SPECIALTY COFFEE IN COSTA RICA: EFFECT OF ENVIRONMENTAL FACTORS AND MANAGEMENT OPTIONS ON SOIL CHEMISTRY AND MICROBIAL COMPOSITION

A Thesis

by

LINDA SUSAN STURM-FLORES

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2012

Major Subject: Agronomy

Specialty Coffee in Costa Rica: Effect of Environmental Factors and Management Options on Soil Chemistry and Microbial Composition

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Committee Members,	Terry J. Gentry
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May 2012

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ABSTRACT

Specialty Coffee in Costa Rica: Effect of Environmental Factors and Management Options on Soil Chemistry and Microbial Composition. (May 2012)

Linda Susan Sturm-Flores, B.S., The College of William and Mary;
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Co-Chairs of Advisory Committee: Dr. William A. Payne
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In the Central Valley of Costa Rica in the Department of Heredia, I investigated the soil chemical properties and microbial communities under four native shade tree species in a coffee agroforestry system. In the second year of the study, Effective Microorganisms®, a microbial inoculant, was applied to examine its effect on soil chemistry. The shade tree species included in this study were *Anonna muricata* L., *Diphysa americana* Mill., *Persea americana* Mill., and *Quercus* spp. L.

Plots measured 20 by 30 meters and were replicated three times for each shade tree species except for *Quercus* spp., which only had two replications. Twelve composite soil samples were collected from each plot in 2008 and again in 2009, and twelve composite foliar samples were taken from the coffee plants in each plot in 2008. The results of this study indicated that the species of native shade tree had a significant effect on soil ammonium-N, nitrate-N, total dissolved nitrogen and magnesium. Sun or shade position had a significant effect on dissolved organic nitrogen and dissolved

organic carbon. The species of native shade tree also had a significant effect on the composition of soil microbial communities. PLFA analysis revealed a significant difference in soil fungi abundance in soil samples from *Annona* plots relative to those from *Persea* plots. Effective microorganisms in combination with the tree species, as well as in combination with species and sun or shade position, had a significant interaction effect on soil ammonium-N, with the EM-treated plots showing higher concentrations of soil ammonium-N. There was a significant positive correlation between soil pH and foliar calcium, as well as soil dissolved organic nitrogen and foliar %N, at $p< 0.01$.

This study suggests that *Quercus* spp. is a tree species that may help to regulate the cycling of nitrogen in the coffee agroecosystem. *Annona muricata* appears to inhibit the action of some fungal species and may reduce the occurrence of fungal pathogens in the soil, although the present study did not explore this issue. Although *Diphysa americana* is a legume, it does not appear to increase the amount of soil nitrogen in the vicinity of the coffee plants themselves. All four tree species in this study improve coffee soils by increasing soil concentrations of dissolved organic nitrogen and dissolved organic carbon. Coffee yield data and long term observations on the health of the coffee plants would clarify whether one of these species is particularly beneficial, from an agronomic perspective, for the productivity of this coffee agroecosystem.

DEDICATION

This work is dedicated to my Lord and Savior Jesus Christ. Thank you, Lord God, for filling my life with purpose and joy. Thank you for giving me strength during hard times. Thank you for blessing me with wonderful people who assisted me along the way to the completion of this thesis. My greatest desire is that my daughters, my work and my life may glorify You.

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I would like to thank my committee co-chairs, Dr. William A. Payne for his support and encouragement of my professional career over the past eight years, and Dr. Jacqueline Aitkenhead-Peterson, for her guidance and enthusiasm for this project for the past four years. I am also appreciative of the valuable guidance I received from committee members Dr. Terry Gentry, Dr. Andrew Klein, and Dr. Richard Woodward.

This study would not have been possible without the support of my Costa Rican research collaborator, Dr. Edgardo Alpizar Vargas of the Doka Estate Coffee Company in Heredia, Costa Rica, and Dr. Panfilo Tabora of Earth University, Costa Rica. I would also like to thank Dr. Fernando Casanovas at the Center for Tropical Research and Education (CATIE) in Turrialba, Costa Rica who provided excellent advice as to the experimental design of this study.

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CHAPTER I

INTRODUCTION

I.1 Factors affecting coffee production

Coffea arabica L., an evergreen shrub whose native habitat is the forest understory of the high plateau regions of Ethiopia, is the world's second largest dollar value commodity traded following petroleum. It is estimated that 25 million small-scale coffee farmers and their families from tropical, developing countries worldwide produce about 70% of the world's coffee (ICO, 2002; Wintgens, 2004).

Although the genus *Coffea* includes approximately 70 species, there are two main species cultivated for commercial purposes: *Coffea arabica L.,* grown between 1200 and 2000 m above sea level in Africa, Asia, and the Tropical Americas, and *Coffea canephora* Pierre ex A. Froehner, grown in tropical Africa below 1000 meters (Wintgens, 2004). Approximately 90% of the coffee beverage consumed worldwide comes from *Coffea arabica* L. When this species is grown with the correct suite of environmental conditions, the coffee bush can produce a beverage with an outstanding flavor (Wintgens, 2009). The manner in which coffee is grown is critically important from an environmental standpoint since coffee thrives in some of the most biologically diverse regions on the planet.

The majority of coffee is produced by farmers in tropical, developing countries with holdings of 20 or fewer hectares of land. Traditionally, farmers have grown their

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This thesis follows the style of Soil Biology and Biochemistry.

coffee in combination with leguminous and fruit producing shade trees. In addition to providing fruit, firewood, and mulch, the shade from their canopies benefits coffee bushes by reducing diurnal fluctuations in air temperature, protecting coffee plants from intense wind and rain, and minimizing soil erosion. In the Mérida State in the Venezuelan Andes, Ataroff and Monasterio (1997) reported that over a two-year period, soil loss in the <4 mm soil fraction on 31% slopes was doubled for an established full sun plantation when compared to a shade plantation. Shaded coffee plantations also provide habitat for a variety of insectivorous birds and wildlife (Gallina et al., 1996; Mogul and Toledo, 1999; Perfecto et al., 1996; Raghuramulu, 2005). Coffee plantations with a cover of diverse, native shade trees conserve biodiversity by serving as biological corridors between forested and protected areas (Estrada et al., 2006).

Shade trees play a vital role in maintaining water availability for the coffee crop as well. In Chiapas, Southern Mexico, Lin (2010) compared four sites, one with high shade cover (60-80%), two sites with medium shade cover (30-65% and 45-65%), and one site with low shade cover (10-30%). She found that with a shade cover of 30% or more, there was a significant 32% reduction in evaporative transpiration demand when compared to the low shade cover site. During both the wet and dry seasons, soil sensor measurements showed that the soil moisture in the medium and high shade cover sites was significantly higher than in the low shade cover site (Lin, 2010). Coffee transpiration demand was also significantly higher in the low shade site relative to the two medium shade sites and the high shade site and this pattern was especially pronounced during the dry season (Lin, 2010).The benefit of water availability to coffee

bushes can be expected to become even more important as climate change reduces the area suitable for coffee cultivation, in large part to due to issues relating to water availability (Laderach et al., 2010). Shade trees also promote nitrogen availability in coffee agroecosystems. Babbar and Zak (1994) investigated nitrogen cycling in the Central Valley of Costa Rica and found an average net mineralization rate of 14.8 g N m^{-3} yr^{-1} in shaded plantations, relative to 11.1 g N m^{-1} yr^{-1} in full sun plantations.

In the late 1970's, highly productive coffee varieties that thrived in full sun began to replace traditional coffee plantations. These new varieties of coffee were promoted for two reasons: a) there was an increasing demand for coffee beans worldwide and b) both industry experts and rural farmers alike believed that full sun coffee would be less likely to contract coffee leaf rust (*Hemileia vastatrix*), a fungal disease that had been accidentally introduced to Latin America (Perfecto et al., 1996). Researchers in Chiapas, Mexico, however found that the incidence of leaf rust was negatively correlated with the number of strata in coffee agroforestry systems (Soto et al., 2002), suggesting that inclusion of shade trees in the canopy layer would confer protection against coffee leaf rust. While full sun plantations produce higher yields of coffee beans than shaded plantations, full sun coffee has proven to be ecologically unsustainable. For example, full sun plantations experience more soil erosion and require higher inputs of agrochemicals (Perfecto et al., 1996). Pesticide use in full sun plantations has led to livestock deaths, contaminated aquifers, and illness and death among Central American coffee farmers (Boyce, 1994). Full sun plantations have proven to be economically unsustainable as well. During the 1980's, overproduction

contributed to a sharp decline in coffee prices. This decline was accompanied by an increase in the cost of agrochemicals used for coffee production, and coffee farmers suffered a dramatic decrease in their incomes (Boyce, 1994). In addition, full sun plantations remain productive for only half as long relative to shaded plantations, which increases replacement costs for individual coffee bushes (Perfecto et al., 1996).

The human health problems and associated degradation of the environment, combined with expanding markets for shade grown organic and specialty coffees, have resulted in a great deal of interest in organic methods for coffee production. A persistent obstacle to *"going organic"*, however, is that organically grown coffee has not been as productive as conventionally grown full sun coffee. For example, Lyngbaek et al. (2001) found that as a group, the ten multistrata organic farms they studied were 22% less productive than ten comparable conventional coffee farms, defined as having full sun coffee or coffee shaded by only a single species of shade tree. When farmers transition from conventional farming to organic farming, there is a drop in total and plant available soil nutrients during the first two years, likely due to the time it takes for leaf litter and other forms of mulch to fully decompose (Afrifa et al., 2003). Likewise, in a six-year study conducted at sites in Nicaragua and Costa Rica contrasting four management regimes: 1) moderate organic, 2) intensive organic with chicken manure, 3) moderate conventional, and 4) intensive conventional, in Costa Rica, it took the organic experiments (1 and 2) two years longer to develop levels of productivity similar to that of the conventionally managed plots (Haggar et al., 2011). In Nicaragua the organic plots that were intensively managed produced a harvest equal to that of the

conventionally managed coffee from the first harvest (Haggar et al. 2011). The discrepancies in the results from the pairs of sites in Costa Rica and Nicaragua may be explained by the initial differences in soil fertility, as the Nicaraguan site was used for shade coffee or young secondary forest prior to establishing the experiment, while the Costa Rican site was previously used for sugarcane production.

Nitrogen has been found to be one of the key factors limiting productivity in organically grown coffee. Although it is possible to supply organic matter by means of mulch, crop residues, and organic fertilizers, optimum available nitrogen levels are seldom achieved in practice, and the timing of crop demand and nutrient availability is often not synchronized. In addition, a significant amount of nitrogen is lost during the process of composting of manures and plant residues due to volatilization as N_2, N_2O, or NH_3, or by transformation into stable organic compounds (Berry et al., 2002).

Organic farming does not necessarily reduce the occurrence of diseases and pests below economically harmful thresholds (Van Der Vossen, 2005). Furthermore, Van der Vossen (2005) found that to sustain economically viable yields of 1 Mg ha^{-1} yr^{-1} green coffee additional composted organic matter would have to be brought in from external sources, a requirement that most smallholder farmers cannot meet using current organic farming methods.

I.2 Effective Microorganisms®

Dr. Teruro Higa of the University of Ryukyus, Okinawa, Japan coined the term *"Effective Microorganisms®"* (EM) to describe a group of microorganisms which work synergistically to promote and maintain healthy plant growth (Higa and Parr, 1994). The

use of EM may have the potential to increase the productivity of organic farming systems (Subadiyasa, 2003). Although it has been reported that most of the organically certified coffee farmers in Costa Rica and Guatemala use EM (Lotter, 2005), there are no published studies concerning the use of EM and its effect on soil chemistry in coffee plantations.

Originally, EM was cultivated from 80 species of bacteria belonging to 10 genera (Higa, 1993). Previous efforts to change the microflora of soils by introducing single cultures of microorganisms have, for the most part, been unsuccessful. EM works differently in that it combines microorganisms which are ecologically compatible and work in a synergistic manner (Higa and Parr, 1994). EM technology has been modified over time to include large populations of lactic acid bacteria (*Lactobacillus plantarum*, *L. casei*, and *Streptococcus lactis*, among others), and yeasts (*Saccharomyces spp.*), with smaller numbers of photosynthetic bacteria (*Rhodopseudomonas palustris* and *Rhodobacter sphaeroides*), Actinomycetes (*Streptomyces spp*.) as well as other types of microorganisms (Javaid, 2010).

In an EM culture, both photosynthetic bacteria and aztobacters coexist symbiotically by an exchange of food sources between them. The aerobic azotobacters consume organic matter and produce waste products which are the ideal food source for photosynthetic bacteria (Higa, 1993). When there is an over proliferation of azotobacters, anaerobic conditions are created. In the absence of photosynthetic bacteria, putrefaction, the phenomenon of organic substance decomposition into inorganic substances, would begin to occur. Putrefaction produces unstable, often

harmful intermediate products and a large amount of heat (Higa, 1989; Higa and Parr, 1994). In the presence of photosynthetic bacteria, however, the intermediate waste products produced by the azotobacters and other microorganisms are used by the photosynthetic bacteria. Higa and Parr (1994) claim that these microbes use heat as an energy source to produce useful organic compounds from these intermediate waste products that can be absorbed directly by plants, although there is an absence of scientific consensus supporting this theory.

Proponents of EM state that it benefits plants producing antioxidants (Higa, 1993). These investigators theorize that antioxidants in the soil prevent the harmful reactions brought about by oxidation. As a result, plant roots become stronger and more capable of carrying on functions essential to their growth and health, such as absorbing nutrients from the soil. Higa postulates (Higa 1993) that the synergistic activity of the microorganisms in EM promote an antioxidized soil condition in which beneficial plant hormones persist for a longer period of time.

Amino acids and organic acids are also affected by the oxidative state of the soil. When the predominant condition is oxidative, amino and organic acids are changed into molecules that are useless or harmful to plants. Amide, an intermediary produced by the breakdown of amino acids, is highly toxic and can cause inertia in plant cells (Higa, 1993). In addition, amino acids in oxidized soils may be broken down and absorbed by plants in the form of ammonium in wet soils or nitric acid in dry soils. In order for the plant to synthesize an amino acid from these sources of inorganic nitrogen, the plant needs to attach a sugar molecule to it. This places a burden on the process of

photosynthesis, using up sugar molecules to reform amino acids rather than using them for other plant functions. Proponents of EM theorize that when antioxidants are present in the soil, the oxidation of amino acids by soil bacteria is retarded, allowing the amino acids to persist in the soil for a longer period of time, making them more available to plant roots where they can be absorbed and converted into vegetable protein. Furthermore, under antioxidative conditions, organic acids are converted into sugars in the soil (Higa, 1993), becoming an energy source for the soil food web. Again, there is a lack of scientific consensus in support of the theories developed by Higa (1993).

The major objectives of my study were to: a) to examine the effect of shade trees on soil chemistry and microbial community diversity, b) to examine relationships between soil and foliar chemistry and c) to investigate the effect of adding Effective Microorganisms® on soil chemistry in the vicinity of different shade tree species and in sun and shade positions.

CHAPTER II

THE EFFECT OF SHADE TREE SPECIES AND POSITION ON SOIL CHEMICAL PROPERTIES AND MICROBIAL COMMUNITY COMPOSITION

II.1. Introduction

Beginning in 1840, coffee was Costa Rica's first export product and the backbone of its socioeconomic development. In recent decades coffee's role in the Costa Rican economy has declined due to microprocessor production replacing coffee and bananas as the country's leading source of export revenue (Nelson, 2008). However, coffee still generates essential income for approximately 5% of the nation's work force, most of which are small farmers with five or fewer hectares of land (Ronchi, 2002).

The volcanic soils and ideal altitudes of the Central Valley of Costa Rica produce exceptionally high quality coffee. *Coffea arabica* L. is the only species cultivated in Costa Rica, with Caturra Vermelho being the predominant cultivar used. It is a relatively small variety with dark green leaves and abundant secondary branches, with fragrant white flowers approximately 3 cm in diameter, and fruits and seeds slightly larger than the Bourbon variety (Krug et al., 1947). The coffee beverage produced by Caturra Vermelho is characterized by a good body with a light, acidic flavor. Caturra Vermelho was discovered in Brazil in 1937 as a mutant of the Bourbon variety and has been used to develop high-density coffee-growing practices (Wintgens, 2004).

The manner in which coffee is grown in Costa Rica and elsewhere is critically important from an environmental standpoint since the zones appropriate for coffee cultivation are potentially among the most biologically diverse regions on the planet.

Traditionally, farmers have grown their coffee in combination with leguminous and fruit producing shade trees, although with the introduction of more highly productive full sun varieties, many coffee farmers converted to full sun coffee monocultures in the 1980's. About 30% of Costa Rican coffee grown today is considered full sun coffee, although the percent of shade cover is quite low on many so-called shaded coffee farms (Znajda, 2000). In addition to providing fruit, firewood, and mulch, shade benefits coffee plants by reducing diurnal fluctuations in air temperature, protecting coffee plants from intense wind and rain, minimizing soil erosion, and providing habitat for a variety of insectivorous birds and wildlife (Gallina et al., 1996; Mogul and Toledo, 1999; Perfecto et al., 1996; Raghuramulu, 2005). Shade has been found to promote the production of larger coffee beans, especially when coffee is grown at lower altitudes, perhaps by lowering the temperature of the microclimate in which coffee grows (Muschler, 2001). Vaast et al. (2006) also found shade to increase the size and improve the chemical composition of coffee beans grown in the Central Valley of Costa Rica, considered an ideal coffee growing region, by delaying bean maturation by up to one month. An additional incentive for farmers to grow native shade trees within their coffee plantations is that doing so qualifies the coffee for Rainforest Alliance Certification, which increases coffee's appeal to socially and environmentally conscious consumers and allows the product to command a higher price in the marketplace (www.rainforest-alliance.org/agriculture/certification). Coffee plantations with a cover of diverse, native shade trees conserve biodiversity by serving as biological corridors between forested and protected areas (Estrada et al., 2006).

It has long been recognized that not only must a soil be suitable for a tree species to flourish, but the tree itself affects the formation of the soil in which it is growing (Remezov and Pogrebayak, 1969). Studies in North America have shown that the chemical characteristics of forest floors differ with the dominant tree species present, in part due to differences in the chemical composition of the leaf litter produced (Binkley and Menyailo, 2005b). However, in a natural setting, it is often difficult to determine whether soil chemistry is the result of site characteristics such as soil parent material, local topography and aspect, local climate or the age of the soil, which may confer a competitive advantage on certain species as opposed to others, or whether the soil properties observed are the product of the changes that followed as a result of the establishment of the tree species themselves (Binkley and Menyailo, 2005a). For example, cedar forest floors tend to have higher concentrations of calcium, relatively alkaline pH, and inorganic nitrogen predominantly in the form of nitrate. In contrast, forest floors of western hemlock-dominated forests tend to have a more acidic pH, lower concentrations of calcium, and a higher proportion of inorganic nitrogen in the form of ammonia (Binkley and Menyailo, 2005a).

Some effects of tree species on soil chemistry have been well documented as due to the influence of the tree species themselves. For example, there is moderately strong evidence that the amount of nitrogen mineralized per year in a hectare of forest surface soil is inversely related to the lignin:N ratio of the litterfall of the dominant tree species (Binkley and Giardina, 1998). At least 10 separate studies suggested that nitrogen-fixing trees increase the concentration of carbon in the soil and enhance the cycling of other

soil nutrients as well (Binkley and Giardina, 1998). The bulk of the literature on the interactions between tree species and soils has been conducted in temperate forested or boreal ecosystems (e.g. Menyailo et al., 2002; Vesterdal et al., 2008; Smolander and Kitunen, 2011).

