentration of caffeine from the coffee leaf samples.

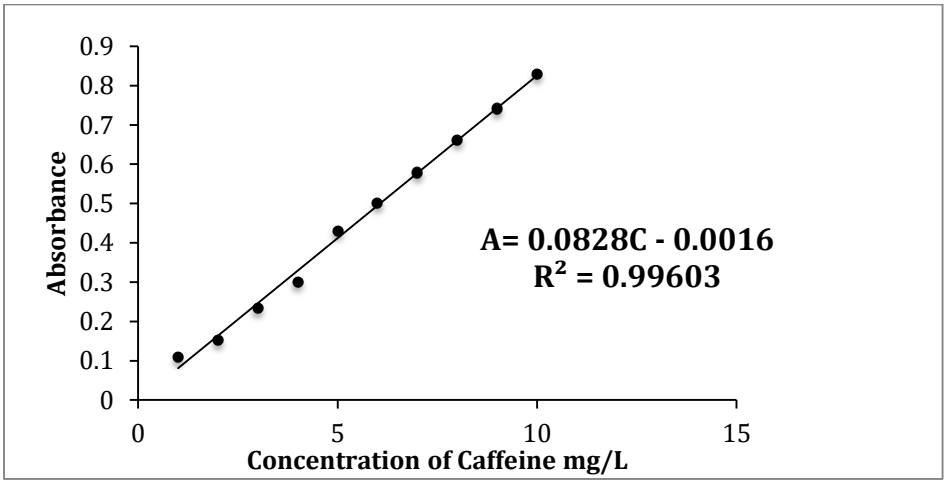


Figure 1. The caffeine standard curve generated by a serial dilution of a pure caffeine solution. The equation A=0.0828C-0.0016 is the y=mx+b equation for the regression line between the ten dilutions. This equation is used to solve for concentration (C) in Equation 1.

EQUATION 1: Absorbance to concentration caffeine

$$\frac{A + 0.0016}{0.0828} = \text{Concentration mg/L}$$

(A= average absorbance at 274 nm)

SPECTROPHOTOMETER SAMPLE PREPARATION:

I refrigerated the leaves collected from the farm until they were placed in a drier no more than 24 hours after collection. Leaves were heated until crisp and dry. I then blended each of the forty samples of 15 leaves until finely shredded (about 15 seconds). I then placed 5 grams of the shredded leaves into a 100 ml Erlenmeyer flask and added 100 ml of water. Caffeine is very

soluble in boiling water (66 g/100 ml) (Sigma- Aldrich). I heated flasks in a water bath for one hour to extract the caffeine. Once the flasks were cooled in a refrigerator, I strained the solution with a coffee filter and refilled the aliquot to 100 ml of distilled water.

Since the spectrophotometer could not detect large concentration of caffeine particles, I had to greatly dilute the coffee leaf samples. To prepare the sample for spectrophotometer analysis I used a syringe to add 0.2 ml of the leaf solution to 40 ml of deionized water. I rinsed the syringe with deionized water and flushed the syringe with the solution three times before removing the 0.2 ml. Once I diluted the leaf solution in a graduated cylinder I used a clean glass rod to mix the new solution to ensure a uniform concentration.

SPECTROPHOTOMETER ANALYSIS:

I used the same UV-200 RS spectrophotometer for all samples and the standardized curve. For the caffeine samples I rinsed the quartz cuvette with tap water twice, with deionized water once, and then twice with the prepared sample. Since the peak absorbance for caffeine is around 274 nm, I measured the absorbance of each sample between 280 nm and 270 nm. I tested each sample twice, zeroing the blank at 280 nm with the same sample of distilled water each time and refilling the cuvette with a fresh sample.

Using Equation 1 and Equation 2, I calculated the percent caffeine of the original dry sample from the average absorbance of each sample. From Equation 1, I found the concentration (mg/L) of caffeine in the sample and then entered this into Equation 2 to incorporate the dilution factor, leaf mass, and unit conversions to get a final percentage of caffeine per sample.

