THE GREATER UNSEEN: ON THE IDENTITIES, DISTRIBUTIONS, AND IMPACTS OF FOLIAR BACTERIA ON TROPICAL ARBOREAL SPECIES

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Bacteria have been called the "unseen majority" in nature. Leaves of higher plants comprise perhaps the largest bacterial substrate on earth, yet we know surprisingly little about the bacteria that occupy these spaces. The shaded understory of tropical forests is likely a "hotspot" for bacteria because water availability and humidity are high and UV radiation is low. Ultimately, these communities may be critical mediators of plant performance among co-occurring woody species and ultimately contribute to plant species distributions at the community level. In this dissertation, I (Chapter 2) review the ecology and behavior of bacteria that reside on the phyllosphere (on and inside leaves) and outline testable hypotheses to empirically evaluate the potential ecological implications of foliar bacteria. Moreover, I conducted a major effort to test interrelated hypotheses regarding the distribution, impact, and identity of foliar bacteria with replicated manipulations of N, P, and K in large experimental forest plots in Panama. To determine the net effect of foliar bacteria, I experimentally reduced bacterial *in situ* via the application of standard antibiotics for nearly three years. Specifically, I (Chapter 3) evaluated the degree to which soil nutrients and foliar bacteria impacted seedling growth among co-

occurring woody species. Additionally, I (Chapter 4) evaluated the degree to which soil nutrients and foliar bacteria mediated leaf traits and enemy impacts among species and soil nutrient additions. Finally, I (Chapter 5) conducted a major sequencing effort to determine the degree to which host species, soil nutrients, and commercial antibiotics caused variation in bacterial endophyte community structure. Overall, my results demonstrate that there are frequent interactions between soil nutrient and foliar bacteria on plant performance and enemy impacts, which differ among host species. Further, metagenomic sequencing revealed that host species, soil nutrient additions, and antibiotics caused significant variations in bacterial community composition. For every metric, plant-bacterial interactions are largely dependent on host species and soil resource supply, a classic niche axis for species coexistence. Ultimately, my work provides evidence that foliar bacteria are an entirely independent plant functional trait that can cause critical species-specific performance outcomes, which may have important implications for plant diversity maintenance.

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PREFACE

First, I would like to thank my advisor, Walt Carson, for his constant support, encouragement, and never-ending willingness to provide honest comments and critique. He has become a valued collaborator and friend. I also offer my great thanks to the rest of my committee. I thank Jonathan Pruitt for being a model scientist and offering such great support for my work and work out of the Carson lab. Joint lab meetings between the Pruitt and Carson lab at the University of Pittsburgh became the best stage to propose new ideas and receive tough and always constructive feedback on talks and manuscripts. I also thank Mark Rebeiz and Nate Morehouse, who have been gracious to step in recently as the most recent members of my committee. Further, I offer my gratitude to Betsy Arnold, who many times has been the lone source of support for some of the microbial and sequencing work. She was always willing to take and quickly return frantic emails from Panama. Finally, I'd like to recognize my three emeritus committee members, Rick Relyea, Michael Grabe, and Brian Traw. Brian's collaborative efforts and advisement during my first-year rotation inspired my entire dissertation work.