Results from studies on temperate forests cannot be necessarily be extrapolated to tropical forests, which are distinctive in climate and rainfall patterns and house a greater diversity of plant and animal species. For example there are typically 4 tree species per acre on average in a temperate forest versus 20-86 per acre in a tropical forest which supports a variety of vines, lianas, and epiphytic plants (Lowman and Bouricius, 2003). Powers et al. (2004) conducted a study in the rain forest of Costa Rica at the La Selva Biological Station to examine the effects of four trees from four different families on the forest floor material. The investigators contrasted the forest floor soil chemistry under mature *Pentaclethra macroloba* Kuntze (Mimosaceae), which they considered to be the background or baseline trees for the study, with forest floor soil chemistry beneath the canopy of four target species: *Hyeronima alchorneoides* Allemão (Euphorbiaceae), *Lecythis ampla* Miers (Lecythidaceae), *Depteryx panamensis* Record & Mell (Fabaceae), and *Balizia elegans* Ducke (Mimosaceae). They found no significant differences in soil concentrations of calcium, potassium, magnesium, phosphorus, carbon, nitrogen, or pH suggesting that tree species had no effect on soil chemistry. Payán et al. (2009) compared ten paired conventional and organic coffee fields shaded by the commonly used legume shade tree *Erythrina poeppigiana.* The researchers found that soil carbon and nitrogen concentrations declined significantly

with increasing distance from the tree's stem. Samples taken within one meter of the shade trees contained significantly higher soil carbon and nitrogen concentrations than did samples taken from the same depth two or more meters from the trees. It is likely that the active root zone, as well as the litterfall from the trees contributed the carbon and nitrogen. This was not the case with the organic farms, where carbon and nitrogen soil concentrations remained relatively constant throughout the farm, presumably due to the use of organic inputs and the purposeful mulching and distribution of the trees' pruning residues. The work of Pavan et al. (2009) supported the findings of Beer (1988) who reported that leguminous shade trees in coffee fields can contribute between 5000 and 10,000 kg ha^{-1} $yr^{-1}$of organic material to the soil in the form of leaf litter and pruning residues. Corroborating the idea that leaf litter and pruning residues may supply organic C and N to soil beneath shade tree canopies, Palm and Sanchez (1990) reported that the polyphenolic compounds in three different species of tropical legumes in an alley cropping experiment in the Peruvian Amazon influenced the rates of decomposition of the leaf litter more than the percent nitrogen or lignin in the leaflets. They concluded that nitrogen release by legumes with high polyphenolic concentrations will be slower than that by legumes with low polyphenolic concentrations.

Polyphenolic compounds are typically present in leaf litter, whereas the C produced by roots as exudates or sloughing of dead cells tends to have no polyphenols and therefore a greater biodegradability. In a later study by Palm and Sanchez (1990) the decomposition and nitrogen release patterns from the leaves of 10 tropical legume trees commonly used in agroforestry systems were investigated in a laboratory

experiment and compared to the lignin, nitrogen, and phenolic content of the leaflets. Rice straw was used as a control. Palm and Sanchez (1990) concluded that net mineralization was not correlated to % N or % lignin in the leaf material but was found to be negatively correlated to the polyphenolic concentration or the polyphenolic-to-N ratio. The researchers concluded that mineralization in excess of the control soil only occurred when the materials had a polyphenolic:N ratio of less than 0.5. Costa Rican farmers are interested in learning about these and other effects of shade trees on soils in their coffee fields, as such information may facilitate their choice of shade trees for their coffee agroforestry systems (Albertin and Nair, 2004; Alpizar Vargas, *personal communication,* 2008).

Because the quantity and chemical composition of leaf litter varies among tree species, it follows that different tree species provide different substrates upon which microorganisms can act (Pastor and Post, 1986). When released from the leaf litter, phenolic substances, which serve to protect plants against herbivory and parasitism, influence the rates at which mineralization and nitrification occur by affecting the soil microbes responsible for these processes (Kuiters, 1990). Polyphenols can also complex with proteinacious substances, making the nitrogen unavailable to the system. Polyphenols produced by different tree species have different protein-complexing capabilities. This may allow the plant to regulate the form that nitrogen may take and give the plant a competitive advantage for the uptake of nitrogen in organic form (Hättenschwiler and Vitousek, 2000). In an experiment using purified tannins from oak leaf litter in an artificial aqueous medium, fourteen of nineteen fungal species tested

were inhibited, four of which were considered important decomposer species (Harrison, 1971).

Plants often have associated bacteria in their rhizospheres that promote the healthy growth of the host plant by providing nutrients or other growth factors, or by producing antibiotics and other substances which hinder the growth and survival of harmful microbes (Burr et al., 1984; Davidson, 1988). Ushio and colleagues (2008) investigated the composition of microbial communities in the top 5 cm of soil under the crowns of two conifers and three broadleaved trees in the tropical montane forest of Mt. Kinabalu, in Malaysian Borneo. They found that the abundance of specific microbial biomarker lipids corresponded with soil pH, total carbon and total nitrogen. Thus, tree species may affect the soil microbial community indirectly through their effects on these soil factors.

The objectives of this study included examining the effects of native tree species and sun or shade positions of the soil sample on soil chemistry and microbial community composition as well as determining if specific groups of soil microbes have a significant relationship to soil chemistry.

II. 2. Materials and methods

II.2.1. Site description

The study was carried out in the Central Valley of Costa Rica in the Department of Heredia at Finca La Hilda, a Rainforest Alliance Certified coffee farm owned by the Doka Estate Coffee Company. The study site is at an altitude ranging from 1300 and 1400 m above sea level. Rainfall in the region varies between 2400 and 3600 mm yr^{-1}

with approximately 90% of the rainfall occurring during the months of June through November followed by a dry season December through May (Meteorological Station, Finca la Hilda, 2007-2010). The soils at the study site were classified as Udands, which are Andisols with an udic moisture regime.

II.2.2. Experimental design

A three factorial arrangement in a split-split plot design (Figure 2.1) was used to study the effects of tree species and sun or shade position on soil chemistry and soil microbial community composition. Within this experimental design, experimental plots were selected based on the presence of native shade tree species, which included a) *Annona muricata* L. (Annonaceae), b) *Diphysa americana* Mill. (Fabaceae), c) *Quercus* spp.L. (Fagaceae), and d) *Persea americana* Mill. (Lauraceae). These species were of interest due to either their nutritional value for wildlife and humans (Pers*ea* spp. and *Annona* spp.) or their value as a fine wood (*Quercus* spp. and *Diphysa americana*). The shade tree species were not pruned. The plots measured 20 x 30 m. A two factorial arrangement was used to study effect of tree species and sun/shade position with three completely randomized replications for *Anonna muricata, Diphysa americana*, and *Persea americana*, and two replications for *Quercus* spp., in a split-split plot design (Figure 2.1). The eleven experimental plots were delineated and soil samples were collected from the plots influenced by the shade of *Persea* on May 17, 2008, one week after the onset of the rainy season and before the application of any fertilizers. Two weeks later, soil samples were taken from the *Quercus, Diphysa* and the *Annona* plots.

These samples were taken after the first round of fertilizer applications for the growing season and three weeks into the rainy season.

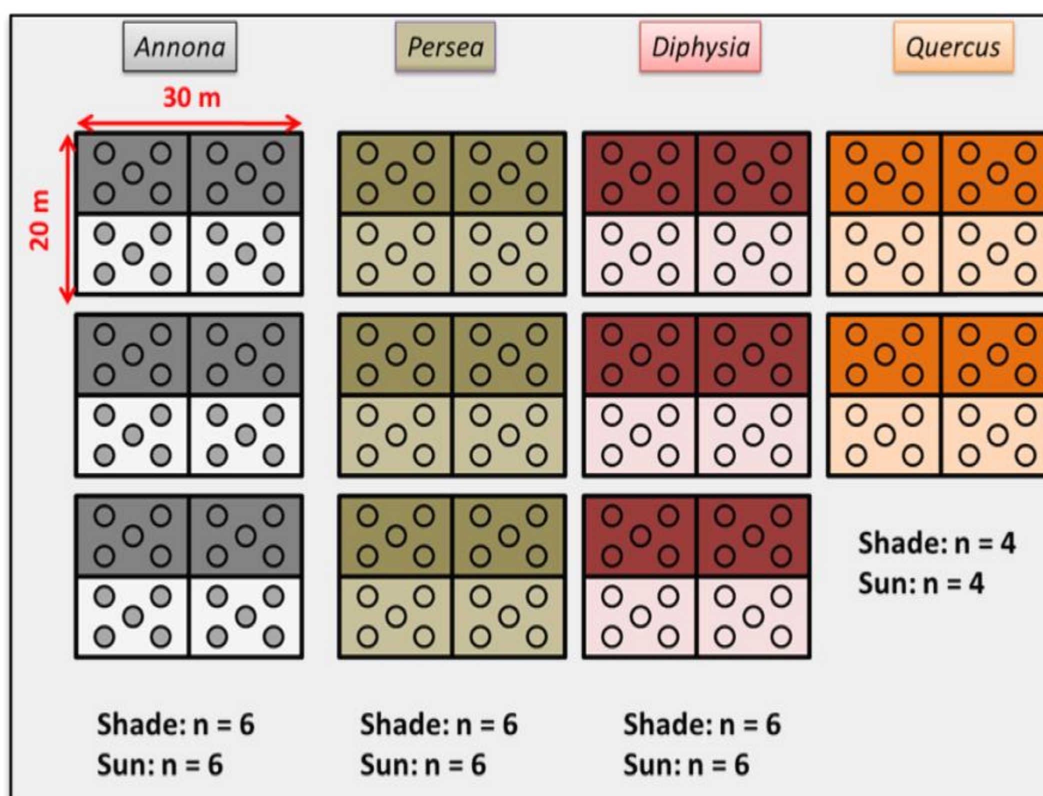

Figure 2.1. Experimental design showing two factorial and split-split plot arrangement.

II.2.3. Sample collection and processing

Four composite soil samples were taken from each plot (Figure 2.1). For the soil samples collected, one soil sample was the product of five 2.5 cm diameter soil cores extracted with an auger 40 cm from the base of individual coffee bushes. Soil cores

were taken to a depth of 20 cm and mixed together in a plastic three gallon bucket. A composite sample was scooped with a metal hand spade into a quart-sized zip lock bag. Before the next soil core was taken, both the soil auger and the mixing bucket were disinfected with iodine and then carefully rinsed to prevent transfer of microorganisms from one area into another. A total of 44 composite soil samples were collected and frozen within four hours of sampling at -20° Celsius in a freezer located at the study site and shipped to Texas A&M on dry ice for analyses.

For each tree species foliar samples were taken from five representative coffee bushes in the shade, and from 5 representative coffee bushes in the sun. Four leaves were sampled per coffee bush from the third fully opened leaf pair (Gitimu, 1998). Two shade and two sun samples were taken per plot, resulting in a total of 44 foliar samples. Foliar samples were collected from each plot on the same days that the soil samples were collected and were place in brown lunch-size, labeled paper bags. The bagged leaf samples were then taken to the CAFESA lab in Heredia, Costa Rica for chemical analyses.

II.2.4. Chemical analyses of soil samples

Water extractions were performed on each of the 44 soil samples. Aliquots of 3.5 g of air-dried, sieved (2mm) soil were placed in 50 mL high density polyethelene (HDPE) centrifuge tubes and combined with 35 mL of ultra-pure water to achieve a soil:water ratio of 1:10. The centrifuge tubes were shaken for 90 minutes at 70 rpm. The soil:water units were then centrifuged at 19,600 g-force for 20 min (Sorval RC6 with SS34 rotor) at 4°C prior to removal of the supernatant using a canula and syringe.

pH and electrical conductivity were recorded on the supernatant prior to its filtration through ashed (500° C at 4 h)Whatman GF/F filters (nominal pore size 0.7 μm) and transfer to acid-washed, ultra-pure water rinsed, 50 mL HDPE bottles and frozen until chemical analyses.

Dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) were measured using high temperature Platinum-catalyzed combustion with a Shimadzu TOC-VCSH and Shimadzu total measuring unit TNM-1 (Shimadzu Corp. Houston, TX, USA). Dissolved organic carbon was measured as non-purgeable carbon using USEPA method 415.1 which entails acidifying (1N HCl) the sample and sparging for 4 min with C-free air. Ammonium-N was analyzed using the phenate hypochlorite method with sodium nitroprusside enhancement (USEPA method 350.1) and nitrate-N analyzed using Cd–Cu reduction (USEPA method 353.3). Alkalinity was quantified using methyl orange (USEPA method 310.2). All colorimetric methods were performed with a Westco Scientific Smartchem Discrete Analyzer (Westco Scientific Instruments Inc. Brookfield, CT, USA). Dissolved organic nitrogen (DON) is the product of TDN—(NH_4-N + NO_3-N).

For analysis of base cations and anions, aliquots of extracts were filtered through 0.2 μm Pall filters. Calcium, magnesium potassium and sodium were quantified by ion chromatography using an Ionpac CS16 analytical and Ionpac CG16 guard column for separation and 20 mM Methanosulfonic acid as effluent at a flow rate of 1 mL min^{-1} and injection volume of 10 mL using a DIONEX ICS 1000 (DIONEX Corp. Sunnyvale, CA, USA). Fluoride, chloride, bromide and sulfate were quantified on a Dionex ICS 2000

using an Ionpak AS20 and Ionpak AG20 analytical and guard columns for separation with 35 mM KOH as effluent at a flow rate of 1 mL min^{-1} and an injection volume of 25 µL (Dionex Corp, Bannockburn, IL). For all analyses a NIST traceable standard, check standard, replicate sample and blank were run every 10th or 12th sample for quality control purposes.

II.2.5. Chemical analyses of foliar samples

The chemical analyses of the foliar samples were carried out in the CAFESA laboratory located in Heredia, Costa Rica under the supervision of Ing.Marco Corrales. Total nitrogen was determined the Micro-Kjeldahl method (Emmert, 1935). Boron concentrations were analyzed using dry digestion and spectrophotometry with Azomethine-H (Zaijun et al., 2006). Sulfur and phosphorus were investigated using dry digestion with spectrophotometric analysis of turbidity with $BaCl_2$ and ammonium molybdate, respectively. Potassium, calcium, magnesium, copper, zinc, and manganese concentrations were determined by dry digestion with spectrophotometric analysis of atomic absorption.

II.2.6. Analyses for microbial community composition

Phospholipid fatty acid (PLFA) analysis was used to assess the composition of the microbial communities inhabiting the collected soil samples. Microbial cells have membranes comprised of a bi-layer of phospholipids. The fatty acid composition of this bilayer, that is the types of phospholipid fatty acids present, can serve as biomarkers for different microbial functional groups. PLFA analysis can be used to analyze both bacterial and fungal biomass (Frostegard and Baath, 1996). Since phospholipids are

rapidly degraded following cell death, PLFA analysis is a good way to assess living biomass in a soil sample. PLFA analysis is often the method of choice for microbial analyses, since one of the limitations of traditional dilution plating and culturing methods is that it is estimated that only a small fraction (less than 1%) of the soil microbial community is culturable by known techniques (Hill et al., 2000). It is assumed that all PLFA's are equally accessible to the extracting agent and that the PLFA profile of the extract is representative of the *in situ* soil system (Tate, 2000). The specific method employed for this study is described by Ushio et al. (2010).

Twenty-two composite soil samples were prepared for PLFA analysis by thoroughly mixing and then removing approximately 10 g of soil using a small metal spatula from each of the 44 air-dried composite soil samples from the twelve experimental plots. The two sun position samples and the two shade position samples from each plot were then combined, mixed together, and approximately 5 grams of this composite sample were put into a glass test tubes for freeze-drying. These 22 composite samples were freeze-dried using liquid nitrogen and then shipped on frozen gel packs to the University of Wisconsin in Madison, Wisconsin.

Briefly, PLFA analysis involves fractionating the lipids into neutral lipids, glycolipids, and phospholipids. The first two classes of lipids are discarded, while the phospholipids are methylated to give fatty acid methyl esters (FAME's). The FAME's are analyzed by a gas chromatograph coupled with a mass spectrometer, which generates peaks whose area corresponds to the amount of a particular FAME present (Tate, 2000). Upon receiving the lipid data from the University of Wisconsin, a conversion factor of

2.87 E -06, which was derived from the internal standards on the gas chromatograph, was used to convert from peak area to µgrams lipid g soil $^{-1}$. I then divided by the unit weight of the soil and converted to µmol lipid g soil^{-1} by dividing by the molecular weight of the lipid, to arrive at the absolute abundance of the lipid biomarker. Finally, I divided the absolute abundance by the total mol g $^{-1}$ of lipid extracted and multiplied by 100 to get mol%. This final step normalized the data and indicated the relative abundance of any given lipid (Balser, 2007).

I reduced my dataset by excluding any FAMEs that were present in the blanks or in the standards, FAMEs that appeared in less than 0.5% of the dataset, unknown FAMEs, and any lipids containing more than 20 carbons. The result was a complete lipid biomarker dataset consisting of over 100 FAMEs. Although a plethora of lipid biomarkers have been cited (Table 2.1), I included just 21 of these biomarkers because they were well-supported in the literature in the analysis of shade tree species versus abundance of microbial groups. Lipid biomarkers representing Gram+ bacteria were the following iso-branched lipids: *i*14:0, 15:0, i15:0, a15:0, i16:0, 17:0, i17:0, and a17:0. 19:0 10 Me represented Actinomycetes. Mono-unsaturated, alcohols, and cyclopropyl lipids indicated Gram $^{-}$ bacteria and included 16:1ω7c, 12: X OH, 16: X OH, *cy*17:0, *cy*19:0 c11-12, SF 8 (18:1ω7c/18:1ω9t). Biomarkers for fungi included 18:3ω6c, SF19 (18:2ω6c/T:18:0), 18:1ω9c, and 16:1ω5c (Frostegard and Baath, 1996; Sinsabaugh et al., 1999; Ushio et al., 2010; Zelles et al., 1992).

II.2.7. Statistical analyses

Means and standard deviations for each soil chemical constituent were calculated for each shade tree species and under sun or shade position. Univariate analysis of variance with two factors (tree species and sun or shade position) was performed to test the hypothesis that shade tree species and sun or shade position have no effect on soil chemistry. Univariate analysis of variance with two factors (tree species and sun or shade position) was also used to test the hypothesis that shade tree species have no effect on the composition of soil microbial communities. Pearson bivariate correlation analysis was used to examine correlations between soil chemistry and individual microbiological groups using the 21 lipid biomarkers listed above. Cluster analysis with Euclidean distance was used to examine similarity or dissimilarity among microbial community compositions among shade tree species. The complete dataset of over 100 lipid biomarkers was used to perform the cluster analysis and to generate diversity indices using the software program SPSS v. 16.

Table 2.1. PLFA biomarkers, microbial group to which they belong, and source of information.

PLFA Biomarker	Microbial Group	Reference
14:0	Common Bacterial Signature	Gonzalez-Chavez et al., (2010)
i14:0	Gram + bacteria	O'Leary and Wilkinson, (1988)
15:0	Common Bacterial Signature	Tunlid and White, (1992)
i15:0	Gram + bacteria	Zak et al., (1996); Zogg et al., (1997); Ringelberg et al., (1997); Bardgett et al., (1996); Frostegård and Bååth, (1996)
a15:0	Gram + bacteria	Zak et al., (1996); Ringelberg et al., (1997); Bardgett et al., (1996); Frostegård and Bååth, (1996)
i15:1G	Gram + bacteria	Gonzalez-Chavez et al., (2010)
15:0 3OH	Gram - bacteria	Gonzalez-Chavez et al., (2010)
Br 2OH-15:0	*Flavobacterium balustinum*	Tunlid and White, (1992)
16 : 0	Gram + bacteria	Gonzalez-Chavez et al., (2010)
i16:0	Gram + bacteria	Zak et al., (1996); Zogg et al., (1997); Bardgett et al., (1996); Frostegård and Bååth, (1996)
16:1 ω 9	Common Bacterial signature	Hill and al., (2000)
10Me 16:0	Sulfate reducer (Gram +; actinomycetes)	Tunlid and White, (1992)
16 : 1 ω 5	Arbuscular mycorrhizal fungi; Common Bacterial signature; Eucaryotes?	Nordby et al., (1981); Tunlid and White, (1992)
16 : 0 10	Nocardioform fungi	Frostegard et al., (1993b)

Table 2.1. Continued.