EQUATION 2: Concentration caffeine to percent caffeine

$$\textbf{Percent Caffeine} = \frac{(\text{Concentration x Initial Volume})}{\text{Mass of leaf}} \text{ x } 100\% \text{ x Dilution Factor of Cuvette Sample}$$

$$= \frac{Concentration\ mg}{L\ solution}\ x\ \frac{1L}{1000\ ml}\ x\ \frac{100\ ml\ total\ sample}{1}\ x\ \frac{1\ g}{1000\ mg}\ x\ \frac{1}{5\ g\ leafs}\ x\ 100\%\ x\ \frac{40\ ml\ cuvette\ sample}{0.2\ ml\ sample}$$

$$\textbf{= Concentration x } \mathbf{0.4}$$

I averaged the two samples to generate the spectrophotometry curve for each of the forty samples. If any peak absorbance were at wavelengths greater than +/- 2 nm from 274 nm I excluded these points from the data set because there were presumably contaminated. I used StatPlus to run ANOVA tests to compare farm site to each variable (all three diseases and soil moisture, temperature, and light).

RESULTS

The main focus of this study is the relationship between soil pH and caffeine content in coffee leaves. I found no statistically significant correlation between the pH of the soil in which a tree grows and the caffeine content in its leaves (Figure 2). Caffeine averaged 2.72% of the

sample weight and ranged from 3.97% to 1.97%. There was a significant difference ($F_{3,36}$=3.87, p=0.017) in soil pH between the farm sites with an average soil pH range of 4.107 to 4.705 between farm sites and with a whole farm average of 4.506.

Additionally, the R^2 values for the comparison of caffeine concentration to the prevalence of *Hemileia vastatrix* (R^2 = 0.0177), *Mycena citricolor* (R^2 = 2.8E-06), and herbivory (R^2 = 0.02007) suggested caffeine was not a good indicator of disease prevalence (Figures 3-5). One unusual finding is that *Hemileia vastatrix* prevalence was significantly different between sites ($F_{3,36}$=16.80, p<0.01). At site 1, 2, and 3 the percent of average affected leaves per tree was 1.09%, 3.04%, and 3.63% respectively. Site 4 was an outlier with 20.22% of its leaves affected by *Hemileia vastatrix.* There was no significant correlation between soil pH and disease prevalence (Figures 6-8). Furthermore there was no significant difference between the plots for moisture and temperature, but there was a significant difference between plots for light levels ($F_{3,36}$ =9.75, p<0.01).