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1.0 INTRODUCTION

Until recently, microbial communities have been called "the great unseen majority" globally (Whitman *et al.* 1998, van der Heijgen *et al.* 2008, Klotz 2010). Indeed, studies on the human microbiome have estimated that microbial cells outnumber human cells by a factor of 1.3:1, begging interesting debates as to whether we are actually more bacteria than human (reviewed by Sender *et al.* 2016). These microbes are comparably abundant in nature, occurring in densities of up to 10^8 cells/g of soil and 10^7 cells/cm^2 on plant tissues (Horner-Divine *et al.* 2003, Lindow & Brandl 2003, Gans *et al.* 2005, Delmotte *et al.* 2009, Innerebner *et al.* 2011). With the ever-increasing quality and cost-effectiveness of molecular technologies, we are beginning to unlock the mysteries of these highly cryptic yet important organisms. For example, it recently has become clear that microbes (fungi and bacteria) mediate important plant phenotypes across many biomes (Friesen *et al.* 2011, Turner *et al.* 2013). Thus, it may be impossible to understand the mechanisms that drive plant distributions and species coexistence at larger scale without considering microbial communities that associate with plant hosts. For example, mycorrhizal fungi associate with roots of over 80% of plant species globally and are essential for nutrient exchange, drought, and even pathogen resistance (reviewed by Wang & Qiu 2006, Brundrett 2009). More recently, scientists have discovered living bacteria (endobacteria) within fungal cells in roots and are now thought to be major drivers of plant-fungus symbioses (Bianciotto *et al.* 1996, Bonfante & Anca 2009). Thus, bacteria may in fact be driving many of

interactions that have large implications for plant performance and fitness. The overall picture of how bacteria interact with plant hosts, particularly those that associate with above-ground portions of plant hosts, is still limited and fragmentary.

In my dissertation, I first review the literature to date on the distribution and impacts of foliar bacteria on plant hosts in agroecosystem and in nature (Chapter 2). Next, I empirically test a series of hypotheses to quantify the degree to which foliar bacteria mediate seedling growth in a tropical understory across soil nutrient additions and host tree species (Chapter 3). In Chapter 4, I test the degree to which soil nutrient addition, foliar bacteria, and interactions between the two mediate leaf traits and enemy impacts among co-occurring host plant species. Finally, I used high-throughput sequencing techniques to empirically evaluate how variations in soil resource supply and antibiotic applications structure bacterial endophyte communities among tree species. I provide abstracts for the dissertation chapters below.

1.1 CHAPTER 2: ECOLOGY AND NATURAL HISTORY OF FOLIAR BACTERIA

Leaves of higher plants comprise perhaps the largest bacterial substrate on earth, yet we know very little about the bacteria that occupy these spaces. In this review, we first examine the ecology and behavior of bacteria that reside on leaf surfaces. Next, we discuss the ecological implications of foliar bacteria that reside in interior portions of leaf tissues. Later, we consider the studies on foliar bacteria in tropical habitats to date. Finally, we examine evidence regarding the potential roles of foliar bacteria in structuring tropical plant communities. Bacteria colonize the phyllosphere via animal vectors or passively from soil, wind, or rain, though there are too

few data to determine the relative contributions of these sources to the phyllosphere. Additionally, the degree to which parent plants transmit bacteria to offspring via seed remains unknown. We predict that high temperature, high humidity, low UV radiation, and leaf architecture in the tropical understory enable tropical leaves to support more abundant and diverse bacterial communities compared to temperate leaves. While the extent of competitive interactions among bacteria remains poorly resolved, evidence from agricultural crop species and *Arabidopsis thaliana* suggests that these interactions cause niche partitioning based on carbon use. The degree to which phyllobacteria and endophytes of tropical plants are pathogenic versus mutualistic or neutral remains unexplored. We hypothesize, however, that the detrimental impact of bacterial pathogens ultimately increases as the abundance of single host tree species increases, which can promote and maintain plant diversity in tropical forests.

1.2 CHAPTER 3: SOIL NUTRIENTS MEDIATE PLANT-MICROBE INTERACTIONS

The phyllosphere (leaf surface) is one of the world's largest microbial habitats and is host to an abundant and diverse array of bacteria. Nonetheless, the degree to which bacterial communities are benign, harmful, or beneficial to plants *in situ* is unknown. We tested the hypothesis that the net effect of reducing bacterial abundance and diversity would vary substantially among host species (from pathogen to mutualist) and this would be strongly mediated by soil resource supply rates. To test these hypotheses, we monitored tree seedling growth responses to foliar bacteria among replicated resource supply treatments (N, P, K) in a tropical forest in Panama for 29 months. We applied either antibiotics or control water to