PLFA Biomarker	Microbial Group	Reference
16:3 ω 3	Microalgae	Hill and al., (2000)
17:0	Common Bacterial Signature	Tunlid and White, (1992)
i17:0	Gram + bacteria	Zak et al., (1996); Ringelberg et al., (1997); Bardgett et al., (1996); Frostegård and Bååth, (1996)
a17:0	Gram + bacteria	Zak et al., (1996); Ringelberg et al., (1997); Frostegård and Bååth, (1996)
cy17:0	Gram – bacteria (Anaerobe)	Zak et al., (1996); Zogg et al., (1997); Ringelberg et al., (1997); Bardgett et al., (1996); Frostegård and Bååth, (1996)
i17:1G	Gram + bacteria	Gonzalez-Chavez et al., (2010)
Br17:1	Sulfate reducer	Tunlid and White, (1992)
17:1 ω 6	Sulfate reducer	Tunlid and White, (1992)
17:1 ω 8c	Common Bacterial Signature	Gonzalez-Chavez et al., (2010)
i17:1 7 ω c	*Desulfovibrio*	White et al., (1977); Ringelberg et al., (1997); Pinkart et al., (2002)
i17:1 ω 7	*Flavobacterium balustinum* (sulfate reducer)	Tunlid and White, (1992)
18:0	Common Bacterial signature	Gonzalez-Chavez et al., (2010)
18:1 ω 5c	Gram - bacteria	Zak et al., (1996)
18:1 ω 5	Common Bacterial signature	Hill and al., (2000)
18:1 ω 7	Common Bacterial Signature	Tunlid and White, (1992)
18:1 ω7t	Common Bacterial signature; aerobe	Zogg et al., (1997); Hill et al., (2000)

Table 2.1. Continued.

PLFA Biomarker	Microbial Group	Reference
18:1 ω 8c	Methane-oxidizing bacteria, Type 2	Tunlid and White, (1992)
18:1 ω 8t	Methane-oxidizing bacteria, Type 2	Tunlid and White, (1992)
18:1 ω 6c	Methane-oxidizing bacteria, Type 2	Tunlid and White, (1992)
18:1 ω 7c	Gram - bacteria	Zak et al., (1996); Zogg et al., (1997); Ringelberg et al., (1997); Bardgett et al., (1996); Frostegård and Bååth, (1996)
18:1 ω 7	Gram – bacteria	Wilkinson, (1988)
18 : 2 6 ω c	Fungi	Guckert et al., (1985)
18:2 ω 6	Fungi; Cyanobacteria; Plants?	Tunlid and White (1992); Zak et al. (1996); Zogg et al., (1997); Ringelberg et al. (1997); Pinkart et al. (2002); Madan et al. (2002)
18:3 ω 6	Fungi	Tunlid and White (1992); Hill and al., (2000)
18:3 ω 3	Fungi, Plants	Pinkart et al., (2002)
18:3 ω 6	Fungi	Tunlid and White, (1992)
br18:0	Gram + bacteria	O'Leary and Wilkinson, (1988)
10Me18:0	Actinomycetes	Kroppenstedt (1985), White et al. (1977), Ringelberg et al., (1997)

Table 2.1. Continued.

PLFA Biomarker	Microbial Group	Reference
i19:0	Common Bacterial signature	Hill and al., (2000)
cy19:0	Gram – bacteria (Anaerobe)	Wilkinson, (1988); Zak et al. (1996); Zogg et al., (1997); Ringelberg et al., (1997); Bardgett et al., (1996); Frostegård and Bååth, (1996)
cy19c11-12	Gram - bacteria	Gonzalez-Chavez et al., (2010)
19:11 ω 1 c	Gram + bacteria	Gonzalez-Chavez et al., (2010)
20:5	Barophilic/psychrophilic bacteria	Hill and al., (2000)
20:1 ω 11c	*Francisella tularensis*	Tunlid and White, (1992)
20:5 ω 3	Fungi; Plants	Madan et al., (2002); Ringelberg et al., (1997)
20:3 ω 6	Protozoa	Hill and al., (2000)
20:4 ω 6c	Protozoa	Gonzalez-Chavez. et al., (2010)
20:4 ω 6	Protozoa	White et al. (1977); Ringelberg et al., (1997); Pindart et al., (2002)
22:1 ω 13c	*Francisella tularensis*	Tunlid and White, (1992)
22:6	Barophilic/psychrophilic bacteria	Hill and al., (2000)
24:1 ω 5c	*Francisella tularensis*	Tunlid and White, (1992)

II. 3. Results

II.3.1. Soil chemistry under sun and shade positions

Sun or shade position had no significant effect on any soil chemical constituent, with the exception of dissolved organic carbon (DOC) and dissolved organic nitrogen (DON). Shade positions contained higher soil concentrations of both water extractable DOC and DON than did sun positions. Mean water extractable DOC under sun and shade positions were 95.0 ± 35.7 µg g^{-1} soil and 119.5 ± 38.5 µg g^{-1} soil respectively. Mean water extractable soil DON under sun was 4.0 ± 3.2 µg g^{-1}soil and under shade positions was 6.3 ± 3.6 µg g^{-1} soil. Of the other N-species, nitrate-N was higher than ammonium-N, with an average 40.2 ± 17.8 µg g^{-1} soil under sun and 43.0 ± 22.6µg g^{-1} soil under shade. Ammonium-N had an average of 5.8 ± 6.6 µg g^{-1} soil under sun and 4.5 ± 2.6 µg g^{-1} soil under shade. Orthophosphate-P had an average of 0.88 ± .85 µg g^{-1} soil under sun positions and 0.89 ± .98 µg g^{-1} soil under shade positions. Potassium was the dominant base cation with 97.8 ± 51.6 µg g^{-1} soil compared to calcium, magnesium and sodium which had mean values of 74.6 ± 70.2, 18.4 ± 5.04, and 57.4 ± 68.8 µg g^{-1} soil respectively under sun positions. Under shade conditions, potassium had a mean value of 114.6 ± 78.2 µg g^{-1} soil and calcium, magnesium and sodium had mean values of 58.7 ± 17.8, 19.2 ± 5.4, and 54.5 ± 68.9 µg g^{-1} soil respectively. Bicarbonate was the dominant anion followed by chloride. Mean bicarbonate was 139.1 ± 73.4 under sun and 167.6 ± 150.6 µg g^{-1} soil under shade and alkalinity was 6.14 ± .34 under sun and 6.12 ± .25 under shade. Fluoride was the least dominant anion with an average mass of

2.71 ± 10.7 under sun and 0.46 ± 0.59 µg g^{-1} soil under shade positions. There was no significant difference in soil pH among soils taken from sun or shade positions.

II.3.2 Effects of Native Shade Trees on Soil Chemical Constituents under Coffee

No significant treatment effects were observed for species for PO_4^{3-}, HCO_3^-, Na+, Ca^{2+}, F^-, Cl^-, SO_4^{2-}, or K^+ (Table 2.2). However, there was a significant species effect for NO_3-N and NH_4-N. Nitrate-N concentrations in soils in the vicinity of *Quercus* averaged 63.63 ± 20.33 µg g^{-1} soil, significantly higher than in soils from plots influenced by the other three shade tree species. Soils near *Quercus* also showed significantly higher NH_4-N, on average 10.84 ± 9.45 µg g^{-1} soil. Mean total dissolved nitrogen (TDN) concentrations were 77.66 ± 21.35 µg g^{-1} soil in Q*uercus* soils which was also significantly higher than in soils influenced by the other three shade tree species (Figure 2.2). Soil pH was also affected by the shade tree species present; soils beneath *Quercus* had an average pH of 5.95 ± 0.18 and were significantly more acidic than soils beneath *Persea* (pH 6.27 ± 0.14). The pH values for soils under *Annona* and *Diphysa* soils averaged 6.21 ± 0.20 and 6.02 ± 0.19, respectively.

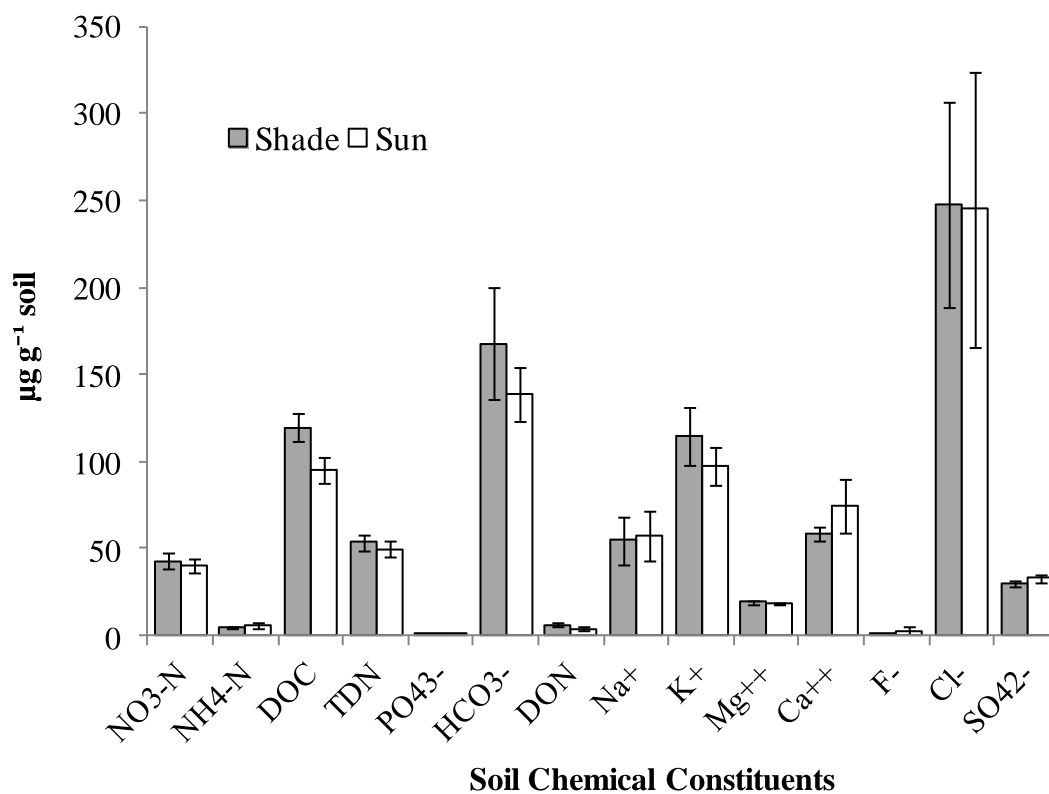

Figure 2.2. Mean soil chemistry under combined tree species in sun and shade positions in a Costa Rican coffee agroforestry system. Error bars are standard error. Sample size is n=22 for sun and n=22 for shade.

Table 2.2. Statistical significance of univariate analysis of variance performed on water extractable soil chemistry of soils beneath coffee plants growing in a Costa Rican coffee agroforestry system. Significance is α < 0.05. Significant effects are in bold. Tree species were *Persea americana* (Aguacatillo), *Annona muricata* (Anona), *Diphysa americana* (Guachipelin), and *Quercus* spp. (Roble).

Effects	NO_3-N	NH_4-N	DOC	TDN	DON	PO_4-P	HCO^{3-}	Na^+	K^+	Mg^{2+}	Ca^{2+}	F^-	Cl^-	SO_4^{2-}
Species (S)	**0.00**	**0.00**	0.62	**0.00**	0.66	0.79	1.00	0.45	0.10	**0.03**	0.32	0.50	0.15	0.10
Sun/Shade (SS)	0.40	0.20	**0.03**	0.35	**0.02**	0.85	0.35	0.98	0.35	0.34	0.39	0.39	0.94	0.26
S*SS	0.09	0.21	0.27	0.18	0.15	0.13	0.08	0.12	0.52	0.18	0.31	0.49	0.90	0.09

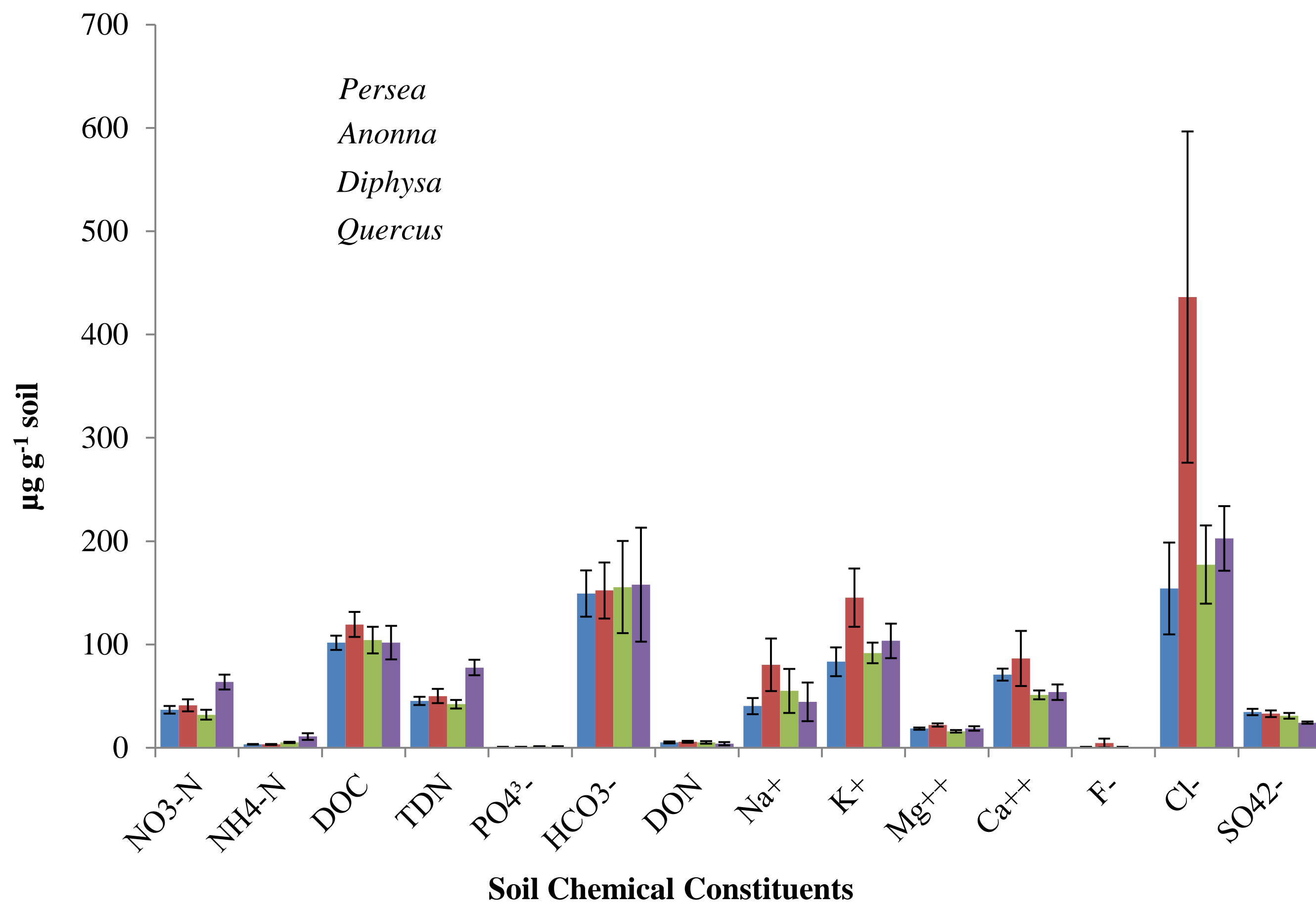

Figure 2.3. Mean water-extractable soil chemical constituents under coffee plants growing near four native tree species in a Costa Rican coffee agroforestry system. Error bars are standard error. Sample size is n =12 for *Persea americana*, *Annona muricata*, and *Diphysa americana*; n = 8 for *Quercus* spp.

There was a significant species effect on water extractable soil magnesium, with *Diphysa americana* having significantly lower soil concentrations than *Annona muricata*, with an average of 15.88 μg ± 4.19 and 21.98 ± 5.26 μg g^{-1} soil, respectively (Figure 2.3).

II.3.3. Relationship between soil chemistry and coffee foliage chemistry

Samples of coffee beans for chemical analysis were unavailable but chemical analysis of coffee bush foliage was available from CAFESA, which I used on the assumption that foliar and fruit chemistry may be similar. Sun or shade position had no effect on any foliar chemistry with the exception of percentage nitrogen (Table 2.3). Foliar N was significantly higher in coffee foliage under *Diphysa* in a full sun position relative to a shade position (Table 2.3). Species of shade tree had a significant effect on all coffee foliar chemistry with the exception of potassium, zinc and percentage carbon (Table 2.3). Phosphorus was significantly greater in coffee foliage adjacent to *Diphysa* relative to that under *Annona* and *Quercus* (Table 2.3). Calcium was significantly higher in coffee foliage adjacent to *Annona* in a full sun position relative to foliage adjacent to *Quercus* (Table 2.3). Iron content of coffee foliage adjacent to *Annona* was significantly greater than that adjacent to *Diphysa* and *Quercus* (Table 2.3) and copper and manganese content were significantly higher in foliage adjacent to *Diphysa* than the other shade tree species (Table 2.3). Using univariate analysis of variance with shade tree species and sun or shade position as factors, I examined their effects on coffee foliage chemistry. Shade tree species had a significant effect on coffee foliage total phosphorus ($p < 001$), calcium ($p < 0.001$), magnesium ($p < 0.01$), sulfate ($p < 0.01$),

iron ($p < 0.001$), copper ($p < 0.001$), manganese ($p < 0.001$), boron ($p < 0.01$) and percentage nitrogen ($p < 0.01$). Sun or shade position had a significant effect on foliar carbon ($p < 0.05$) and foliar nitrogen ($p < 0.001$) but not on any other coffee foliar chemistry. There was an interaction effect of shade tree species and sun or shade position on percent N in coffee foliage.

Because of the interest in the effect of tree species on soil chemistry I examined correlations between coffee foliar chemistry adjacent to shade tree species and soil chemistry obtained below the relevant coffee plant. Soil pH had a significant positive correlation with foliar calcium and magnesium (Table 2.4). Soil nitrate-N had a significant negative correlation with foliar manganese (Table 2.4). Soil ammonium-N content had a negative correlation with foliar potassium, calcium and iron. Soil DOC and DON were negatively correlated with foliar zinc and nitrogen content (Table 2.4). Soil magnesium content was positively correlated with foliar magnesium content and negatively correlated with foliar manganese content (Table 2.4). Soil fluoride was positively correlated with foliar potassium, calcium and magnesium (Table 2.4).

Table 2.3. Chemistry of coffee foliage determined by shade tree species and sun or shade position. Values in parenthesis are standard deviation. Differences in superscript letters indicate a significant difference in foliar chemistry for each chemical constituent.