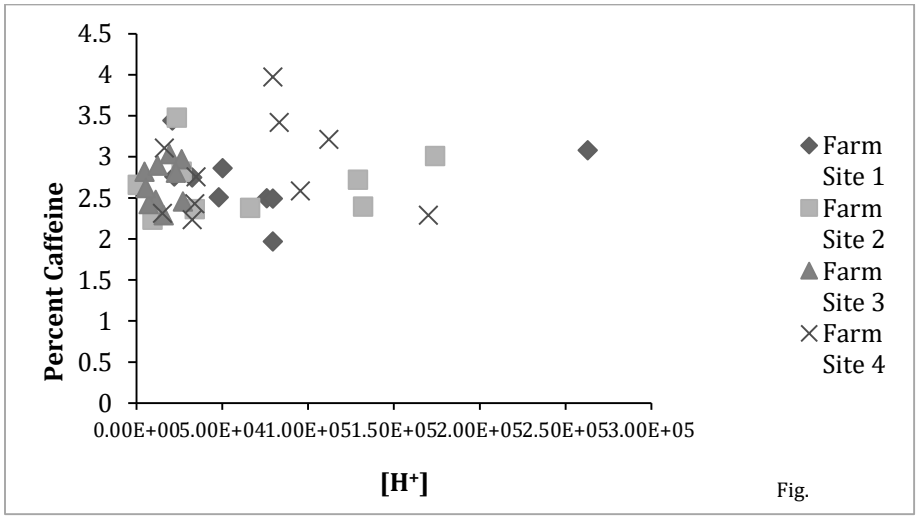
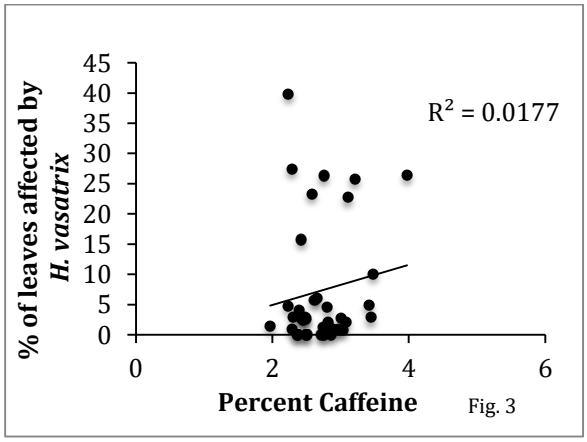
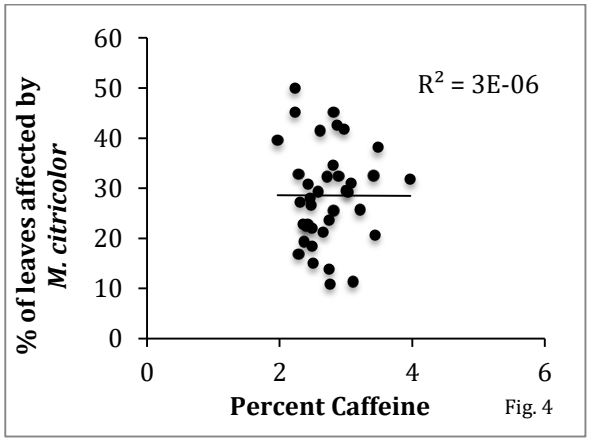


Figure 2: Percent caffeine in leaf sample compared to soil hydrogen ion concentration ($[H^+]$). A comparison of $[H^+]$ to percent caffeine found in each five-gram sample of dried coffee leaves. No correlation was found between $[H^+]$ and percent caffeine concentration, as supported by the low R^2 value of 0.011.