replicated seedlings of five common tree species (*Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*). These antibiotic treatments significantly reduced both the abundance and diversity of bacteria epiphytically as well as endophytically. Overall, the impact of bacteria was highly host specific. Applying antibiotics increased growth for three species by as much as 49% (*Alseis*, *Heisteria*, and *Tetragastris*), decreased growth for a fourth species by nearly 20% (*Sorocea*) and had no impact on a fifth species (*Desmopsis*). Perhaps more importantly, the degree to which foliar bacteria were harmful or not varied with soil resource supply. Potassium enrichment significantly mitigated the impact of foliar bacteria on seedling growth rates. Alternatively, phosphorus enrichment caused bacteria to switch from being primarily harmful to beneficial or vice versa, but this depended entirely on the presence or absence of nitrogen enrichment (i.e. important and significant interactions). Our results are the first to demonstrate that the net effect of reducing the abundance and diversity bacteria can have very strong positive and negative impacts on seedling performance. Moreover, these impacts were clearly mediated by resource supply rates. Though speculative, we suggest that foliar bacteria may interact with soil fertility to comprise an important, yet cryptic dimension of niche differentiation.

1.3 CHAPTER 4: IMPACTS OF SOIL NUTRIENTS AND BACTERIA ON LEAF TRAITS AND ENEMIES

1. Two of the biggest determinants of seedling and sapling performance, particularly in tropical forests, are limiting resources and enemies. Though it has been demonstrated that soil nutrients mediate metrics of plant performance even in deep shade, the degree to

which nutrient addition mediates plant-bacterial interactions remains unknown. Here, we test three hypotheses in an effort to evaluate the degree to which soil resource supply (H1), foliar bacteria (H2), and their interactions (H3) impact leaf traits and enemy impacts for seedlings in a tropical forest in Panama.

2. We experimentally reduced bacterial loads among seedlings of five co-occurring woody species for 29 months and measured leaf number and leaf damage rates for seedlings nested within a 15-yr, N, P, K factorial resource supply experiment.
3. Overall, we found that interactions between of N, P, and K caused substantial species-specific increases or decreases for all performance metrics by up to 100%. Perhaps more importantly, interactions between N, P, and K and antibiotic applications caused species-specific increases or decreases for all metrics by up to 204%.
4. Our data suggest that interactions among soil nutrients and foliar bacteria may have the potential to alter the rank-order performance of coexisting plant species in deeply shaded forests.

1.4 CHAPTER 5: ENDOPHYTE COMMUNITY ASSEMBLAGES

Because it is becoming clear that plant-associated microbes are critical determinants of plant health and performance, the ecology of the plant microbiome is a new area of interest. In particular, bacterial endophytes, or those that reside inside plant tissues, may substantially increase plant performance as mutualists or decrease performance as pathogens, the latter necessitating extensive antibiotic usage in agroecosystems to increase crop yields. The drivers of bacterial endophyte community composition, however, particularly for foliar endophytes, are

poorly understood. Here, we test the degree to which soil nutrients, host species, and commercial antibiotics structure bacterial endophyte community composition in a tropical forest in Panama where bacteria are abundant and diverse. We test the following hypotheses: 1) Bacterial endophyte richness, diversity, and community composition vary substantially among coexisting plant species and 2) soil nutrient availability (N, P, K). Further, 3) There are frequent interactions between soil nutrient supplies and endophyte community composition among host plant species. Finally, 4) Antibiotic applications decrease bacterial richness and diversity and cause substantial changes in community composition. To address these hypotheses, we use high-throughput sequencing to quantify bacterial endophyte community composition among seedlings of five co-occurring woody species (*Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*) nested in a long-term experimental manipulation of soil nutrients (N, P, and K). We applied commercial antibiotics to a subset of *Tetragastris* seedlings for almost three years. We demonstrate that endophyte composition and diversity substantially varied among plant species by as much as 46%. Further, combinations of N, P, and K (specifically, N*K, P*K, and N*P*K) and interactions between P addition and host species cause substantial differences in relative abundances of bacterial endophytes by up to 234%. Last, antibiotic applications increased endophyte richness and diversity by up to 100%. Our results ultimately suggest that endophyte communities are an independent plant functional trait with the potential to alter the rank-order performance of coexisting plant species.