	P	K^{+}	Ca^{2+}	Mg	SO4	Fe	Cu	Zn	Mn	B	N	C
	mg kg^{-1}					µg kg^{-1}					%	
Annona												
Sun	0.11^{a}	2.2	1.4^{c}	0.42^{b}	0.41^{a}	85.5^{b}	14.8^{ab}	10.5	100.0^{a}	57.5^{b}	3.1^{a}	48.4
	(0.01)	(0.2)	(0.1)	(0.06)	(0.06)	(4.5)	(2.0)	(1.8)	(25.7)	(10.1)	(0.1)	(0.2)
Shade	0.12^{ab}	2.0	1.3^{bc}	0.39^{ab}	0.38^{a}	85.5^{b}	13.8^{a}	9.7	108.2^{a}	50.3^{ab}	3.1^{ab}	48.1
	(0.02)	(0.1)	(0.1)	(0.04)	(0.05)	(12.5)	(1.2)	(1.4)	(25.2)	(3.4)	(0.1)	(0.2)
Diphysa												
Sun	0.15^{c}	2.1	1.1^{abc}	0.35^{ab}	0.45^{ab}	67.2^{a}	17.5^{b}	12.0	189.8^{b}	42.0^{a}	3.3^{c}	48.2
	(0.01)	(0.1)	(0.1)	(0.03)	(0.05)	(4.5)	(1.0)	(5.4)	(26.7)	(5.7)	(0.0)	(0.3)
Shade	0.14^{c}	2.2	1.1^{ab}	0.32^{a}	0.42^{a}	70.5^{a}	17.5^{b}	9.7	198.5^{b}	46.0^{ab}	3.0^{a}	48.0
	(0.01)	(0.2)	(0.2)	(0.06)	(0.02)	(5.6)	(1.8)	(3.1)	(23.4)	(5.1)	(0.1)	(0.2)
Quercus												
Sun	0.12^{ab}	2.0	1.1^{ab}	0.36^{ab}	0.56^{b}	67.8^{a}	14.3^{a}	9.8	110.8^{a}	55.3^{b}	3.2^{bc}	48.2
	(0.01)	(0.3)	(0.1)	(0.04)	(0.10)	(10.6)	(1.5)	(1.0)	(16.5)	(5.5)	(0.1)	(0.6)
Shade	0.12^{ab}	2.0	1.0^{a}	0.38^{ab}	0.49^{ab}	73.8^{ab}	13.8^{a}	9.8	103.8^{a}	52.8^{ab}	3.1^{ab}	48.1
	(0.02)	(0.2)	(0.2)	(0.01)	(0.09)	(4.5)	(2.1)	(1.0)	(3.1)	(6.3)	(0.1)	(0.2)

Table 2.4. Correlations between coffee foliage chemistry and underlying soil chemistry. N = 32. *significant at $p < 0.05$ and ** significant at $p < 0.01$. Data is combined sun and shade positions for *Quercus, Annona* and *Diphysa.*

Soil Chemistry (mg kg^{-1})		Coffee Foliar Chemistry (mg kg^{-1})											
		P	K^+	Ca^{2+}	Mg^{2+}	SO_4^{2-}	Fe	Cu	Zn	Mn	B	%N	%C
	pH	-0.23	0.02	**0.44	*0.39	-0.06	0.25	-0.05	-0.20	-0.16	0.16	-0.34	0.17
	NO_3-N	0.01	-0.18	0.00	0.34	0.10	-0.06	-0.41	-0.09	*-0.43	0.06	0.22	-0.07
	NH_4-N	0.11	*-0.37	*-0.36	-0.19	0.16	*-0.39	0.02	-0.11	0.01	0.00	*0.45	0.26
	DOC	-0.13	0.00	0.07	0.13	-0.15	0.04	-0.21	*-0.41	-0.17	-0.07	*-0.43	-0.20
	PO_4-P	0.01	-0.08	-0.05	-0.16	-0.09	-0.10	0.06	-0.28	0.11	0.16	-0.10	0.30
	HCO_3^-	-0.22	0.13	0.11	0.05	-0.28	-0.01	-0.11	-0.32	-0.11	0.03	*-0.36	-0.19
	DON	-0.03	0.16	0.07	0.08	-0.05	0.10	-0.04	*-0.38	-0.04	-0.09	**-0.54	-0.18
	Na^+	-0.23	0.33	0.34	0.17	-0.23	0.17	-0.04	-0.30	-0.17	0.16	-0.27	0.14
	K^+	-0.03	-0.12	0.25	0.23	-0.17	-0.06	-0.20	-0.14	-0.33	-0.01	-0.04	-0.24
	Mg^{2+}	-0.15	0.09	0.16	*0.37	-0.23	0.34	-0.17	-0.07	*-0.43	0.13	-0.17	-0.15
	Ca^{2+}	-0.29	-0.04	0.20	0.32	-0.19	0.13	0.05	-0.11	-0.28	0.09	-0.20	-0.09
	F^-	0.04	*0.37	*0.37	*0.37	-0.14	0.12	-0.03	-0.09	-0.18	-0.09	0.01	0.06
	Cl^-	-0.18	0.01	0.24	0.25	-0.19	0.02	-0.04	-0.14	-0.34	0.03	-0.13	-0.14
	SO_4^{2-}	-0.28	0.23	0.09	-0.06	-0.15	0.29	0.20	0.16	0.05	0.11	-0.35	0.03

II.3.4. Effect of tree species on microbial community composition

Examination of soil microbial community composition using PLFA analysis revealed that soils in *Quercus* spp. plots were the most distinctive in terms of microbial community when the complete dataset of microbial PLFA's, consisting of over 100 PLFA biomarkers, was used for the analysis (Figure 2.4).

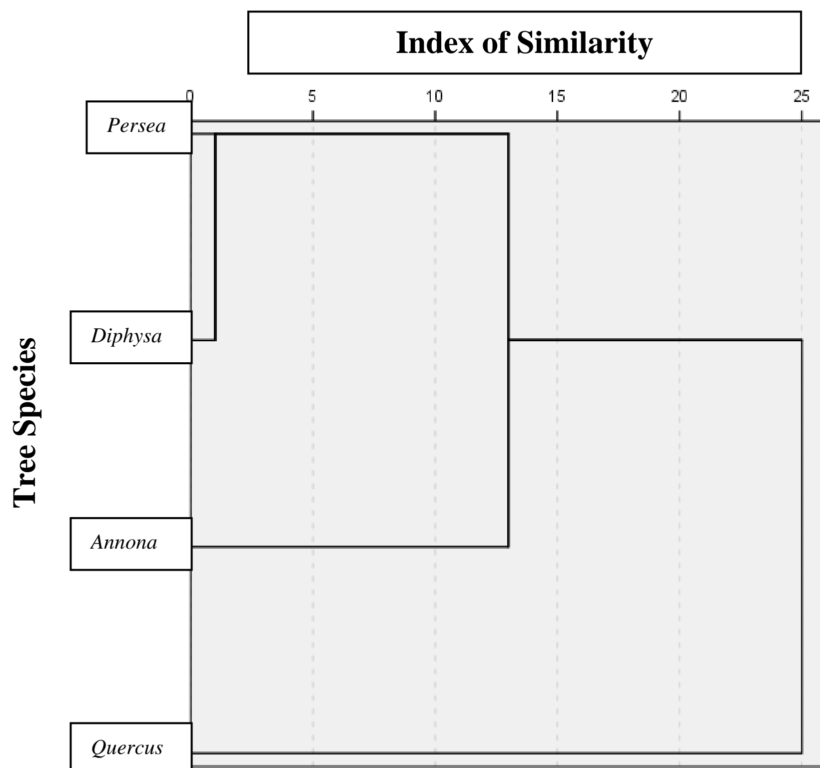

Figure 2.4. Dendrogram showing dissimilarities of soil lipid profiles among four species of native shade trees. Increasing distance represents increasing dissimilarity. Ward linkages used for the Euclidean distance analysis which is based on the complete dataset of 100+ lipid biomarkers.

Annona muricata appeared to inhibit the action of some fungal species. The microbial community composition of *Annona* soils, based on the complete reduced set of over 100 PLFA biomarkers, was dissimilar to the microbial community composition in soils influenced by the other tree species. Soils from *Quercus* spp. also exhibited a set of PLFA biomarkers that were quite distinct from the other three species. The soils whose PLFA profiles were most similar to one another were those from plots with *Diphysa americana* and *Persea americana* trees present.

Table 2.5. Diversity indices of soil microbial communities from samples in the vicinity of four native shade trees from a sunny or shaded position in a Costa Rican coffee agroforestry system. S=Species richness; D=Simpson's index.

Species/Position	S	D
Persea Shade	35	0.914
Persea Sun	34	0.899
Diphysa Sun	47	0.917
Diphysa Shade	46	0.900
Annona Shade	46	0.9086
Annona Sun	51	0.912
Quercus Shade	50	0.9276
Quercus Sun	52	0.9219

Species richness indicates how many different species on average were present in the sample. Samples from sun positions in plots with *Persea americana* trees had the lowest number of microbial species, while samples from sun locations with *Quercus spp*.

had the highest number of microbial species. The Simpson index, an index of dominance, indicates the probability that individuals picked at random will belong to the same species (Colinvaux, 1986). Although the Simpson index shows that the number of organisms per individual species is unevenly distributed in the soils of all four tree species, *Quercus spp.* had the microbial community whose members were the least evenly distributed among the species present, while *Persea americana* had species numbers that were the most evenly distributed. Additional analyses were performed using only the 21 PLFA biomarkers in my dataset where there is strong agreement from previous studies that the biomarker represents a particular microbial functional group (Table 2.6). Using the complete 100+ biomarkers dataset, diversity indices based on the PLFA profiles of the soils from each of the plots were also calculated (Table 2.7).

The species of shade tree present had a significant effect on the microbial community composition of the soils (Table 2.8). *Quercus* spp. had the highest mean mol percentage of Gram positive bacteria (18.15 ± 1.74). Soils under *Annona* and *Persea* had an average of 17.72 ± 1.30 and 17.15 ± 0.87 mol percent Gram positive bacteria, respectively. Soil under *Disphysa* had the lowest mole percentage of Gram positive bacteria with 16.70 ± 2.30. Soil under *Diphysa* also had the lowest mol percentage of Gram negative bacteria with 13.85 ± 1.91. Soil under *Annona* and *Persea* had 14.26 ± 1.03 and 14.50 ± 0.77 mole percent Gram negative bacteria. *Quercus* spp. also had the highest mol percentage of Gram negative bacteria, with 15.28 ± 0.90. There was no significant difference among the four tree species with regard to the mol percentages of

Gram positive or Gram negative bacteria nor the ratios of Gram +:Gram - bacteria, or Fungi:Bacteria. There was a significant tree species effect with regard to the abundance of fungi in the soils sampled from plots influenced by the four different native shade tree species. *Persea* soils were significantly higher in mol percentage of fungi, with a mean value of 18.44 ± 1.13, relative to *Annona* soils, which had a mean mol percentage of 14.47 ± 2.42 fungi. *Quercus* spp. and *Diphysa* soils, with mean mol percentage of fungi of 15.80 ± 1.05 and 17.46 ± 1.45 respectively were neither significantly different from one another nor from the soils of the other two shade tree species in this study. Sun versus shade position had no significant effect on any of the above-mentioned parameters. Total bacterial biomass per species and sun/shade condition is shown in Table 2.8.

There was a significant negative correlation between the abundance of Gram negative bacteria and soil HCO_3^- (R^2=0.20; Figure 2.5; Table 2.10). Yet a similar relationship was not apparent between Gram positive bacteria or fungi and HCO_3^-. There was also a significant negative correlation between Gram negative bacteria and soil concentrations of Na^+ (R^2 = 0.29; Table 2.10; Figure 2.6). Finally, there was a strong positive correlation between the abundance of Gram negative and Gram positive bacteria in the soil samples (R^2 = 0.74; Figure 2.7).

Table 2.6. Average abundance of lipid biomarkers from soil sampled from plots influenced by each tree species. Only lipid biomarkers firmly established as representing a particular microbial group were included in this table; therefore, actual abundances of each microbial group can be expected to be greater than shown here.

Lipid abundance (mol %) Total lipid	*Persea* 100%	*Annona* 100%	*Diphysa* 100%	*Quercus* 100%
Iso-branched lipids (Gram + bacteria)	**17.15**	**17.72**	**16.7**	**18.15**
*i*14:0	0.64	0.78	0.78	0.66
15:0	1.10	1.09	0.99	1.02
*i*15:0	6.18	6.15	6.06	6.92
*a*15:0	2.66	2.57	2.80	2.84
*i*16:0	2.52	3.06	2.42	2.60
17:0	0.76	0.67	0.69	0.69
*i*17:0	2.15	2.25	1.86	2.26
*a*17:0	1.15	1.15	1.10	1.16
Methyl branched lipids (Actinomycetes)				
19:0 10 Me	0.58	0.61	0.76	0.65
Mono-unsaturated, alchohols, and cyclopropyl lipids (Gram-)	**14.50**	**14.53**	**13.85**	**15.28**
16:1ω7c	3.41	3.42	3.37	3.25
12: X OH	0.0	0.66	0.38	0.44
16: X OH	1.64	1.27	1.64	1.37
*cy*17:0	2.17	2.08	1.88	2.17
*cy*19:0 c11-12	2.90	3.71	2.63	4.27
SF 8 (18:1ω7c/18:1ω9t)	4.40	3.12	3.96	3.79
Fungi, non-specific	**5.02**	**3.6**	**3.92**	**3.41**
18:3ω6c	1.36	1.01	1.05	0.88
SF19 (18:2ω6c/T:18:0)	3.66	2.59	2.87	2.53
Ectomycorrhizae/Saprophytic fungi				
18:1ω9c	10.30	7.84	10.60	8.98
Arbuscular Mycorrhizae				
16:1ω5c	3.12	3.04	2.94	3.40
Total Fungi	**18.44**	**14.48**	**17.46**	**15.79**
Fungi/bacteria ratio	0.58	0.46	0.59	0.47
Gm+/Gm- ratio	1.19	1.24	1.21	1.19

Table 2.7. Average total microbial biomass per species and sun/shade condition. Table based on complete reduced dataset of 100+ biomarkers.

Species	Sun/Shade Position	Average Total Biomass (nmol lipid g^{-1} soil)	Standard Deviation
Persea americana	Sun	193	59.45
Persea americana	Shade	206.6	21.72
Diphysa americana	Sun	292	28.06
Diphysa Americana	Shade	262.8	56.4
Annona muricata	Sun	260.8	13.68
Annona muricata	Shade	235	27.24
Quercus spp.	Sun	287	10.8
Quercus spp.	Shade	258.8	126

Table 2.8. Results of analysis of variance for microbial groups in soils from plots influenced by one of four native shade tree species in a Costa Rican coffee agroforestry system. Significance indicated in bold; alpha=0.05.

	Tree species and sun or shade position		
Microbial Group	Species	Sun/shade	Species * Sun/shade
Gram + bacteria	0.55	0.77	0.43
Gram – bacteria	0.42	1.00	0.43
Fungi	**0.01***	0.20	0.98
Gram +/Gram -	0.31	0.56	0.41
Fungi/bacteria	0.07	0.42	0.63

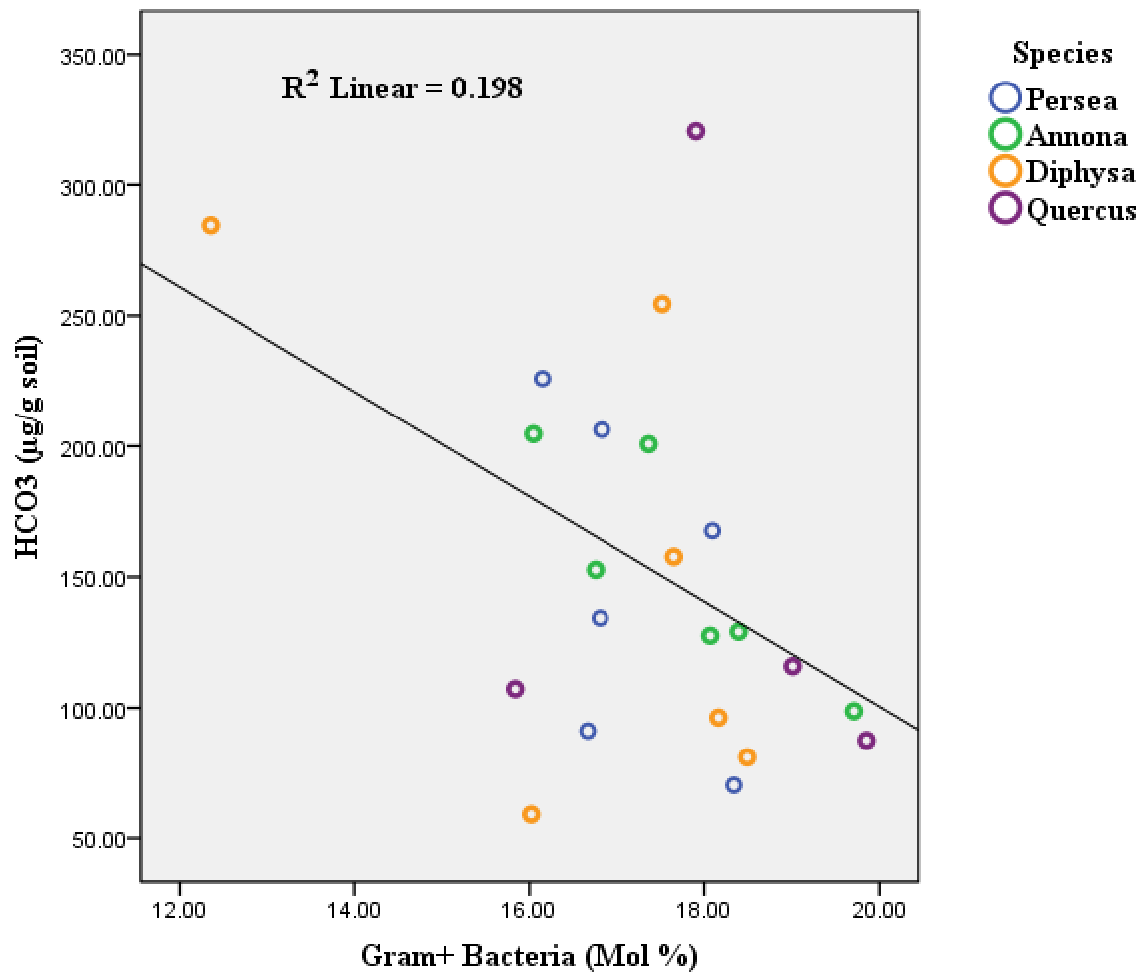

Figure 2.5. Relationship between abundance of Gram negative bacteria and concentration of soil HCO_3^- in soil samples from a Costa Rican coffee agroforestry system influenced by four different species of native shade trees. Alpha = 0.04.

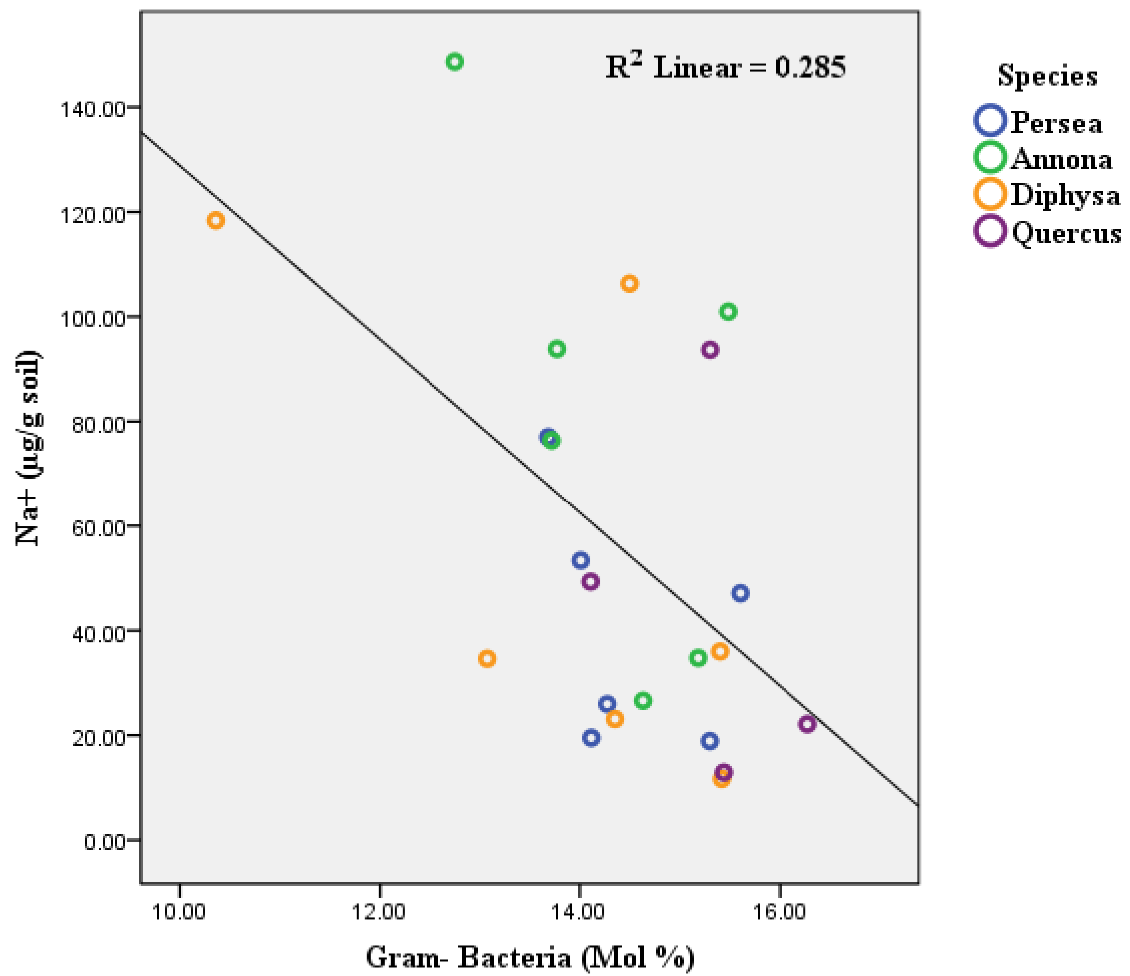

Figure 2.6. Relationship between Gram negative bacteria and soil concentrations of sodium in soil samples from a Costa Rican coffee agroforestry system influenced by four different species of native shade trees. Alpha = 0.01.