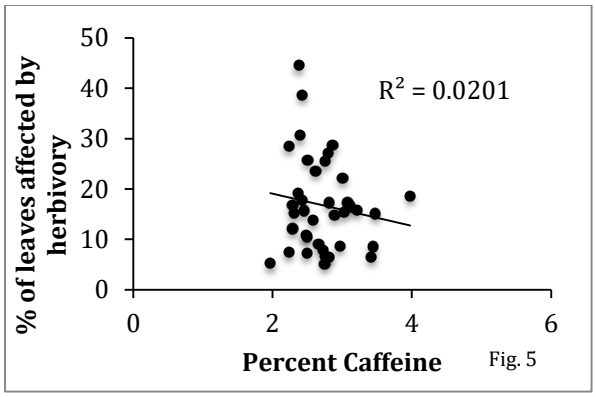
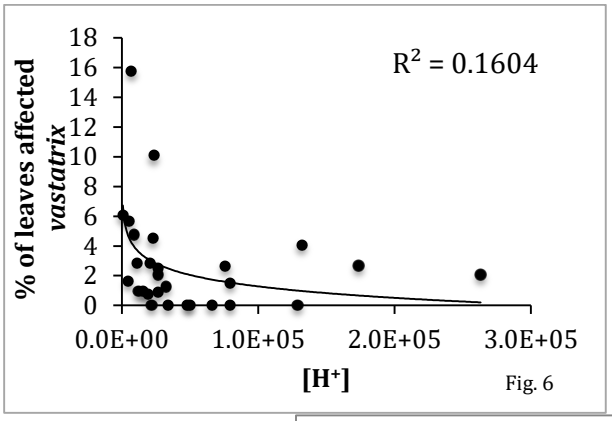
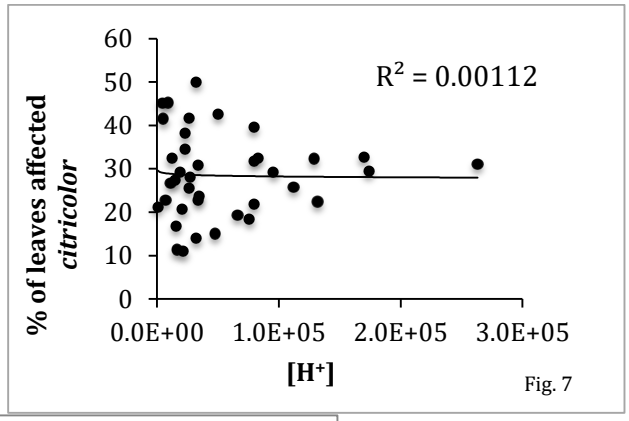
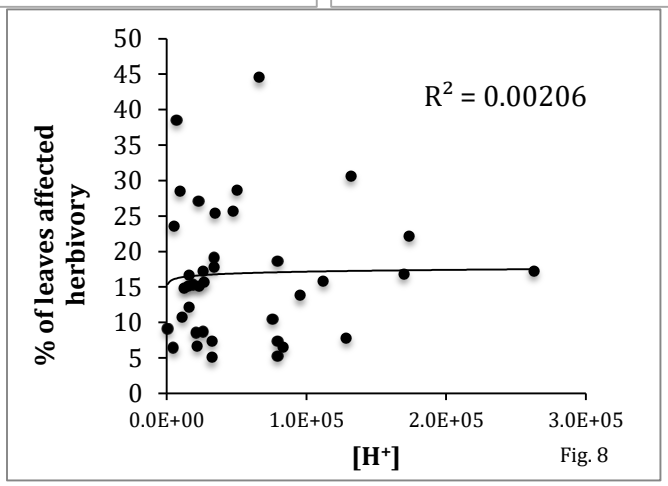


Figure 3-5. *Hemileia vastatrix*, *Mycena citricolor*, and Herbivory vs Percent Caffeine of Leaf Sample. The percent of disease-affected leaves per tree compared to the percent caffeine in the five-gram sample generated from the 15 leaves from each tree.







Figures 6-8. *Hemileia vastatrix*, *Mycena citricolor*, and Herbivory vs soil Hyrdrogen Ion Concentration. Site four was a significantly different outlier for *H. vastatrix* and was not included. Note these are logarithmic curves because hydrogen ion concentration is measured on a logarithmic scale.

DISCUSSION

The purpose of this study was to explore the relationship between soil pH, disease, and caffeine content of in *Coffea arabica* leaves. I predicted that plants growing in a lower soil pH would have relatively less caffeine in their leaves, thus make the plants more prone to disease. Over the soil pH range sampled, the caffeine concentration remains relatively constant. There was no significant difference between caffeine content and soil pH, suggesting that differences in caffeine content are due to other factors.

Interestingly, there was no correlation between disease prevalence and caffeine concentration found in this study. Other research findings support the theory that caffeine is a potent protective alkaloid (Kim et al, 2006). However there are a few studies that show that caffeine is not effective at preventing certain insects from eating the coffee plant tissues, such as with the leaf miner *Perileucopter coffeella* (Filho and Mazzafer, 2000). While conducting the herbivory sample, I noticed the presence of leaf miner tracks in some of the leaves. Another of the most destructive sources of herbivory not addressed in this study (but present on the farm) is the coffee berry borer, *Hypothenemus hampei.* These beetles have gut microbes that can break down the caffeine, rendering the insects unharmed from the normally lethal doses (Ceja-Navarro et al, 2015). Insects specially adapted to tolerate caffeine could have skewed by herbivory count. Although there are expectations such as *P. coffeella* and *H. hampei*, caffeine is generally considered a highly effective insecticide (Frischknecht, 1986). Thus I find it is curious that there is no correlation between disease and caffeine content was found in this study.