2.0 THE ECOLOGY AND NATURAL HISTORY OF FOLIAR BACTERIA WITH A FOCUS ON TROPICAL FORESTS AND AGROECOSYSTEMS

2.1 INTRODUCTION

Sometimes referred to as the 'great unseen,' bacteria are by far the most abundant organisms on Earth ($4\text{-}6*10^{30}$ individuals) and represent the largest organic pool of nitrogen and phosphorus (Whitman *et al.*, 1998). Comprising a global biomass of 350,000-550,000 million tonnes (Whitman *et al.*, 1998), bacteria outweigh invertebrates by orders of magnitude and exceed the biomass of all plants and animals on Earth (Groombridge & Jenkins 2002, Hogan 2010). If, as Wilson (1987) argued, invertebrates are the "little things that run the world," we argue that by their sheer abundance and biomass alone, bacteria have as much if not more of a function in worldwide ecology.

Whereas soil microbial communities and their effects on plants have received extensive attention, (Mills & Bever, 1998; Packer & Clay, 2000; Reynolds *et al.*, 2003; Bever, 2003; Falkowski *et al.*, 2008; Van der Heijden *et al.*, 2008; Mangan *et al.*, 2010; Maron *et al.*, 2011;

Schnitzer *et al.*, 2011; van der Putten *et al.*, 2013), relatively little is known about foliar bacteria and their interactions with plants in nature. Yet bacteria are by far the most abundant colonizers of the leaf surface, occurring at densities of up to 10^7 cells/cm^2 on leaves (Lindow & Brandl, 2003; Delmotte *et al.*, 2009). Moreover, the global leaf surface area (upper and lower leaf surface) may be one of the largest microbial habitats at over 1 billion km^2 (Morris & Kinkel, 2002; Vorholt, 2012), which is two times larger than the earth's surface area (510 million km^2: CIA, 2010).

Though studies from agricultural systems and plantations are more common, the degree to which foliar bacteria benefit or are detrimental to *wild* plants remains little studied, particularly in the tropics (see Gilbert, 2002; Ghazoul and Sheil 2010). The degree to which bacteria-plant interactions are comparable between non-wild and wild systems remains unclear. For example, major differences in the phylogenetic diversity and species composition of plant hosts likely exist between temperate agroecosystems and the understory of tropical forests. In addition, major differences may exist between these systems, including microbial communities, canopy structure, and disturbance regimes. Nevertheless, we use findings in agricultural systems as a means to guide our hypotheses and predictions to inform us about the diversity, abundance, and potential impact of foliar bacteria in tropical forests. That being said, only a handful of studies have examined tropical foliar bacteria in the wild (see Lambais *et al.*, 2006; Furnkranz *et al.*, 2008; Li *et al.*, 2008; Qin *et al.*, 2012; Kembel *et al.*, in press), and even the basic ecology of these organisms remains fertile ground for research. Here, we (1) examine the ecology and behavior of bacteria that reside on the leaf surface; (2) discuss the ecological implications of foliar bacteria that reside in interior portions of leaf tissues; (3) consider studies on foliar bacteria

in tropical habitats to date; and (4) examine evidence regarding the potential roles of foliar bacteria in structuring tropical plant communities.