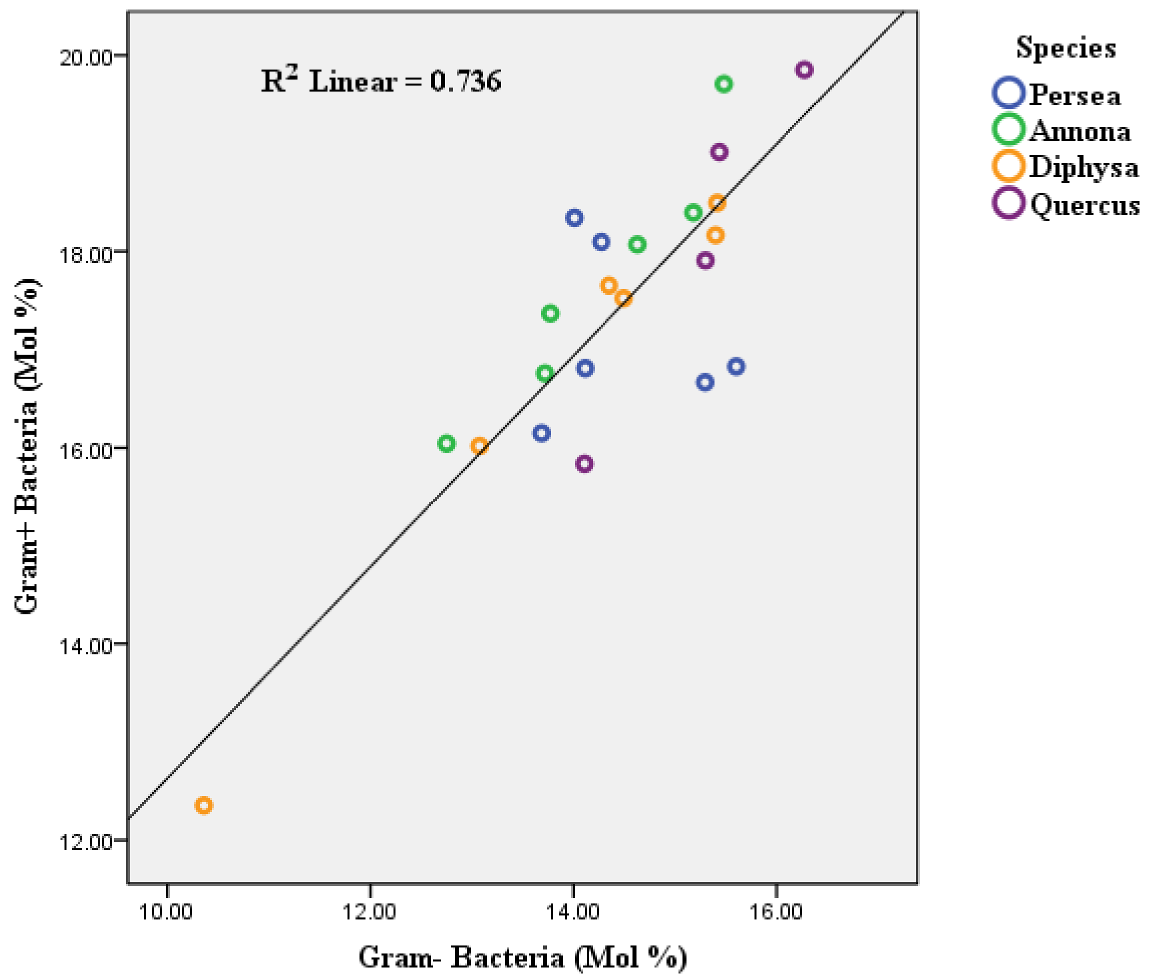

Figure 2.7. Relationship between abundance of Gram negative bacteria and Gram positive bacteria in soil samples from a Costa Rican coffee agroforestry system influenced by four different species of native shade trees. Alpha = 0.00.

Table 2.9. Correlations between soil chemistry and abundance of microbial groups. Pearson Correlation Coefficient is shaded; level of significance is in clear area. Based on data set of 19 well-referenced lipid biomarkers and averages for *Persea*, *Annona*, *Diphysa* and *Quercus* spp. Significant correlations in bold. * indicates significant correlation at $p < 0.05$.

Microbial Group	Soil Chemical Constituents														
	pH	NO_3^-	NH_4-N	DOC	TDN	PO_4^{2-}	HCO_3^-	DON	Na^+	K^+	Mg^{++}	Ca^{++}	Fl^-	Cl–	SO_4^{2-}
Gram+	.09	-.08	-.10	-.13	-.11	-.20	**-.45***	-.07	-.41	.13	.17	-.03	-.22	.01	.00
Fungi	.03	-.11	-.19	-.03	-.11	.14	-.02	.25	-.20	-.12	-.32	-.30	-.06	-.22	.04
Gram-	-.08	.14	.09	-.15	.11	-.15	-.38	-.27	**-.53***	.01	.18	-.11	-.32	-.18	-.13
Gm+/Gm-Ratio	.34	-.41	-.38	.06	-.41	-.09	-.15	.39	.20	.24	.01	.17	.20	.35	.25
Fungi/Bact Ratio	-.07	-.06	-.14	.07	-.06	-.06	.26	.24	.15	-.13	-.28	-.19	.15	-.12	.05

II.4. Discussion

II.4.1. Effect of tree species and sun and shade position on soil chemistry

Sampling of soil for comparative purposes should be taken under similar climatic and management conditions. Soils from positions under *Quercus, Diphysa* and *Annona* were taken three weeks into the rainy season and after the first round of fertilization. Soils from under *Persea* were taken two weeks earlier which was one week into the rainy season and prior to fertilizer application. The difference in timing for soil collection may have had an effect on soil chemistry, particularly nitrogen and phosphorus, although this was not apparent when comparing soil chemistry among those collected prior to and after fertilization and any effect of fertilization may well have been disguised by the strong effect of shade tree species on soil chemistry.

Soils retrieved under shade positions contained higher water extractable DOC and DON content than did soils under sun positions. Microbial processes slow down with cooler soil temperatures (Hoorman and Islam, 2010), resulting in slower mineralization of organic carbon and nitrogen. In addition, the rate of soil organic matter (SOM) decomposition is faster in soil exposed to continuous wetting and drying cycles relative to soils consistently dry or consistently wet (Hoorman and Islam, 2010). During the rainy season, shaded soils would remain saturated longer than soil in sunny areas, maintaining anaerobic conditions for a longer period of time, slowing down the decomposition process. However, anaerobic conditions under shade positions did not appear to lower nitrate-N through denitrification or increase ammonium-N through

dissimilatory nitrate reduction and nitrite ammonification which would be expected if soils were saturated for long periods of time.

Pedologists and ecologists have long recognized that tree species have an effect on the development of soil properties (Jenny, 1942; Zinke, 1962). More recent research has shown that tree species and plants can change soil properties relatively rapidly (Wedin and Tilman, 1990; Pastor et al., 1993; Binkley and Giardina, 1998). There is however no real consensus on the mechanisms controlling a tree's effect on soil chemistry (Reich et al., 2005). Incomplete knowledge on these mechanisms is confounded because vegetation distribution tends to respond to landscape-scale soil variability that arises from other state factors such as climate, parent material and topography (Van Breemen et al., 1997; Binkley and Giardina, 1998; Finzi et al., 1998). While tree species have been shown to have an effect on soil chemistry in humid temperate forest ecosystems (Finzi et al., 1998; Ayres et al., 2009), evidence of effects have been conflicting in tropical forest ecosystems (e.g. Power et al., 2004; Russell et al., 2007: Dinesh et al., 2010). Powers et al. (2004) found no effect of tree species on soil chemistry to a depth of 15 cm under four different tropical tree species when each was compared to *Pentaclethra*; they concluded that the lack of significant statistical difference in the soils was due to a small sample size which may have confounded their statistical power. Dinesh et al. (2010) examined soil chemistry under four tree species used as live stake support for growing black pepper in India. They reported significant differences in soil DOC, DON, potassium, calcium and magnesium under the four tree species.

The difference between the two climatic regimes may be that of the state factor of time. For example, most of the studies examining tree species and soil chemistry in temperate ecosystems have examined landscapes that have undergone glaciation and as a result the age of the soil is quite young relative to the deeply weathered, un-glaciated soils of tropical ecosystems. In my study, the chemistry of the soils influenced by four different species of native shade trees was significantly different in that soils under *Quercus* had significantly higher concentrations of NO_3-N, NH_4-N, and TDN, and soils under *Diphysa* had significantly lower soil concentrations of Mg^{2+} than did soils under *Annona.* Soil pH was also affected by the tree species present, with soils under *Quercus spp* significantly more acidic than soils under *Persea.* Reich et al. (2005) in a study examining fourteen tree species in Poland found that the calcium content of leaf litter was most responsible for changes in soil properties particularly soil C:N ratio. For example, as leaf litter calcium increased, soil pH significantly increased and soil C:N ratio significantly decreased in the organic horizon and mineral soil to 40 cm depth. The proportion of cellulose in leaf litter had a significant and positive correlation with soil C:N ratio (Reich et al., 2005); this would make sense in that cellulose is relatively recalcitrant and not easily broken down by soil macro and micro invertebrates and microbiology. A further study in a temperate spruce forest in the Czech Republic reported that both foliar N and the foliar lignin:nitrogen ratio had a strong and significant relationships with soil DOC (Aitkenhead-Peterson et al., 2006).

While I did not examine the foliage of specific shade trees, I did examine the data of coffee bush foliar chemistry that related to soil samples taken. Unfortunately

only foliar data for coffee plants beneath *Annona*, *Quercus* and *Diphysa* was available and the assumption was made that foliar and bean chemistry would be similar.

Higher total dissolved nitrogen in soil under *Querc*us may be partially explained by the presence of higher tannins and phenolic compounds in the foliage of *Quercus* which may inhibit the breakdown of proteins by forming complexes with casein and protecting the protein from the action of the protein-cleaving enzyme, trypsin (Feeny, 1969). The condensed tannins and hydrolysable tannins in leaves of *Quercus spp* also inhibit the activities of the nitrifying bacteria *Nitrosomonas* and *Nitrobacter*, even at small concentrations (Rice and Pancholy, 1973). However, because nitrate-N was the dominant N species under all shade tree species it is unlikely that nitrifying bacteria was inhibited.

It is possible that polyphenolic substances produced by *Quercus spp.* form complexes with proteinaceous organic matter, prolonging the availability of organic nitrogen in the soil and possibly giving the shade tree a competitive advantage for the uptake of organic nitrogen. Indeed, the roots of some plants have been found to contain a range of amino acid transporters (Jones et al., 2005), although it is not known whether the roots of *Quercus spp*. or *Coffea arabica* contain such machinery.

The presence of elevated soil NO_3-N under *Quercus* perhaps is due to substances in the oak litterfall, root exudates or root decomposition products that promotes oxidized soil conditions. Soil nitrate content and its mobilization under oak relative to spruce in temperate forests was suggested to be due to slower growing rates, and thus N uptake of oak is slower relative to spruce. Furthermore, the decomposability of oak litter is faster

than spruce resulting in a store on N that is not immediately used by the tree (Rosenqvist et al., 2007). The species effect on soil magnesium, with *Diphysa* having significantly lower soil concentrations than *Annona*, may be simply due to the differences in chemical composition of the litterfall produced by these two species. Data on foliar chemistry also showed significantly higher magnesium content in the coffee leaves from *Annona* plots relative to *Diphysa* plots.

It would be reasonable to expect that *Diphysa americana*, a leguminous tree, would benefit the coffee plants by converting atmospheric nitrogen (N_2) to ammonium (NH_4) through a symbiotic relationship with *Rhizobium* bacteria. The observation that soils influenced by *Diphysa Americana* did not show higher nitrogen levels than did soil samples from plots influenced by the other three species of shade tree is reasonable, since samples were taken between coffee plants, more than two meters from the base of the shade tree. This result supports the findings of Payán et al. (2009) who concluded that there were significantly greater concentrations of soil carbon and nitrogen within one meter as compared to two or more meters from legume shade trees.

II.4.2. Effect of position and tree species on microbial community composition

Quercus spp. are ectomycorrhizal trees which Morris et al. (2008) found to host multiple species of fungi on their root tips in a tropical montane forest. These fungi increase the soil volume that can be exploited by the host plant, and they aid in the uptake of relatively immobile ions such a phosphate, zinc, copper, molybdenum, potassium, and ammonium (Bowen, 1980; Javaid, 2009). The multiple species of ectomycorrhizal fungi may help to explain the distinctive microbial composition of soil

samples in the vicinity of *Quercus.* Although the mol percent of fungal PLFA biomarkers was not the highest for this species, the microbial community composition diversity index was. The fact that soil under *Quercus* had the second lowest abundance of fungi of the four shade tree species supports the findings of Harrison (1971) who suggested that oak tannins inhibit the growth of many fungal species. It is interesting to note that soils beneath *Annona* were found to have the lowest abundance of total fungal biomarkers and had a significantly lower mol percentage of fungi than soil under *Persea.* This finding lends support to the conclusions of Vieira et al. (2010) who reported that water-based extracts from the *Annona* fruit, when tested in-vitro, acted as a bactericide toward both Gram positive (*S. aureus*) and Gram negative (*V. cholera* and *E. coli*) bacteria. It is suggested that these substances limited the abundance of fungi in soils influenced by *Annona* trees in this Costa Rican coffee agroforestry system. Indeed, extracts from the pericarp of the *Annona* fruit are used in rural communities for their antileishmanial (a Leishmania parasite) activity, and a study by Jaramillo et al. (2000) showed the extracts to be more effective than the commercial antiprotozoal drug Glucantime®. It is likely that these anti-protozoal substances in the pericarp of *Annona muricata*, as well as the relatively high sodium, fluoride, and chloride content of the *Annona muricata* soils (Figure 2.2) selected for a unique array of soil microbes.

The soils whose PLFA profiles were most similar to one another were those from plots with *Diphysa americana* and *Persea americana* trees present (Table 7 and Figure 4). *Persea americana* has been found to host an endophyte forming endomycorrhizae of the vesicular-arbuscular type (Ginsburg and Avizohar-Hershenson,1965), while *Diphysa*

americana is a legume. Legumes often form symbiotic relationships with bacteria of the genus *Rhizobium*, which are housed in nodules on the plants' roots, enabling them to convert atmospheric nitrogen to plant-available inorganic forms. In addition to this nitrogen fixing symbiosis, legumes often form mycorrhizal relationships as well.

PLFA analysis is often the method of choice for microbial analyses, since one of the limitations of traditional dilution plating and culturing methods is that it is estimated that only a small fraction, (less than 1%) of the soil microbial community is culturable by known techniques (Hill et al., 2000). Even so, bacterial counts have given an estimate of 3.4 times higher that the recovery measured by PLFA (Frostegard and Baath, 1996). Therefore, it is likely that the estimates of microbial functional groups given in this study were substantially lower than the numbers that actually exist in the coffee agroecosystem. In order to be conservative, only 21 PLFA markers which were the most well established in the literature were used to indicate the composition of the microbial communities groups in the soil samples. It is possible that the magnitude of these functional groups could have changed had I used my complete reduced data set of 100+ biomarkers for this analysis.

II.5. Conclusions

- The study was carried out at a farm with shade trees and was not the typical full sun coffee plantation. Nevertheless, position of coffee bushes in sun or shade positions resulted in some significant differences in soil DOC and DON.
- Species of shade tree was generally more important for soil chemistry and microbial community composition than sun or shade position.

- Stronger conclusions could be reached as to the effects of the shade trees on the coffee plants themselves by acquiring coffee yield data and information on the flavor and quality of the coffee produced in soils influenced by the four different species of shade trees studied.

CHAPTER III

THE EFFECT OF EFFECTIVE MICROORGANISMS ON SOIL CHEMISTRY IN A COSTA RICAN COFFEE AGROFORESTRY SYSTEM

III.1. Introduction

Dr. Teruro Higa of the University of Ryukyus, Okinawa, Japan coined the term "Effective Microorganisms®" (EM) to describe a group of microorganisms which work synergistically to promote and maintain healthy plant growth (Higa and Parr, 1994). The use of EM may have the potential to increase the productivity of organic farming systems (Subadiyasa, 2003) or to reduce the amount of inorganic nitrogen fertilizer needed in conventional or low-input farming systems (Khaliq et al., 2006) . Although it is reported that most of the organically certified coffee farmers in Costa Rica and Guatemala use EM (Lotter, 2005), there are no published studies concerning the use of effective microorganisms with coffee.

Originally, EM was cultivated from 80 species of bacteria belonging to 10 genera (Higa, 1993). Previous efforts to change the microflora of soils by introducing single cultures of microorganisms have, for the most part, been unsuccessful. EM works differently in that it combines microorganisms which are ecologically compatible and work in a synergistic manner (Higa and Parr, 1994). EM technology has been modified over time to include large populations of lactic acid bacteria (*Lactobacillus plantarum*, *L. casei*, and *Streptococcus lactis*, among others), and yeasts (*Saccharomyces spp.*), with smaller numbers of photosynthetic bacteria (*Rhodopseudomonas palustris* and

Rhodobacter sphaeroides), Actinomycetes (*Streptomyces spp*.) as well as other types of microorganisms (Javaid, 2010).

Results of studies on the benefits of using EM as a soil amendment in order to increase the productivity of organic farming systems or to decrease the amounts of chemical fertilizers needed in conventional farming systems have been inconclusive. Some have documented the positive results on the growth and productivity of crops when EM has been used in combination with organic matter. For example, Hussain et al. (1993) reported that in extensive field trials, EM increased crop yields while allowing farmers to reduce their dependence on chemical fertilizers. In a another field experiment investigating the effects of integrated use of organic and inorganic nutrient sources with effective microorganisms, researchers found that using organic matter and EM together resulted in a 44% increase in the growth and yield of seed cotton over the control. Furthermore, they found that the integrated use of OM + EM with ½ the recommended mineral NPK could substitute for 85 kg N per ha, reducing the mineral fertilizer requirements by 50% (Khaliq et al., 2006). In a field experiment at the Mhasrakham University in Thailand, Vetayasuporn (2006) reported that biological fertilizer containing EM was just as effective as chemical fertilizer at improving the growth and yield of shallots (*Allium cepa var. ascolonicum*). Bhatti and Qureshi (2005) found that with the application of EM, the soil's porosity and water holding capacity increased, although no statistical analyses were done to determine if water holding capacity increases were statistically significant. On organic farms in Canterbury, New Zealand, EM increased onion yields by 29% and the proportion of highest grade onions by 76%.