The lack of correlation might stem from my sampling technique: I selected only healthy leaves to test for caffeine. There is research showing that plants can redirect their secondary metabolites to injured tissues (Coley and Barone, 1996), so I avoided damaged leaves to control for this phenomenon. However, this method may have caused me to miss trends in caffeine and disease because coffee trees may not systemically increase total caffeine content when under attack, but is rather a localized redistribution of caffeine (Frischknecht et al, 1986). This could be a way to allocate secondary metabolites in an energy efficient manner (Madera, 20014). The interaction between detrimental insects and fungi with coffee's natural defense systems is a complex phenomenon that could provide valuable insights for better pest management. If we understand how to plant distribute its secondary metabolites while we could avoid removing the plant tissues with the most caffeine or cultivate strains that produce the most caffeine in the most damage prone areas of the plant. The distribution of the plant's natural insecticide resources is an important direction for future research.

Hemileia vastatrix is a massive problem for coffee farmers, destroying up to 90% of crops in some regions of the world (McCook, 2006). One unusual finding in this study was that farm site four had a relatively large amount of *H. vastatrix,* since there is no significant correlation between soil pH and the fungi's prevalence in this location, the high *H. vastatrix* prevalence must be due to other factors. The spores of this fungus are spread through wind and rain, but also through human movement (McCook, 2006). This plot was also located next to the roaster, parking lot, and road; it is possible that human traffic helps spread *H. vastatrix* faster there than in other parts of the farm. To decrease infection rates, it is essential we critically examine how our behaviors promote the spread of disease, not only in regards to *H. vastatrix* but to all afflictions of the coffee tree.

I conducted the brief survey of light, soil moisture, and temperature conditions to develop a general sense of what other factors were actively affecting the health of the coffee trees. There was no significant difference between the plots for moisture and temperature, but there was a significant difference between plots in light levels ($F_{3,36} = 9.85$, $p<0.01$). Differences in light levels could be related to varying amount of shade from windbreaks, time of day, and weather conditions. However there was no apparent pattern between disease prevalence or caffeine concentration and light levels. It would be necessary to collect light level data on many more days and times to get a reliable estimate of lights effects on caffeine and disease. For these three variables I assumed they were similar enough amongst the forty trees to have negligible affects on leaf caffeine content and disease prevalence.

Various sources of error could have skewed my results. It is possible that I could have biasedly selected the trees, branches, and leaves I randomly sampled. Additionally for light, soil moisture, soil pH, and temperature I only sampled once at each tree in the morning; to increase the quality of this data I could sample many times and take an average over time. With more time and more accurate instruments I could collect more reliable and detailed data.

There are many avenues for future research in the realm of caffeine, soil, and disease. By studying other factors (i.e. age, pruning times, water contaminants, etc.) that might affect caffeine content in coffee plants, coffee growers can get a better sense of how to provide an ideal environment in which coffee plants can produce the most secondary metabolites. Research on soil components, such as soil nutrient levels and texture, could provide valuable insights into the best growth substrates to obtain higher coffee yields. If people wish to minimize profit losses due to disease it is important to understand how the many environmental factors affect the growth of the coffee plant.

With coffee being a billion dollar industry it is essential that scientists and farmers learn how to control the spread of fungi such as *Hemileia vastatrix* and *Mycena citricolor* and reduce the impacts of herbivory to minimize job and profit losses. Although this study found no correlation between soil pH, leaf caffeine content, and disease, further research is needed to understand the factors that influence the health of Coffea *arabica* plants. By promoting the production of the coffee plant's protective secondary metabolites and other defense mechanisms, the use of traditional insecticides, herbicides and fungicides may be reduced: increasing production and minimizing loses through environmentally friendly methods.

ACKNOLEDGEMENTS

Many thanks to Sofía Arce Flores for the immense amount of time and guidance you put into this project. Also thank you to Frank Joyce and all the UCEAP staff that helped make this project possible.