2.1.1 Definitions

We modify Beattie & Lindow's (1999) definition of "phyllobacteria" and restrict it to those bacteria that live and persist on the leaf surface without being harmful or parasitic. This includes mutualistic and commensal taxa. They are true epiphytes (Kricher, 2011), functionally defined in part by not colonizing the interior of leaf tissues. Thus we distinguish epiphytic phyllobacteria from bacterial endophytes and pathogens: Bacterial endophytes (Table 4) reside inside leaves and are commensal, mutualistic, or pathogenic. Pathogens and endophytes may colonize the leaf surface via horizontal transmission (e.g., passively, by factors such as wind or rain, or via animal vectors), or via vertical transmission (from a parental plant to offspring via seed or by raining down from a mother plant to offspring: Ewald, 1987), but they must reach the leaf's interior before they can cause disease or function as mutualists (Beattie & Lindow, 1995; Gnanamanickam, 2006). Last, we define "core microbiome" as a subset of ecologically important microbial taxa commonly shared among individuals of a single plant species or among multiple plant species living in the same habitat, community, or region (Shade & Handelsman, 2012).

2.2 BACTERIAL COLONIZATION AND RECRUITMENT TO THE PHYLLOSPHERE

2.2.1 From soil to seed to seedling

The origin of the bacteria that colonize the phyllosphere in tropical habitats remains unclear (Bulgarelli *et al.*, 2013), but some may originate from the surface of the seed. The seed surface harbors bacteria, which are transmitted to the emerging cotyledon during germination (Maude, 1996; Nelson, 2004; Gitaitis & Walcott, 2007). Seeds reside in soil, which is a rich habitat for bacteria. For example, Herner-Divine *et al.* (2003) estimated the abundance of bacteria to be 10^8 in a single gram of soil. In another study, 10 grams of soil hosted $8.3 * 10^6$ bacterial species (Gans *et al.*, 2005). Indeed, evidence suggests that seeds "recruit" bacterial populations via seed exudates, some of which may make up the core microbiome that reach the phyllosphere upon germination (Vorholt, 2012). For instance, seeds release a variety of exudates, many of which can either inhibit bacterial pathogens or attract beneficial bacteria to ward off pathogens (reviewed by Nelson, 2004; for seed endophytes that colonize the rhizosphere see Johnston-Monje & Raizada, 2011; Links *et al.*, 2014). Additionally, bacterial pathogens are known to be seedborne (Maude, 1996; Gitaitis & Walcott, 2007). For example, over 60 species of pathogenic bacteria representing 5 genera of (clade/clades) transmit from seed to seedling in the 100 host crop species studied to date, however these studies have been largely concentrated in temperate regions (Neergaard, 1979; Phatak, 1980). Though tropical soils are often acidic and because of this may harbor less abundant and diverse bacterial communities compared to temperate soils (Bath & Anderson, 2003; Fierer & Jackson, 2006; Lauber *et al.*, 2009; Rousk *et al.*, 2010), tropical seeds are still exposed to an enormous abundance of soil bacteria. The few studies

characterizing tropical seed microbiomes focus exclusively on fungal communities and all but ignore bacterial communities (reviewed by Gilbert, 2002; Dalling *et al.,* 2011; but *cf* Gallery *et al.*, 2010; Garcia *et al.*, 2013; Zalamea *et al.*, in review). Empirical studies are needed to determine the degree to which soil bacteria infect seeds and go on to colonize the phyllosphere after germination.

2.2.2 Mechanical chauffeuring

When a young developing shoot emerges from the soil it may contact bacteria mechanically on the surface of the seed or in the soil and incidentally transported onto the phyllosphere. We term this *mechanical chauffeuring*. Free-living bacterial pathogens in temperate soils in agroecosystems may fail to survive for more than a few days (Schaad & White, 1974; Schuster & Coyne, 1974; McCarter *et al.*, 1983; Goodnow *et al.*, 1990; Kocks *et al.*, 1998). Bacterial persistence, though, may be longer in tropical soils, which remain moist and warm throughout much of the year. Indeed, bacterial pathogens of plants can survive on crop residues (e.g., litter, leaf stems) on the forest floor for up to 8 months and can even overwinter (Ark, 1958; Jones *et al.*, 1986; Legard & Hunter, 1990; Maude, 1996; Zhao *et al.*, 2002). For example, Ark (1958) showed that the gram-negative pathogen *Xanthomonas campestris* overwintered in Oklahoma on cotton debris on the soil surface and infected newly emerging plants the following year. Overall, we suggest that mechanical chauffeuring may be a common avenue by which bacteria colonize plant species in the emerging seedling stage when these plants may be particularly vulnerable to pathogens or when they need their bacterial mutualists early in development.