Pea yields were increased by 31% and sweetcorn cob weights by 23%. Under laboratory conditions, the use of EM increased the mineralization of carbon by 8% (Daly and Stewart, 1999). In a study of rice and wheat production in Pakistan, Hussain and colleagues (1999) found that significantly higher grain and straw yields were obtained when fertilizer and organic amendments were combined with EM. The highest yields resulted from NPK + EM, followed by green manure + EM, and farm yard manure + EM. The green manure + EM treatment resulted in grain and straw yields for both the rice and wheat crops that approached those for the NPK treatment alone. A comparative economic analysis of the treatments showed a significantly higher net return when using EM relative to using the other treatments alone.

Pei-Sheng and Hui-Lian (2002) investigated the effects of EM bokashi organic fertilizer on the nodulation and yield of peanut plants at the International Nature Farming Research Center in Nagano, Japan. Compared to chemical fertilizer, EM bokashi fertilizer significantly increased both the nodule numbers per plant and fresh weight per nodule. The EM bokashi treated plants also showed higher rates of photosynthesis, transpiration, and mesophyll conductance. In addition, their total pod number and pod dry weight were significantly higher than those receiving the chemical fertilizer treatment.

Not all studies show positive results from using EM, however. Van Vliet and colleagues (2006) from the Netherlands used DNA fingerprinting to investigate whether bacteria present in EM could remain dominant after being added to slurry manure. They found that the addition of EM had no measurable effects on the bacterial diversity or the

chemical composition of the slurry manure. They also found that EM had no effect on nitrogen uptake or grass biomass production in a greenhouse experiment. The authors did note, however, that different EM stocks prepared by different users showed large variation in microbial make-up and demonstrated low reproducibility (van Vliet et al., 2006). In a four-year field experiment conducted in Zurich, Switzerland, EM did not improve yields or soil quality; furthermore, treatments with living EM compared with its sterilized control treatments showed no significant differences in crop yields, soil respiration, microbial biomass, dehydrogenase activity or microbial community structure (Lyngbaek et al., 2010). In Germany, researchers conducted experiments to evaluate the impact of EM when combined with organic amendments on soil microbial-biomass content and activity, net N mineralization in soil, and the growth of *Lolium perenne*. They reported none or only marginal effects of EM on organic C, total N, and mineral N in the soil. When EM was used alone, it slightly enhanced microbial activity quantified by CO_2 evolution. However, when combined with easily degradable plant residues, EM actually had a suppressive effect on microbial biomass (Schweinsberg-Mickan and Muller, 2009).

A three-year field experiment was carried out beween 2005 and 2007 to study the influence of EM on the yield and quality of organic Arlet apple production in Graz in Styria, Austria. In 2006, the EM-treated apples trees showed a significantly lower incidence of apple scab infection. At the end of three years, the EM treated trees showed a significantly higher growth rate and yielded larger fruit, although EM treatment did not seem to affect yield per tree. There was no effect on fruit quality or fruit longevity in

storage; however the EM treated trees yielded significantly more seeds per apple (Flipp, 2009).

The objectives of my study were:

i. To investigate if the application of Effective Microorganisms has a significant effect on soil chemistry;

ii. To examine if there are any interactions between shade tree species, sun or shade position and presence or absence of EM on soil chemistry;

III.2. Materials and methods

III.2.1. Study site

Finca la Hilda is located in the Central Valley of Costa Rica, at an altitude ranging from 1300 and 1400 meters above sea level. Rainfall in the region varies between 2400 and 3600 mm per year (Meteorological Station, Finca la Hilda, 2007-2010). Approximately 90% of the rainfall occurs during the months of June through November, followed by a dry season December through May. In all plots, soils were classified as Udands.

III.2.2. Experimental design

Experimental plots were chosen based on the presence of four native shade tree species, which included a) *Annona muricata L. (*Annonaceae*)*, b) *Diphysa americana* Mill. (Fabaceae), c) *Quercus spp*.L. (Fagaceae), and d) Pers*ea americana Mill.(Lauraceae*). These species were of interest due to their nutritional value for wildlife and humans (Pers*ea spp.* and *Annona spp.*), or their value as a fine wood (*Quercus spp*. and *Diphysa americana*). The shade tree species were not pruned.

Experimental plots measured 20 x 30 m. A three factorial arrangement was used, the three factors being the shade tree species, whether the sample was taken from a sunny patch or a shady patch, and whether EM was present or absent. There were three completely randomized replications for *Annona muricata, Diphysa americana*, and *Persea americana*, and two replications for *Quercus* spp., in a split plot design (Figure 3.1). Soil data were compared in two ways a) samples collected in 2008 were compared with samples collected in 2009 after EM had been applied and b) control samples were compared to EM samples for year 2009 only. The eleven experimental plots were delineated and soil samples collected from the plots influenced by the shade of *Persea* on May 17, 2008, one week after the onset of the rainy season and before the application of any fertilizers. Two weeks later, soil samples were taken from the *Quercus, Diphysa* and the *Annona* plots. These samples were taken after the first round of fertilizer applications for the growing season and three weeks into the rainy season. All soil samples were collected before EM was applied.

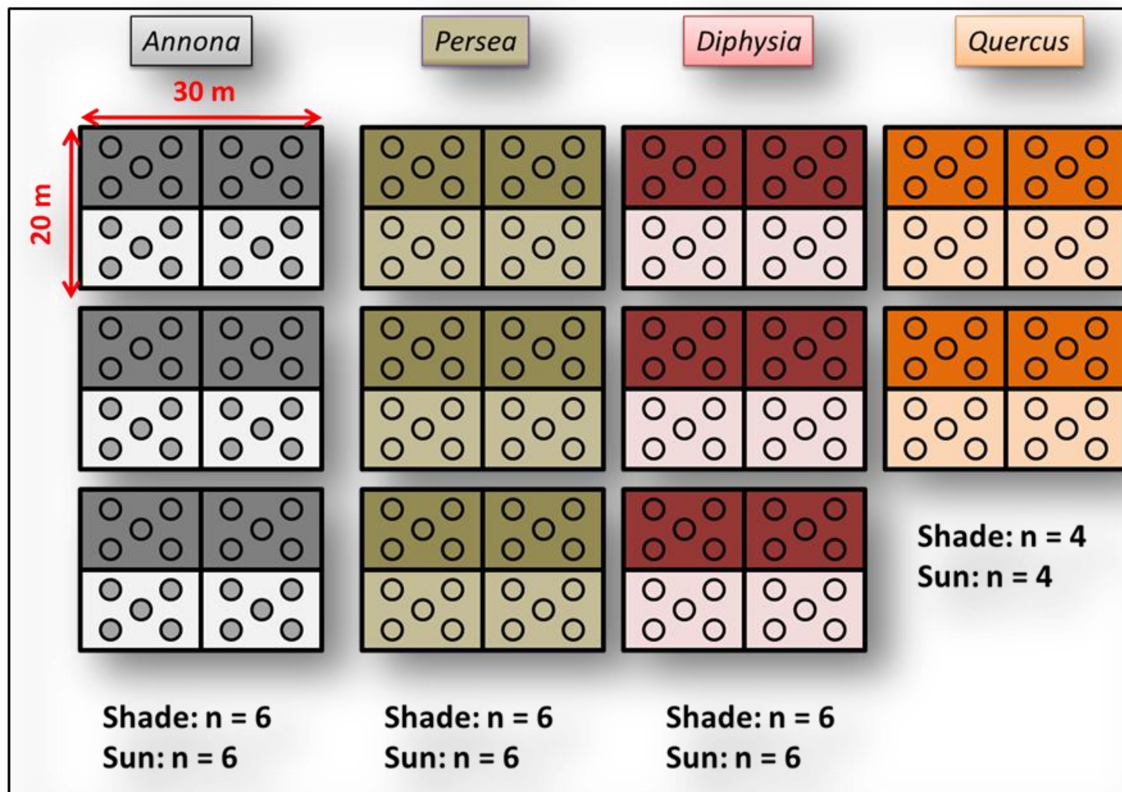

Figure 3.1 Experimental design showing pre-treatment arrangement for EM study.

Effective microorganisms were activated, diluted, and applied, according to the manufacturer's directions, to half of the subplots (Figure 3.2) as follows. EM-1 was donated by EARTH University in Costa Rica, a licensed manufacturer of the product. EM-1 contains microbes that have been multiplied in 5% molasses and water. EM-1 is activated by combining one part EM-1 with one part molasses and 20 parts water. This mixture is left in a sealed, clean plastic container to ferment at room temperature for one week. Before applying the EM to the plants, it was diluted in the field by combining 50 ml of the activated EM in five liters of water in a clean, plastic five gallon bucket. 100

ml of this mixture was scooped out with a clean plastic cup and applied approximately 10 cm from the base of each coffee plant. As recommended, EM was applied three times during the growing season. Post-treatment soil samples were then collected in June 2009. Both pre- and post-treatment samples were collected using the procedures described below.

III.2.3. Sample collection and processing

One soil sample was the product of five soil cores extracted with a 2.5 cm diameter auger 40 cm from the base of individual coffee bushes. Soil cores were taken to a depth of 20 cm and were mixed together in a plastic three gallon bucket. A composite sample was scooped with a metal hand spade into a quart-sized zip lock bag. Before the next soil core was taken, both the soil auger and the mixing bucket were carefully rinsed and disinfected with iodine to prevent carrying microorganisms from one area into another. The bucket was then rinsed a second time with water. With 4 composite samples taken from each plot, I collected a total of 44 composite soil samples. The composite soil samples were frozen within four hours of sampling at -20° Celsius in a freezer located at the study site and shipped to Texas A&M on dry ice for analyses.

III.2.4. Chemical analyses of soil samples

Water extractions were performed on each of the 44 samples collected during pre and post treatment (n=44 pre-treatment and n=44 post treatment). Aliquots of 3.5 g of sieved (2mm) soil were placed in 50 mL plastic centrifuge tubes and combined with 35 mL of ultra-pure water to achieve a soil:water ratio of 1:10. The centrifuge tubes were shaken for 90 minutes at 70 rpm. The soil:water units were then centrifuged at 19,600 g-

force for 20 min (Sorval RC6 with SS34 rotor) at 4°C prior to removal of the supernatant using a canula and syringe. pH and electrical conductivity were recorded on the supernatant prior to its filtration through ashed (500° C at 4 h) Whatman GF/F filters

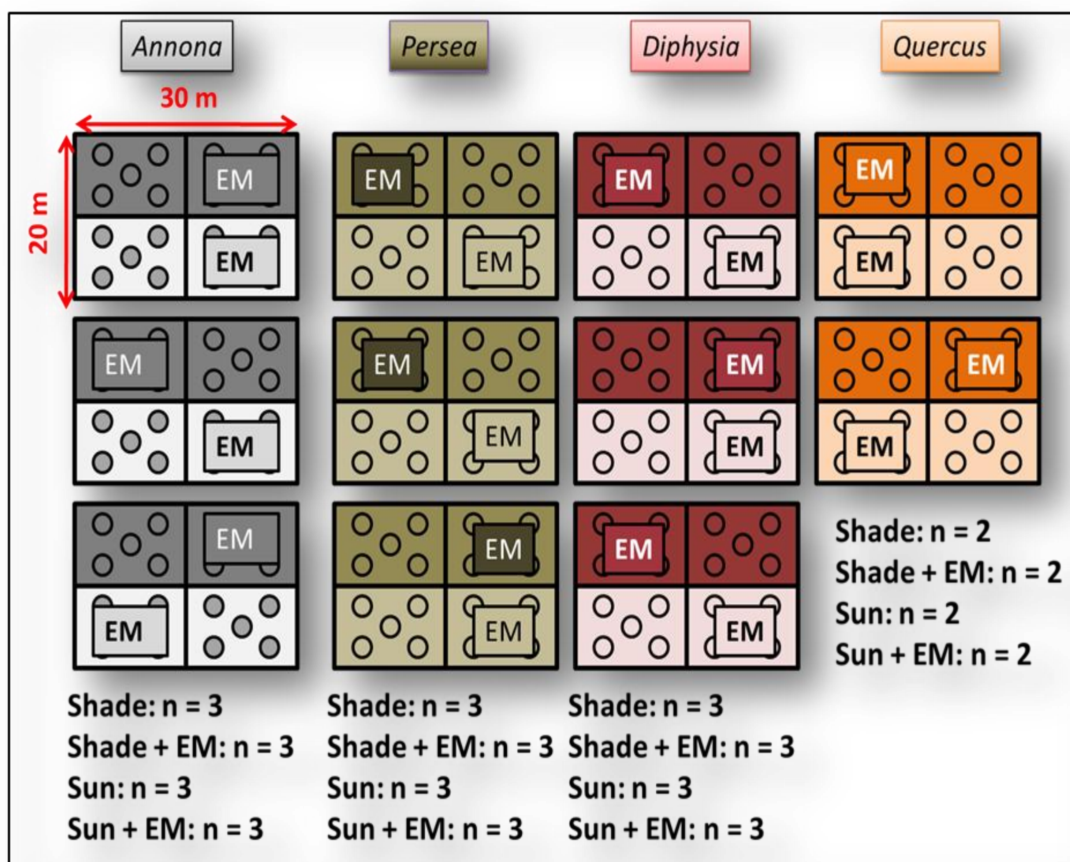

Figure 3.2. **Experimental design showing post-treatment arrangement for EM study.**

C at 4 h) Whatman GF/F filters and transfer to acid-washed, ultra-pure water rinsed, 50 mL high density polyethelene (HDPE) bottles and frozen until chemical analyses.

Dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) were measured using high temperature Pt-catalyzed combustion with a Shimadzu TOC-VCSH and Shimadzu total measuring unit TNM-1 (Shimadzu Corp. Houston, TX, USA). Dissolved organic carbon was measured as non-purgeable carbon using USEPA method 415.1 which entails acidifying (1N HCl) the sample and sparging for 4 min with C-free air. Ammonium-N was analyzed using the phenate hypochlorite method with sodium nitroprusside enhancement (USEPA method 350.1) and nitrate-N analyzed using Cd–Cu reduction (USEPA method 353.3). Alkalinity was quantified using methyl orange (USEPA method 310.2). All colorimetric methods were performed with a Westco Scientific Smartchem Discrete Analyzer (Westco Scientific Instruments Inc. Brookfield, CT, USA). Dissolved organic nitrogen (DON) is the product of TDN—(NH_4-N + NO_3-N).

In order to quantify base cations and anions using ion chromatography, aliquots of sample were filtered through 0.2 μm Pall filters. Calcium, magnesium potassium and sodium were quantified by ion chromatography using an Ionpac CS16 analytical and Ionpac CG16 guard column for separation and 20 mM Methanosulfonic acid as effluent at a flow rate of 1 mL min^{-1} and injection volume of 10 mL using a DIONEX ICS 1000 (DIONEX Corp. Sunnyvale, CA, USA). Fluoride, chloride, bromide and sulfate were quantified on a Dionex ICS 2000 using an Ionpak AS20 and Ionpak AG20 analytical and guard columns for separation with 35 mM KOH as effluent at a flow rate of 1 mL min^{-1} and an injection volume of 25 μL (Dionex Corp, Bannockburn, IL). For all analyses a

NIST traceable standard, check standard, replicate sample and blank were run every 10th or 12th sample for quality control purposes.

III.3. Results

Because there was a significant effect of the years the samples were taken on the soil chemical constituents, my results only include a comparison of the EM treated plots and the control plots for the second year soil samples. Consequently, my samples sizes were smaller than originally planned. The average concentrations of chemical constituents for soil samples taken from treatment and control plots for each tree species is shown in Table 3.1. It is interesting to note that although there were some differences between the treatment and control groups (Figures 3.3 and 3.4), most of these differences were not significant at alpha<0.05 (Table 3.2). Effective microorganisms did not have a significant effect on any soil chemical constituent with the exception of NH_4-N, for which there was a significant EM*species*sun/shade interaction effect as well as a significant EM*species interaction effect, and for Cl^-, for which the interaction effect between EM and sun/shade was significant (Table 3.2). Figure 3.5 provides a closer look at the interaction effect of species*EM*sun/shade position on average soil ammonium concentrations. In a sun position with EM, *Annona* soils had lower and less variable concentrations of NH_4-N than did *Annona* soils in a sun position without EM.

Table 3.1. Average soil concentrations with standard deviations (italics) of water extracted chemical constituents from coffee soils in a Costa Rican agroforestry system. Sample sizes for Persea, Annona, and Diphysa = 6; sample size for Quercus = 4. Includes data from Year 2 only.

	Trees	pH	NO_3-N	NH_4-N	DOC	TDN	PO_4-P	HCO_3^-	DON	Na^+	K^+	Mg^{2+}	Ca^{2+}	F^-	Cl^-	SO_4^{2-}
		Soil Concentrations ($\mu g\ g^{-1}$ soil)														
With EM	*Persea*	6.15	53.2	4.9	119.0	66.0	0.8	73.4	29.6	50.5	82.9	24.2	84.7	0.4	305.7	34.9
		0.33	*13.0*	*1.7*	*32.6*	*19.8*	*1.0*	*67.0*	*4.2*	*71.7*	*12.2*	*8.4*	*30.4*	*0.6*	*98.7*	*8.5*
	Annona	6.05	92.6	15.7	143.4	115.5	0.4	91.6	42.1	22.5	83.8	26.1	132.8	0.2	333.0	26.1
		0.24	*16.0*	*3.8*	*46.5*	*20.3*	*0.3*	*104.7*	*3.6*	*10.3*	*16.6*	*3.5*	*36.7*	*0.4*	*104.6*	*10.4*
	Diphysa	6.26	88.9	8.6	260.0	113.2	2.4	60.2	18.2	19.7	88.4	33.6	128.7	0.4	215.9	24.4
		0.36	*42.1*	*2.1*	*88.3*	*41.2*	*2.7*	*56.3*	*3.2*	*3.3*	*23.0*	*14.0*	*23.6*	*0.6*	*78.3*	*8.8*
	Quercus	6.07	51.6	10.0	134.2	67.1	0.3	68.3	9.2	45.5	100.2	19.0	96.9	0.5	520.5	40.2
		0.3	*48.0*	*9.3*	*55.4*	*55.6*	*0.1*	*79.0*	*2.6*	*40.2*	*33.5*	*4.6*	*35.2*	*0.7*	*324.6*	*26.8*
Without EM	*Persea*	5.98	48.5	4.4	130.6	58.8	0.5	122.6	29.9	14.4	77.9	22.5	89.0	0.2	228.5	35.9
		0.38	*9.5*	*1.0*	*32.9*	*9.4*	*0.4*	*61.3*	*3.3*	*2.6*	*17.5*	*3.2*	*39.7*	*0.3*	*104.8*	*7.5*
	Annona	6.06	125.9	26.7	156.4	160.7	0.4	60.3	41.4	16.5	101.1	31.3	149.1	0.0	403.8	18.6
		0.22	*51.0*	*17.1*	*22.4*	*62.3*	*0.2*	*68.6*	*3.9*	*5.3*	*22.4*	*8.1*	*43.5*	*0.0*	*171.5*	*7.1*
	Diphysa	6.27	97.8	11.4	215.9	123.9	1.5	139.4	19.2	14.8	98.4	33.5	131.2	0.3	259.2	25.8
		0.21	*51.5*	*3.6*	*41.1*	*54.7*	*0.9*	*120.0*	*3.2*	*2.2*	*26.7*	*14.8*	*56.1*	*0.4*	*79.0*	*8.3*
	Quercus	5.72	68.4	9.4	174.7	86.5	0.3	42.5	10.2	30.0	82.1	29.0	94.1	0.5	469.5	36.7
		0.57	*35.2*	*2.9*	*58.7*	*34.3*	*0.2*	*59.8*	*2.6*	*29.6*	*17.0*	*5.7*	*12.9*	*0.3*	*242.4*	*25.9*

Table 3.2. Statistical significance for effects of EM, EM*sun/shade position, and interaction of EM*species*sun/shade position on concentrations of soil chemical constituents. Significant EM interaction effects are indicated in bold. Includes data for Year 2 only.