WORK CITED

Ashihara, H., Sano, H., Crozier, A. 2008. Caffeine and related purine alkaloids: Biosynthesis, Catabolism, function and genetic engineering. Phytochemistry, Vol. 69. 841-856.

Ceja-Navarro, J. A., Vega, F. E., Karaoz, U. Hao, Z., Jenkins, S., Lim, H. C., Kosina, P., Indante, F., Northern, T. R., Brodie, E. L. 2015. Gut microbiota mediate caffeine detoxification in primary insect pest of coffee. Nature Communications, Vol. 6.

Coley, P. D. Barone, J. A. 1996. Herbivory and plant defenses in tropical forests. Department of Biology, University of Utah. Vol 27. 305-335.

Cooper, J. E. 2002. The influence of soil pH on denitrification: progress towards the understanding of this interaction over the last 50 years. European Journal of Soil Science, Vol. 53. 3:345-354.

Deparment of Agriculture, Foresty, and Fisheries. 2012. Production Guideline Coffee. 1-28.

Dicum, G., Luttinger, N. 1999. The coffee book: anatomy of an industry from the crop to the last drop. The New York Press, New York.

Filho, O. G., Mazzafer, P. 2000. Caffeine does not protect coffee against the leaf miner *Perileucopter coffeella*. Journal of Chemical Ecology, Vol. 26. 6:1447-1464.

Food and Agriculture Organization of the United Nations (FAO). 2007.The state of food and agriculture.

Freeman, B. C., Gwyn, G. A. 2008. An overview of plant defenses against pathogens and herbivores. Plant Pathology and Microbiology, 1-12.

Frischknecht, P. M. Ulmer-Dufek, J. Baumann, T. W. 1986. Purine alkaloid formation in buds and developing leaflets of *Coffea arabica*: expression of an optimal defense strategy? Phytochemistry. Vol. 25. 3:613-616.

Ibrahim, S. A., Slameh, M. M., Phetsomphou, S., Yang, H., Seo, C. W. 2006. Application of caffeine, 1,3,7-trimethylxanthine, to control *Escherichia coli* O157:H7. Food Chemistry, Vol. 99. 4:645-650.

Kemmitt, S.J., Wright. D., Goulding, K. W.T., Jones, D. L. 2006. pH regulation of carbon and nitrogen dynamics in two agricultural soils. Soil Biology and Biochemistry, Vol. 38. 5:898-911.

Kim, Y.K., Uefuji, H., Ogita, S., Sano, H. 2006. Transgenic tobacco plants producing caffeine: a potential new strategy for insect pest control. Transgenic Research, Vol. 15. 6:667-672.

Kumar, N. S., Hewavitharanage, P., Adikaram, N. K. B. 1995. Attack on tea by *Xyleborus fornicates*: Inhibition of the symbiote, *Monacrosporium ambrosium*, by caffeine. Phytochemistry, Vol. 40. 4:1113-1116

Madera, M. 2014. Comparison of caffeine concentrations in pairs of *Coffea arabica* leaves exhibiting insect damage and *Hemileia vastatrix*. UCEAP Spring 2014.

McCook, S. 2006 Global rust belt: *Hemileia vastatrix* and the the ecological integration of world coffee production since 1850. Journal of Global History, Vol. 1. 2:177-195.

Nathanson, J.A. 1984. Caffeine and related methylxanthines: possible naturally occurring pesticides. Science, Vol. 226. 184-187

Simga-Aldrich. Product information for caffeine. Sigma-Aldrich publications.

Zuchowski, W. 2007. Tropical Plants of Costa Rica. A Zona Tropical Publication. 194-195.

APPENDIX

Table 1. The raw data for percent caffeine, percent of leaves affected by disease and herbivory, and soil hydrogen ion concentration for each tree sampled. Note two samples from site one and site two were omitted because their spectrophotometer curve was presumably contaminated.

Figure 9. A leaf with signs of herbivory.

Figure 10. A leaf affected by *Mycena citricolor*.

Figure 11. A leaf affected by *Hemileia vastatrix*.