2.2.3 Wind and rain

Wind, rain, and overland flow passively carry bacteria, particularly pathogenic ones, to new plant hosts on small and large scales. Faulwetter (1917) was the first to propose that windborne rain was the primary dispersal agent of angular leaf spot (caused by *X. campestris*) among cotton plants in the southern United States. This has since been confirmed for *X. campestris*, as well as *X. citri and X. axonopodis* in citrus and gram-negative *Erwinia carotova* in potato (reviewed by Fitt *et al.*, 1989; Bock *et al.,* 2005; Bock *et al.*, 2010). Stall *et al.* (1980) showed that wind and rain dispersed the pathogen *X. axonopodis* (causal agent of citrus canker) up to 32 meters from infected grapefruit trees. In fact, wind and rain caused by hurricanes are thought to be primary causes of the recent outbreak of *X. axonopodis* in citrus orchards in Florida (Gottwald *et al.*, 2002; Graham *et al.*, 2004; Irey *et al.*, 2006).

Foliar bacteria may also be dispersed via the water cycle at regional and continental scales. The prominent gram-negative plant pathogen *Pseudomonas syringae* has been found in rain, snow, streams, lakes, and clouds in remote regions throughout Europe, the United States, and New Zealand (Amato *et al.*, 2007; Christner *et al.*, 2008; Morris *et al.*, 2008; Morris *et al.*, 2010). Morris *et al.* (2008) hypothesized that water runoff regionally disseminates *P. syringae* in both agricultural and natural systems and taken up by aerosols that later precipitate bacteria into other non-adjacent ecosystems. Additionally, Williams (2013) proposed that bacteria in tree pollen can de dispersed via the water cycle. If this is true, it's possible that many plant-associated bacteria may disseminate via the global water cycle at scales much greater than for the vast majority of larger biota. This strongly suggests that many taxa of plant-associated bacteria will have nearly worldwide distributions, which could lead to much broader host ranges than for taxa with more limited dispersal.

2.2.4 Animal vectors

Animals, particularly insects, may passively spread bacteria using a variety of mechanisms. We suggest that herbivores and other animals (e.g., birds and reptiles) vector bacteria via defecation, however to our knowledge there have been no studies for larger animal species. We hypothesize that this is particularly prominent in the tropics, where long-distance seed dispersal via defecation functions to promote tropical woody species diversity (Howe & Smallwood, 1982; Nathan & Muller-Landau, 2001; Muller-Landau & Hardesty, 2005). Here, we focus on how insects vector bacteria because this has been the focus of much research. Hemipterans (particularly xylem and phloem tappers) account for more than two thirds of the known examples of insect-vectored bacterial pathogens (Nadarasah & Stavrinides, 2011). Their specialized piercing mouthparts often insert bacteria directly into the plant, allowing them to circumvent defenses or the inhospitable environment on the phyllosphere (e.g., Bruton *et al.*, 2003). Chrysomelid beetles also can directly deposit pathogenic bacteria inside leaves but in some cases the bacteria migrate from frass deposited on the leaf and pass through wounds caused by the herbivores (Yao *et al.*, 1996). In other cases, bacteria may be ingested by their insect vector and later spread via saliva (Stavrinides *et al.*, 2009). Finally, bacteria can ride on the surface of insects (*e.g.*, antennae and legs) and be deposited mechanically as a byproduct of feeding or pollination (Yao *et al.*, 1996; Hildebrand *et al.*, 2000).