Effects	NO_3-N	NH_4-N	DOC	DON	PO_4-P	HCO^{3-}	Na^+	K^+	Mg^{2+}	Ca^{2+}	F^-	Cl^-	SO_4^{2-}
EM	0.25	0.08	0.88	0.73	0.31	0.34	0.13	0.99	0.27	0.66	0.45	0.89	0.62
EM*Species	0.67	**0.05***	0.17	0.95	0.62	0.15	0.61	0.33	0.51	0.95	0.88	0.64	0.88
EM*Sun/Shade	0.92	0.29	0.96	0.95	0.72	0.18	1	0.12	0.33	0.74	0.78	**0.03***	0.79
EM*Species *Sun/Shade	0.74	**0.02***	0.11	1	0.44	0.1	0.16	0.59	0.84	0.92	0.25	0.54	0.48

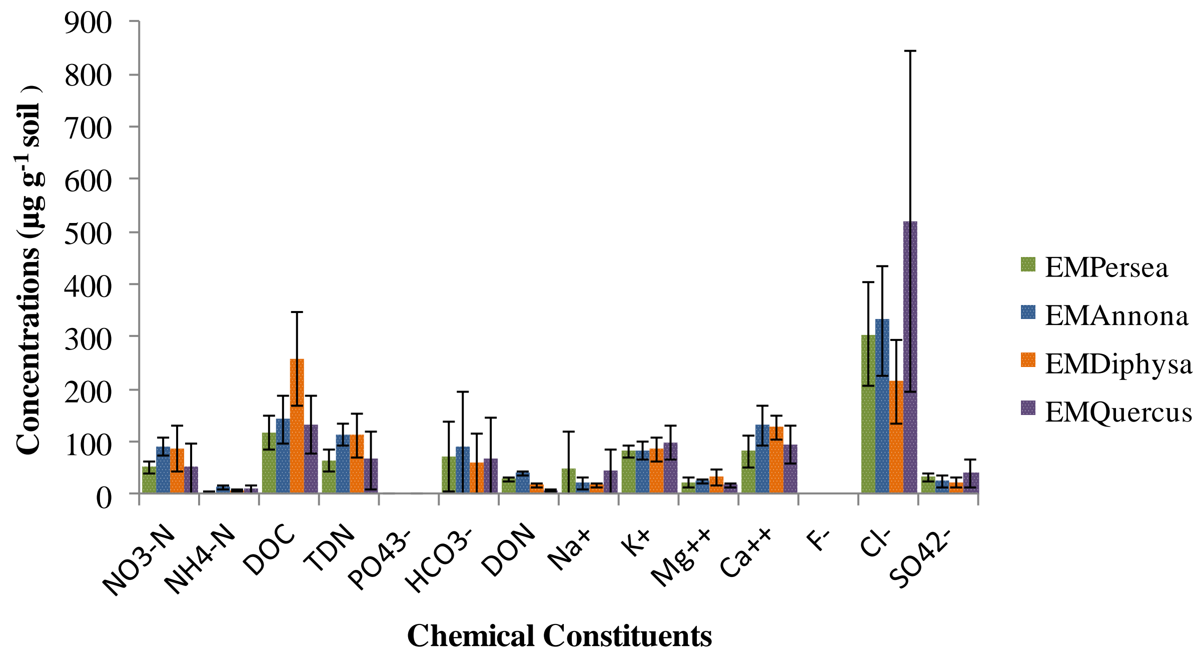

Figure 3.3. EM treated plots: average concentrations of water extractable chemical constituents from soils influenced by one of four native tree species and treated with Effective Microorganisms in a Costa Rican coffee agroforestry system. *Persea americana* and *Annona muricata*, N=6; for *Diphysa americana*, N=5; for *Quercus spp.*, N=4. Error bars are standard error.

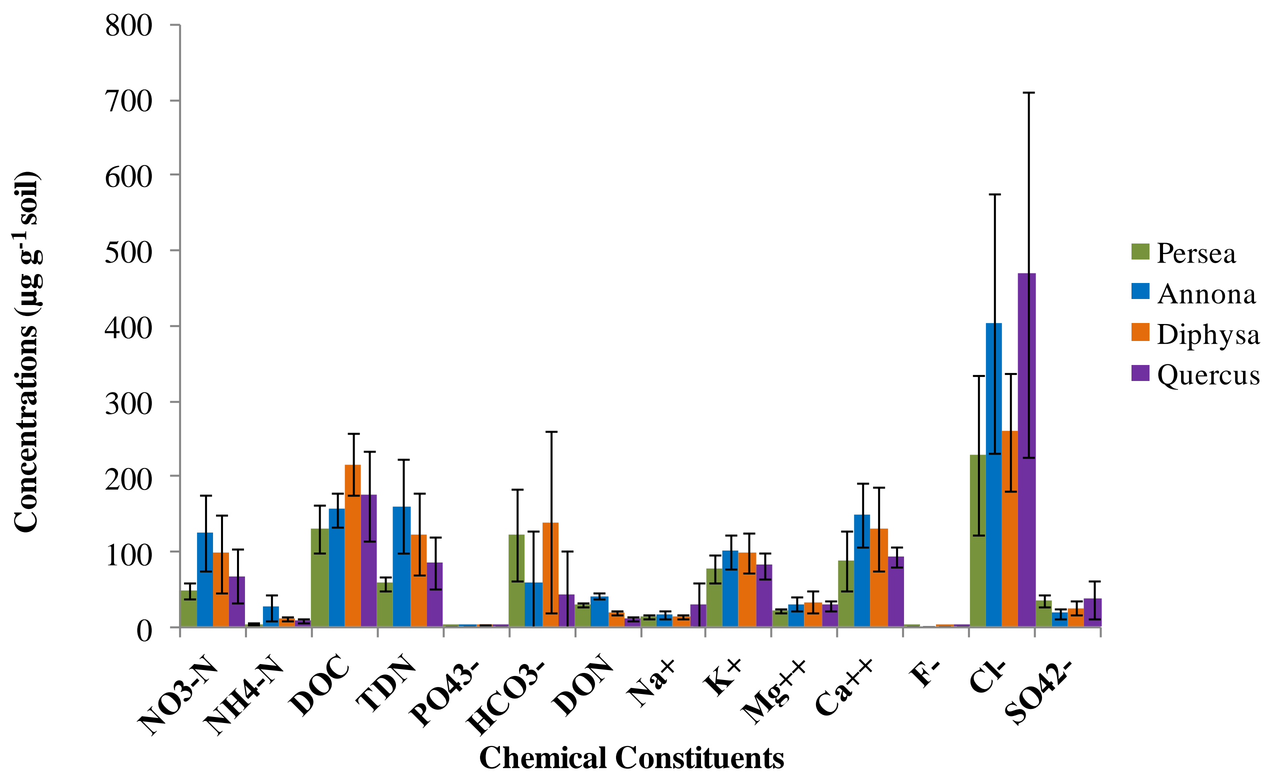

Figure 3.4.Control plots: average concentrations of water extractable chemical constituents from soils influenced by one of four native tree species in a Costa Rican coffee agroforestry system. For *Persea Americana* and *Annona muricata*, N=6; for *Diphysa americana*, N=5; for *Quercus spp.*, N=4. Error bars are standard error.

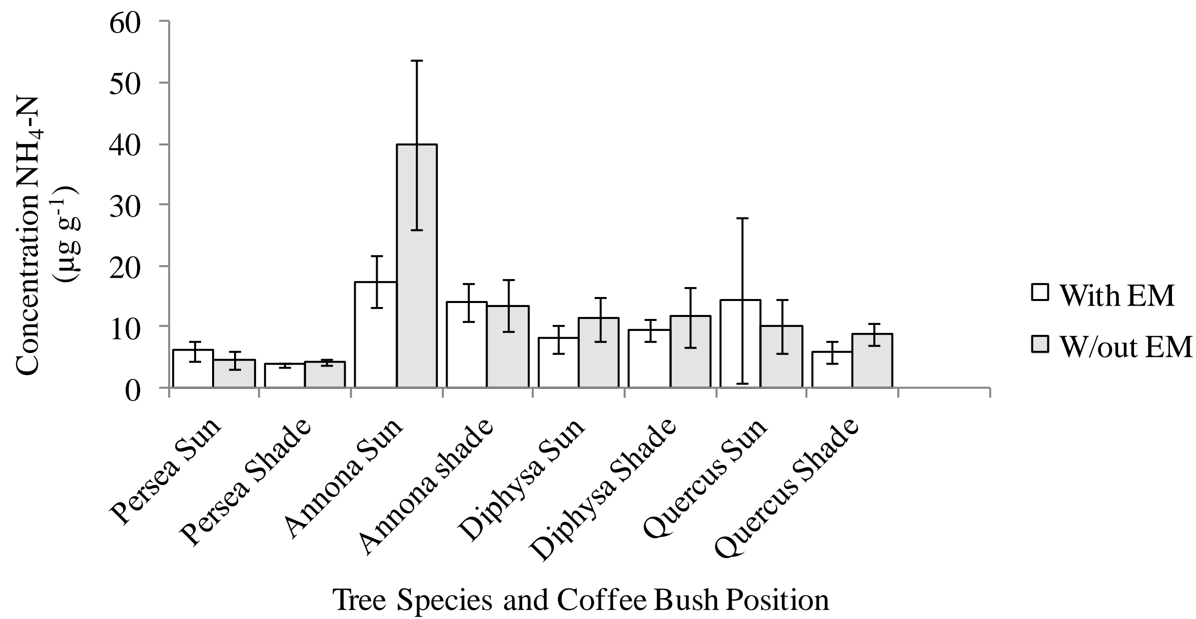

Figure 3.5. Species, sun/shade position and with or without EM in relation to concentration of soil ammonium. For *Persea americana* N=3; for *Annona muricata* N=3; for *Diphysa americana* N=3, 2, 3, 2 respectively, and for *Quercus spp.* N=2.

III.4. Discussion and conclusions

There was a significant interaction effect between EM and shade tree species, as well as between EM, shade tree species and sun or shade position, on soil ammonium. It is possible that the microorganisms in EM were able to overcome the bacterial-inhibiting substances in the *Anonna* litterfall, thus facilitating the process of nitrification, decreasing the amount of NH_4-N in the soil samples from treated plots relative to the soil samples from the *Anonna* control plots. Another possibility is that the antioxidants produced by EM (Higa 1993) promoted an antioxidized soil state, allowing the oxidative process of nitrification to proceed unhindered by an otherwise rate-limiting equilibrium of high concentrations of oxidative products.

Because of the significant effect of the year the soil samples were taken, each year's samples were analyzed separately reducing the sample size. This decreased the power of my statistical tests and increased the probability of making a Type II error; that is, failing to reject the null hypothesis when it really is false. Consequently, Effective Microorganisms may have had a greater effect on soil chemistry than was detected by my statistical tests.

I was not able to discern what caused the changes in the soils between the two years. The fertilization regime was the same for both years, and no unusual weather events occurred (Meterological Station data, Finca la Hilda, 2007-2009). One possibility is that given the steeply sloping terrain and torrential rains at the study site, the EM may have flowed into all of the plots, effectively eliminating the control plots. It would be worthwhile to repeat this investigation in a more controlled environment and with coffee

yield data included in order to more conclusively determine the effects of Effective Microorganisms on soil chemistry and coffee production.

CHAPTER IV

SUMMARY

In the Central Valley of Costa Rica in the Department of Heredia, I investigated the soil chemical properties and microbial communities under four native shade tree species in a coffee agroforestry system. In the second year of the study, Effective Microorganisms®, a microbial inoculant, was applied to examine its effect on soil chemistry. The shade tree species included in this study were *Anonna muricata* L., *Diphysa americana* Mill., *Persea americana* Mill., and *Quercus* spp. L.

The study was carried out at a farm with shade trees and was not the typical full sun coffee plantation. Nevertheless, position of coffee bushes in sun or shade positions resulted in some significant differences in soil DOC and DON. Species of shade tree was generally more important for soil chemistry and microbial community composition than sun or shade position. Stronger conclusions could be reached as to the effects of the shade trees on the coffee plants themselves by acquiring coffee yield data and information on the flavor and quality of the coffee produced in soils influenced by the four different species of shade trees studied.

There was a significant interaction effect between EM and shade tree species, as well as between EM, shade tree species and sun or shade position, on soil ammonium. It is possible that the microorganisms in EM were able to overcome the bacterial-inhibiting substances in the Anonna litterfall, thus facilitating the process of nitrification, decreasing the amount of NH4-N in the soil samples from treated plots relative to the soil samples from the Anonna control plots. Another possibility is that the antioxidants

produced by EM (Higa 1993) promoted an antioxidized soil state, allowing the oxidative process of nitrification to proceed unhindered by an otherwise rate-limiting equilibrium of high concentrations of oxidative products.

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APPENDIX I

PH, ELECTRICAL CONDUCTIVITY AND EXTRACTABLE NUTRIENTS

Table A.1. Ph, Electrical Conductivity and Extractable Nutrients from Soils under Different Shade Tree Species and Sun or Shade Positions. Species 1 = *Persea americana*, Species 2 = *Annona muricata*, Species 3 = *Diphysa americana* and Species 4 = *Quercus spp.* Is Shade Position and 1 = Sun Position

		pH	EC	NO_3-N	NH_4-N	DOC	TDN	PO_4-P	HCO_3^-	DON
Species	Sun Shade		µS cm^{-1}				µg g^{-1} soil			
1	0	6.59	180	27.5	3.7	124.5	38.9	0.8	275.3	7.7
1	0	6.42	90	17.8	1.9	61.6	21.3	0.6	94.8	1.6
1	0	5.9	110	34.6	2.7	77.1	39.6	0.2	80.1	2.4
1	0	6.31	100	30.4	2.3	88.1	37.3	0.5	60.1	4.6
1	0	6.32	180	24.5	2.5	122.2	40.3	0.8	45.7	13.4
1	0	6.19	100	42.4	3.1	139.5	51.0	0.6	102.2	5.6
1	1	6.34	130	32.3	1.9	98.0	39.6	1.3	193.7	5.4
1	1	6.6	150	67.4	5.6	129.9	77.8	0.9	207.7	4.9
1	1	6.25	120	39.2	2.8	100.4	46.9	1.0	105.8	4.9
1	1	6.04	130	32.2	5.7	75.4	40.1	0.4	258.1	2.2
1	1	6.07	140	47.2	4.9	95.7	56.3	1.0	204.9	4.3
1	1	6.26	130	45.0	2.3	107.3	53.9	0.7	162.8	6.6
2	0	6.28	130	25.9	2.2	91.8	32.3	0.8	287.1	4.3
2	0	6.02	140	53.0	3.5	142.2	63.0	0.8	182.2	6.6
2	0	5.84	250	65.0	3.1	138.4	74.4	0.7	151.5	6.2
2	0	5.73	110	29.2	3.8	73.7	34.4	0.1	18.3	1.4
2	0	6.49	100	51.6	2.7	117.9	60.3	1.2	76.0	6.0

2	0	6.28	170	53.6	7.2	172.8	68.9	0.5	103.7	8.1
2	1	6.25	100	12.7	2.1	60.2	18.1	0.2	78.8	3.3
2	1	5.91	110	15.2	2.2	55.1	19.1	0.2	118.7	1.6
2	1	6.21	120	36.0	3.2	116.9	44.4	0.4	89.1	5.2
2	1	6.49	150	82.1	3.6	178.5	98.7	1.5	320.3	13.0
2	1	6.67	240	32.9	2.6	159.1	43.4	2.3	265.1	8.0
2	1	6.36	110	35.9	2.4	125.5	43.5	1.0	136.5	5.2
3	0	6.1	100	23.2	7.6	206.4	45.3	3.4	421.0	14.4
3	0	5.97	80	25.9	7.4	82.3	36.8	0.4	88.0	3.6
3	0	5.75	150	60.9	3.4	99.9	69.3	0.4	520.8	5.0
3	0	6.08	100	36.6	3.9	115.3	47.2	0.6	48.2	6.6
3	0	6.35	110	15.5	4.2	114.8	28.0	0.8	114.8	8.3
3	0	6.21	130	23.0	4.8	164.2	37.5	4.1	200.6	9.7
3	1	5.52	100	18.2	3.0	62.8	24.6	0.2	115.8	3.4
3	1	5.93	100	39.4	3.6	47.6	40.8	0.3	46.2	0.0
3	1	5.71	180	66.1	4.6	71.9	70.4	1.1	93.7	0.0
3	1	6.58	110	24.9	10.4	94.1	37.9	0.3	98.9	2.6
3	1	5.87	90	25.8	4.3	77.3	34.1	0.4	69.3	3.9
3	1	6.22	90	23.9	4.7	115.0	34.3	1.0	48.9	5.7
4	0	5.98	120	56.5	11.5	100.8	76.5	0.2	97.0	8.5
4	0	5.72	140	73.2	9.8	69.4	79.6	0.2	78.1	0.0
4	0	6.1	140	63.5	4.6	160.4	74.0	0.5	542.2	5.9
4	0	6.09	160	109.0	3.8	164.9	121.8	1.3	98.8	9.1
4	1	5.9	110	54.0	28.5	44.1	81.7	3.9	87.9	0.0
4	1	5.61	140	59.7	21.5	93.5	80.1	0.2	126.4	0.0
4	1	5.92	110	45.1	3.1	55.6	48.4	0.2	126.2	0.1
4	1	6.28	120	48.1	3.8	125.2	59.2	0.7	105.7	7.3

1	0	6.19	100	51.5	4.7	112.8	58.9	0.3	33.0	25.2
1	0	5.74	180	60.5	4.0	84.3	70.2	0.4	169.0	29.2
1	0	5.77	130	34.4	3.9	123.8	43.8	0.3	99.6	32.2
1	0	6.58	70	34.0	3.4	85.6	39.0	0.1	108.3	24.2
1	0	5.86	130	44.8	3.8	85.6	51.6	0.2	68.9	28.2
1	0	5.69	180	68.7	3.9	119.9	92.8	0.3	88.6	33.2
1	1	6.48	170	52.2	3.0	172.7	63.6	1.2	211.1	27.2
1	1	5.47	90	52.0	5.9	125.8	63.7	0.2	110.7	31.2
1	1	6.23	120	40.3	4.5	164.1	52.7	0.4	112.3	34.2
1	1	6.19	110	59.1	7.7	127.6	74.3	0.5	0.0	26.2
1	1	6.43	110	63.6	6.3	173.7	79.3	0.7	0.0	30.2
1	1	6.14	160	49.2	4.3	121.7	59.1	2.8	174.5	35.2
2	0	6.17	150	96.3	14.7	176.0	122.7	0.4	0.0	36.2
2	0	5.93	230	156.5	17.1	164.3	181.5	0.3	135.8	42.2
2	0	6.34	130	50.1	8.8	175.0	71.4	0.3	73.4	45.2
2	0	5.83	150	78.1	10.4	79.3	90.1	0.2	112.8	37.2
2	0	5.99	180	104.4	15.5	179.5	134.9	0.4	284.9	43.2
2	0	5.98	130	88.4	15.9	127.8	107.0	0.1	89.1	44.2
2	1	6.22	200	102.1	25.3	124.4	134.3	0.9	146.6	38.2
2	1	5.8	220	171.0	52.9	166.4	224.3	0.3	5.9	40.2
2	1	5.88	250	179.4	41.4	132.0	229.9	0.3	0.0	46.2
2	1	6.44	200	94.5	22.2	169.0	127.2	1.0	57.7	39.2
2	1	6.22	160	116.2	14.7	198.7	137.3	0.4	5.0	41.2
2	1	5.84	140	73.9	15.2	105.8	96.5	0.2	0.0	47.2
3	0	6.23	80	35.3	8.1	183.2	54.1	1.1	317.2	17.2
3	0	6.57	240	165.0	15.0	191.0	195.3	1.2	177.7	21.2
3	0	6.39	170	101.8	10.8	361.8	132.9	6.4	130.9	16.2