Insects vector bacteria that commonly occur on the phyllosphere in the tropics, hence insects may commonly mediate phyllosphere community composition. For example, leaf-cutting ants (*Acromyrmex* and *Atta* spp.) in the Neotropics culture and harbor gram-positive *Streptomyces*

bacteria; these bacteria function to protect their fungal gardens from pathogenic fungi (Currie *et al.*, 1999; Haeder *et al.*, 2009; Schoenian *et al.*, 2011). Furthermore, *Streptomyces* strains frequently produce secondary metabolites, including antibiotics that are widely used in agriculture to kill bacterial pathogens (Schrey & Tarkka, 2008 for roots; Lauber *et al.*, 2009). *Streptomyces* is the predominant genus in the class Actinobacteria, which is the fifth most common class of bacteria on the phyllosphere among 57 tropical tree species in Panama (Kembel *et al.*, in press). We hypothesize that during foraging these leaf cutter ants may disperse *Streptomyces* to the plant surface and even high into the canopy where these bacteria may function to ward off pathogens. This idea is intriguing and should be evaluated empirically. In all, we hypothesize that insect vectors shape foliar bacterial communities on the tropical phyllosphere. Testing this hypothesis would require fairly simple experiments where insects are excluded from plants (e.g., via netting) over relevant time frames and the resulting bacterial communities compared to control plants where insects are present.

2.2.5 Vertical transmission

Phyllosphere bacteria may colonize leaves via vertical transmission, a process by which bacteria are passed from one generation to the next via seed. Bacteria can be transmitted in this manner in three ways: 1. Seeds may be systemically infected via the maternal vascular system 2. Seeds can be indirectly infected from the maternal stigma, where bacteria move through stylar tissues to the embryo. 3. An external infection of maternal flowers or fruits can indirectly infect seeds (Maude, 1996). We also propose that pollen can transmit bacterial pathogens, as pollen is known to contain a variety of antibacterial chemicals that inhibit pathogens *in vitro* (Basim *et al.*, 2006; Carpes *et al.*, 2007; Morais *et al.*, 2011). The extent to which pathogens as well as

mutualists are vertically transmitted and ultimately colonize the seedling phyllosphere is not well understood. Recent studies on grasses and forbes suggest that vertical transmission of fungal mutualists may be common (Cook *et al.*, 2013; Hogsdon *et al.*, 2014), though this area remains contentious and research on woody species is lacking (Rodriguez *et al.*, 2009; Sanchez-Marquez *et al.*, 2012). Among temperate agricultural crops, Schaad (1982) and Vidhyasekaran (2004) collectively listed 20 species of seedborne bacterial pathogens (26 strains). In one of the few tropical examples, Cottyn *et al.* (2001) characterized bacterial communities of crushed seeds from harvested rice from farms in the Philippines. They identified a large proportion of *Pseudomonas* spp. (14 percent), one of the most commonly represented genera on the phyllosphere among temperate crops as well as tropical trees (Vorholt, 2012; Bodenhausen *et al.*, 2013; Kembel *et al.*, in press). More recently, Darrasse *et al.* (2010) demonstrated the transmission of *Xanthomonas* bean flowers from parent to offspring via seed. *Xanthomonas* spp. are particularly inimical to tropical crop species (discussed below). In all, seeds in the tropics may inherit a large portion of their microbiome from the parent plant, and these communities may colonize the phyllosphere after germination. The vertical transmission of bacteria may have huge implications for plant populations as well as community dynamics if pathogenic bacteria are transferred from one generation to the next.