3	0	6.47	130	58.5	8.2	247.1	78.4	0.5	41.4	20.2
3	1	5.97	190	132.4	12.9	184.1	155.8	0.3	0.0	15.2
3	1	6.29	140	67.9	7.0	255.1	92.5	2.3	133.7	19.2
3	1	6.28	160	88.1	13.8	266.0	121.5	2.5	68.2	23.2
3	1	5.69	180	155.2	10.7	144.0	175.4	0.2	107.1	14.2
3	1	6.16	130	78.7	7.0	215.1	99.9	1.2	21.4	18.2
3	1	6.61	110	50.4	6.3	332.0	79.2	4.0	0.0	22.2
4	0	5.86	120	53.8	10.2	183.3	74.2	0.1	42.9	7.2
4	0	5.58	100	50.8	7.5	250.6	75.3	0.5	0.0	11.2
4	0	6.1	110	38.9	7.0	73.9	47.5	0.2	142.3	6.2
4	0	6.49	60	24.5	4.5	207.2	41.5	0.3	130.7	10.2
4	1	5.04	160	121.0	13.1	111.0	136.8	0.1	0.0	9.2
4	1	6.4	90	47.8	6.8	153.9	59.8	0.3	126.9	13.2
4	1	5.89	180	122.5	23.9	119.1	149.8	0.3	0.0	8.2
4	1	5.81	90	20.3	4.7	136.5	29.8	0.4	0.0	12.2

APPENDIX II

CATIONS AND ANIONS IN SOIL EXTRACTS

Table A.2. Cations and anions in soil extracts from soils under different shade tree species and sun or shade positions. species 1 = *Persea americana*, species 2 = *Annona muricata*, species 3 = *Diphysa americana* and species 4 = *Quercus spp*. 0 is shade position and 1 = sun position

Species	Sun Shade	Na^+	K^+	Mg^{2+}	Ca^{2+}	F^-	Cl^-	SO_4^{2-}
					μg g^{-1} soil			
1	0	34.3	91.6	24.1	106.1	0.0	156.2	36.5
1	0	66.6	53.1	13.2	60.0	1.5	135.3	60.2
1	0	10.8	82.2	23.8	52.1	0.8	139.4	39.5
1	0	17.6	37.4	16.6	67.1	0.0	27.4	29.9
1	0	40.1	228.0	13.4	53.6	1.5	621.6	16.5
1	0	26.9	74.1	17.7	50.6	1.9	81.3	32.5
1	1	45.6	55.1	17.0	85.8	0.3	197.5	32.1
1	1	48.8	82.9	23.1	98.7	0.0	67.5	26.6
1	1	22.4	84.3	18.7	57.1	0.0	82.5	33.3
1	1	108.4	61.1	17.3	55.6	0.5	148.9	34.9
1	1	45.4	63.8	18.8	95.7	0.8	108.8	42.8
1	1	16.6	86.2	19.0	67.3	0.0	83.4	29.1
2	0	129.7	148.1	12.7	45.7	0.7	320.0	24.6
2	0	17.2	104.2	26.5	68.7	0.0	90.3	32.4
2	0	18.8	386.7	28.8	73.0	1.0	1273.3	21.9
2	0	23.1	91.8	22.5	46.2	0.1	196.2	36.8
2	0	52.3	92.6	17.2	58.0	0.0	89.0	19.5

2	0	34.4	180.8	19.7	43.5	0.8	333.6	18.7
2	1	186.5	56.4	17.2	41.8	0.0	173.0	42.8
2	1	15.4	78.9	25.3	44.8	0.0	106.1	50.5
2	1	20.0	80.5	18.8	73.3	0.0	118.8	44.1
2	1	277.6	156.3	27.4	106.7	50.6	553.3	24.9
2	1	166.3	281.4	28.5	373.5	0.0	1847.4	44.8
2	1	21.3	85.1	19.2	63.1	1.0	134.0	34.1
3	0	196.3	71.4	11.1	42.0	0.0	306.1	33.7
3	0	16.4	57.6	13.1	37.6	0.0	39.7	33.2
3	0	225.7	116.1	20.1	48.8	0.5	466.3	25.5
3	0	11.1	65.4	16.2	55.8	0.6	64.0	34.9
3	0	29.3	86.9	16.4	61.7	0.0	99.6	35.2
3	0	16.9	65.1	19.5	80.5	0.0	57.8	30.0
3	1	14.3	77.1	17.0	53.4	0.0	138.0	53.2
3	1	9.1	82.5	14.6	46.7	0.0	131.4	27.9
3	1	32.4	160.7	25.0	69.8	0.5	323.8	18.3
3	1	39.7	158.2	9.6	26.0	0.7	252.9	13.9
3	1	43.1	80.3	14.1	48.6	4.2	101.3	33.1
3	1	26.2	80.0	14.0	43.6	0.4	146.3	32.1
4	0	24.9	61.0	20.4	38.5	0.0	110.2	22.4
4	0	19.4	79.7	26.8	53.6	0.0	178.2	26.3
4	0	164.3	180.9	14.4	51.5	0.6	370.3	22.8
4	0	23.1	165.9	28.2	97.5	0.0	288.9	19.2
4	1	79.4	52.5	9.7	26.5	0.7	151.9	23.2
4	1	19.2	77.9	19.6	48.1	0.0	118.3	27.0
4	1	12.0	102.5	14.3	45.6	0.0	187.8	27.5
4	1	13.8	107.5	16.1	69.2	0.0	215.1	25.7

1	0	17.5	64.1	23.2	77.0	0.0	127.9	29.6
1	0	16.3	54.9	21.4	105.5	0.6	146.5	25.8
1	0	15.2	77.4	17.7	63.9	0.0	209.0	39.2
1	0	17.2	78.2	19.9	44.2	0.6	262.6	45.2
1	0	30.6	89.0	25.9	68.5	1.4	327.2	39.7
1	0	11.0	102.9	40.5	131.9	0.0	311.6	42.3
1	1	10.7	76.0	26.5	162.3	0.0	258.0	46.6
1	1	15.2	92.6	20.7	57.9	0.7	210.3	39.6
1	1	11.8	102.2	25.2	67.1	0.0	419.4	34.3
1	1	195.7	66.9	18.2	70.6	0.0	451.8	29.9
1	1	33.1	82.4	22.1	93.5	0.0	149.6	25.7
1	1	15.2	77.7	18.9	99.6	0.6	331.3	26.6
2	0	11.8	70.8	22.8	130.0	0.0	155.2	19.2
2	0	17.2	138.2	40.0	176.4	0.0	464.7	23.4
2	0	13.7	109.4	22.7	71.3	0.0	408.2	14.4
2	0	12.7	61.5	27.0	123.6	0.5	169.2	33.6
2	0	21.9	101.7	32.5	135.1	0.0	414.8	18.6
2	0	23.0	101.1	25.2	80.8	0.0	451.4	20.3
2	1	12.5	89.1	29.0	185.8	0.0	249.1	29.7
2	1	26.1	97.8	31.8	150.9	0.0	558.0	9.7
2	1	17.8	101.3	41.2	180.0	0.0	587.7	15.2
2	1	14.2	79.5	23.8	195.0	0.0	287.8	37.7
2	1	41.6	89.7	25.2	125.4	1.0	387.9	12.0
2	1	21.5	69.6	22.7	137.0	0.0	287.0	34.0
3	0	17.6	79.6	17.1	66.1	0.0	245.7	32.4
3	0	16.1	99.2	42.1	214.9	0.8	271.7	21.6
3	0	23.7	122.8	33.5	155.4	0.0	340.8	18.9

3	0	17.6	83.7	28.1	106.8	0.0	231.5	39.7
3	1	13.8	138.0	54.0	134.9	0.0	223.4	35.1
3	1	11.9	106.3	23.1	97.1	0.7	384.1	14.6
3	1	14.6	69.1	31.3	143.1	0.0	171.3	25.3
3	1	18.7	98.6	57.6	153.2	1.3	205.9	18.8
3	1	22.5	65.3	26.0	114.2	0.0	145.6	23.6
3	1	15.9	71.7	22.7	113.8	0.6	155.8	20.9
4	0	19.5	80.5	32.5	80.2	0.7	327.3	50.0
4	0	13.1	59.7	20.4	86.1	0.0	202.9	24.9
4	0	27.3	61.4	16.3	82.4	1.5	196.9	24.1
4	0	105.6	125.4	16.0	53.8	0.0	921.7	42.2
4	1	13.3	100.0	30.7	106.2	0.7	700.4	6.7
4	1	74.1	88.0	32.3	104.0	0.6	647.3	65.1
4	1	29.2	130.9	25.8	126.3	0.5	635.5	17.2
4	1	20.0	83.2	17.9	125.1	0.0	327.8	77.2

APPENDIX III

CHEMISTRY OF COFFEE BUSH FOLIAGE

Table A.3. Chemistry of coffee bush foliage under different shade tree species and sun or shade position

		Foliar Chemistry (µg g^{-1} dry foliage)						
Species	Position	P	K+	Ca^{2+}	Mg^{2+}	SO_4^{2-}	Fe	Cu
Persea	Shade	nd	nd	nd	nd	nd	nd	nd
Persea	Shade	nd	nd	nd	nd	nd	nd	nd
Persea	Shade	nd	nd	nd	nd	nd	nd	nd
Persea	Shade	nd	nd	nd	nd	nd	nd	nd
Persea	Shade	nd	nd	nd	nd	nd	nd	nd
Persea	Shade	nd	nd	nd	nd	nd	nd	nd
Persea	Sun	nd	nd	nd	nd	nd	nd	nd
Persea	Sun	nd	nd	nd	nd	nd	nd	nd
Persea	Sun	nd	nd	nd	nd	nd	nd	nd
Persea	Sun	nd	nd	nd	nd	nd	nd	nd
Persea	Sun	nd	nd	nd	nd	nd	nd	nd
Persea	Sun	nd	nd	nd	nd	nd	nd	nd
Anonna	Shade	0.12	2.04	1.34	0.35	0.35	80	12
Anonna	Shade	0.12	1.93	1.13	0.40	0.35	105	15
Anonna	Shade	0.14	2.10	1.20	0.35	0.41	70	14
Anonna	Shade	0.10	2.04	1.38	0.36	0.39	93	13
Anonna	Shade	0.12	2.07	1.44	0.44	0.34	88	14
Anonna	Shade	0.14	2.08	1.26	0.43	0.46	77	15
Anonna	Sun	0.11	2.33	1.24	0.34	0.54	93	16
Anonna	Sun	0.11	2.28	1.35	0.37	0.38	84	15

Anonna	Sun	0.10	2.21	1.51	0.43	0.41	83	11
Anonna	Sun	0.13	2.50	1.55	0.48	0.39	83	15
Anonna	Sun	0.10	2.04	1.31	0.44	0.37	81	17
Anonna	Sun	0.11	2.13	1.41	0.47	0.39	89	15
Diphysa	Shade	0.12	2.38	0.96	0.24	0.44	64	15
Diphysa	Shade	0.15	2.18	0.88	0.27	0.42	68	16
Diphysa	Shade	0.14	2.47	1.39	0.42	0.38	80	18
Diphysa	Shade	0.15	2.24	1.11	0.34	0.42	73	18
Diphysa	Shade	0.15	1.90	1.04	0.31	0.44	67	20
Diphysa	Shade	0.14	2.20	0.98	0.31	0.43	71	18
Diphysa	Sun	0.15	2.21	1.09	0.37	0.47	69	19
Diphysa	Sun	0.13	2.02	1.13	0.37	0.45	67	18
Diphysa	Sun	0.16	2.13	1.12	0.31	0.41	61	16
Diphysa	Sun	0.15	2.21	1.31	0.38	0.48	63	18
Diphysa	Sun	0.15	2.11	1.07	0.33	0.38	70	17
Diphysa	Sun	0.14	2.02	1.11	0.34	0.50	73	17
Quercus	Shade	0.13	2.03	0.86	0.38	0.56	74	16
Quercus	Shade	0.13	2.23	0.84	0.39	0.42	80	15
Quercus	Shade	0.10	1.69	1.07	0.36	0.40	71	12
Quercus	Shade	0.13	1.89	1.15	0.37	0.56	70	12
Quercus	Sun	0.13	1.75	1.20	0.37	0.47	73	15
Quercus	Sun	0.12	1.94	0.88	0.31	0.46	52	15
Quercus	Sun	0.12	2.35	1.15	0.37	0.66	71	15
Quercus	Sun	0.11	2.13	1.11	0.40	0.63	75	12

APPENDIX IV

FAME ANALYSIS

Table A.4. Fame analysis: fames used as biomarkers for gram + bacteria, in mol %

Species	Shade/Sun	15:0 ISO	15:0 ANTE	15:0	16:0 ISO	17:0 ISO	17:0	17:0 ANTE	14:0 ISO	Total Gram+
Persea	shade	6.412	2.807	1.049	2.791	2.383	0.755	1.291	0.607	18.09
Persea	shade	6.512	2.706	1.252	2.957	2.178	0.772	1.147	0.816	18.34
Persea	shade	6.096	2.470	1.052	2.338	2.275	0.711	1.126	0.597	16.67
Persea	sun	5.828	2.565	1.161	2.229	1.826	0.832	1.111	0.597	16.15
Persea	sun	6.115	2.736	1.002	2.424	2.113	0.730	1.142	0.567	16.83
Persea	sun	6.109	2.651	1.102	2.369	2.096	0.747	1.076	0.658	16.81
Diphysa	sun	7.627	3.118	0.998	2.492	1.808	0.600	1.060	0.789	18.49
Diphysa	sun	6.766	3.027	0.963	2.696	1.976	0.755	1.268	0.713	18.16
Diphysa	sun	5.506	2.796	0.829	2.316	1.903	0.678	1.129	0.864	16.02
Diphysa	shade	6.424	3.022	1.110	2.606	1.819	0.706	1.093	0.739	17.52
Diphysa	shade	3.686	1.760	1.020	1.901	1.711	0.528	0.860	0.887	12.35
Diphysa	shade	6.353	3.069	1.014	2.485	1.928	0.876	1.213	0.713	17.65
Annona	sun	6.696	2.580	1.006	4.084	2.586	0.610	1.246	0.900	19.71
Annona	sun	5.764	2.332	0.929	2.739	1.942	0.654	0.970	0.714	16.04
Annona	sun	5.687	2.641	1.305	2.848	2.159	0.747	1.229	0.750	17.37
Annona	shade	5.886	2.553	1.071	2.619	2.065	0.739	1.078	0.746	16.76
Annona	shade	6.235	2.799	1.132	3.082	2.425	0.637	1.262	0.819	18.39
Annona	shade	6.659	2.545	1.099	2.966	2.297	0.661	1.098	0.745	18.07
Quercus	shade	7.529	3.638	1.035	2.765	2.027	0.692	1.300	0.866	19.85
Quercus	shade	6.741	2.606	0.919	2.694	2.517	0.681	1.153	0.596	17.91
Quercus	sun	6.114	2.010	1.078	2.210	2.350	0.645	0.964	0.466	15.84
Quercus	sun	7.301	3.092	1.057	2.743	2.159	0.732	1.211	0.716	19.01

APPENDIX V

FAME ANALYSIS

Table A.5. Fame analysis: fames used as biomarkers for gram – bacteria, in mol %

Species	Shade/Sun	16:1ω7c	12:0 2OH	12:0 3OH	16:0 2OH	16:0 3OH	17:0 CYCLO	19:0cy c11-12	Sum In Feature 8	Total Gram-
Persea	shade	3.360	0	0	1.536	0.617	1.535	3.271	3.956	14.27
Persea	shade	3.602	0	0	1.190	0.000	2.174	2.983	4.060	14.01
Persea	shade	3.977	0	0	1.303	0.602	2.462	2.811	4.141	15.30
Persea	sun	2.907	0	0	1.331	0.505	2.057	2.351	4.533	13.69
Persea	sun	3.399	0	0	1.003	0.000	2.268	3.166	5.767	15.60
Persea	sun	3.203	0	0	1.268	0.511	2.531	2.633	3.969	14.11
Diphysa	sun	3.978	0	0.263	1.022	0.526	2.162	3.005	4.461	15.42
Diphysa	sun	3.756	0	0.248	1.215	0.466	2.418	3.049	4.248	15.40
Diphysa	sun	2.964	0.266	0.586	1.108	0.524	2.181	2.246	3.201	13.08
Diphysa	shade	3.630	0	0.262	1.134	0.490	2.278	2.750	3.953	14.50
Diphysa	shade	2.666	0.129	0.507	1.292	0.562	0.000	1.767	3.436	10.36
Diphysa	shade	3.235	0	0	1.031	0.477	2.221	2.953	4.434	14.35
Annona	sun	3.682	0.288	0.507	1.106	0.000	2.580	4.239	3.079	15.48
Annona	sun	3.481	0.175	0.353	0.888	0.000	1.476	3.449	2.930	12.75
Annona	sun	3.559	0.132	0.359	1.149	0.462	2.216	3.254	2.641	13.77
Annona	shade	3.031	0.276	0.335	0.953	0.428	1.924	3.421	3.350	13.72
Annona	shade	3.820	0.249	0.416	0.914	0.314	2.243	3.908	3.318	15.18
Annona	shade	2.945	0.302	0.560	0.860	0.540	2.060	3.976	3.387	14.63
Quercus	shade	3.765	0.255	0.261	0.970	0.434	2.211	3.645	4.737	16.28
Quercus	shade	2.982	0.329	0	0.984	0.000	2.073	5.343	3.589	15.30
Quercus	sun	2.779	0.217	0.423	1.040	0.258	1.801	4.434	3.156	14.11
Quercus	sun	3.454	0	0.274	1.219	0.573	2.588	3.662	3.667	15.44

APPENDIX VI

FAME ANALYSIS FUNGI

Table A.6. Fame analysis: fames used as biomarkers for fungi, in mol %

Species	Shade/Sun	18:3 ω 6c	SF19	18:1 ω 9c	16:1 ω 5c	Total Fungi
Persea	shade	1.233	2.455	10.326	3.164	17.178
Persea	shade	1.423	3.802	10.832	4.051	20.107
Persea	shade	1.330	4.838	9.942	3.294	19.404
Persea	sun	1.257	4.274	9.580	2.596	17.708
Persea	sun	1.534	3.765	9.695	2.692	17.686
Persea	sun	1.367	2.809	11.422	2.929	18.528
Diphysa	sun	1.109	2.445	9.952	3.490	16.996
Diphysa	sun	0.841	2.371	9.152	2.964	15.329
Diphysa	sun	1.243	3.392	10.840	2.373	17.847
Diphysa	shade	0.895	2.601	10.454	2.746	16.696
Diphysa	shade	1.376	3.766	11.154	3.202	19.497
Diphysa	shade	0.853	2.646	12.023	2.874	18.397
Annona	sun	1.385	2.377	6.144	2.623	12.529
Annona	sun	1.202	2.787	9.875	2.891	16.755
Annona	sun	0.851	2.240	6.295	3.104	12.490
Annona	shade	0.865	3.314	7.718	3.857	15.754
Annona	shade	0.906	2.117	6.213	2.718	11.954
Annona	shade	0.861	2.682	10.794	3.025	17.362
Quercus	shade	0.779	2.517	9.282	3.946	16.525
Quercus	shade	0.730	2.671	9.478	2.784	15.662
Quercus	sun	0.808	2.526	7.533	3.478	14.347
Quercus	sun	1.188	2.402	9.628	3.373	16.590

VITA

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