2.2.6 The core microbiome

A recent study demonstrated that a core microbiome (see above) occurs on the phyllosphere among 57 tree species in a tropical forest, which starkly contrasts from a similar study among 56 temperate tree species (Redford *et al.*, 2010; Kembel *et al.*, in press). For adult tropical trees, bacterial OTUs (operational taxonomic units, or the operational definition of

species) representing only 1.4 percent of bacterial diversity were present on over 90 percent of all individuals and made up 73 percent of the total sequences. Simply put, a small subset of bacteria occurred over and over among all species of trees (*sensu* Shade & Handelsman, 2012; Rastogi *et al.*, 2012). This is a surprising result and suggests that a small group of bacteria are either the best at colonizing these tree species, or the best at surviving on the phyllosphere, or a combination of both. Comparatively, Redford *et al.* (2010) failed to identify a core microbiome for bacterial communities on 56 temperate tree species in Colorado using similar techniques. Here, not a single OTU co-occurred on the phyllosphere of all tree species. Regardless, it still remains uncertain the degree to which OTUs in the core microbiome are beneficial or harmful to host plants. Additionally, whether these communities colonize via active recruitment by plant hosts or differential survival of bacteria remains poorly explored. Nevertheless, the finding that a small fraction of bacteria repeatedly co-occur across a large number of tree species in a small area of tropical forest is important and its ecological consequences deserve immediate attention.

A key goal for future research should be to understand the degree to which various sources (e.g., wind vs. insects) contribute to the bacterial phyllosphere, particularly to help us understand plant microbe interactions and how these will likely change. Molecular techniques should be used to determine whether plant hosts actively "recruit" bacterial mutualists to the phyllosphere, as they do for bacterial mutualists in the rhizosphere (reviewed by Mendes *et al.*, 2013). Additionally, understanding the ecology of these taxa is critical, particularly in a changing world where these interactions, as well as the drivers of these interactions, will likely change. Indeed, climate change is projected to significantly alter biogeochemical cycles (Walther *et al.*, 2002; Laurence & Peres, 2006; Lewis *et al.*, 2009) as well as insect herbivores (Bale *et al.*, 2002, Dyer *et al.* 2012) and precipitation (Walther *et al.*, 2002). Future studies that

aim to uncover the contributions of soil, animal vectors, wind and rain to phyllosphere communities will likely enable us to predict the degree to which global climate change will alter these bacterial communities and ultimately their plant hosts.

2.3 FROM THE CANOPY TO THE FOREST FLOOR: TEMPERATURE, HUMIDITY, AND RADIATION STRUCTURE PHYLLOBACTERIAL COMMUNITIES

2.3.1 Temperature and humidity

Temperature and humidity in the tropical understory may be close to optimal for the survival and persistence of a large portion of phyllosphere bacteria. In general, many plant-pathogenic bacteria experience optimal growth at high humidity and at temperatures between 25-30°C *in vivo* (Smirnova *et al.*, 2001). An absence of cold temperatures is also key because winter freezing in temperate zones typically kills more than 99 percent of plant pathogens each year (Burdon *et al.*, 1996). In fact, we predict that because foliar pathogens are more exposed to fluctuations in temperature, most (but not all) will be even more susceptible to freezing. Additionally, high moisture or humidity in tropical habitats likely support more abundant bacterial communities. For example, Monier & Lindow (2004) discovered that populations of the pathogen *Pseudomonas syringae* on bean leaves in agricultural fields decreased by 99% after 8 days under low humidity (<50 percent) whereas this species increased three-fold in 100 percent

relative humidity after 8 days (Monier & Lindow, 2004). Moreover, high humidity may increase the infection rate of foliar bacteria. Leben (1988) found that the infection rate of *P. syringae* on cucumber leaves increased by 48 percent under high humidity (80-100 percent) versus low humidity (30-50 percent). Taken together these results suggest that persistently warm, moist, and humid tropical habitats will allow bacteria to reach densities that are much higher and more persistent than in temperate regions where below freezing temperatures knock back populations each year.

2.3.2 UV radiation

Based on studies in temperate systems, we predict that UV radiation likely stratifies phyllobacterial communities from the canopy to the forest floor in tropical forests. High radiation levels damage bacterial DNA and moreover may restrict phyllobacteria to protected sites on the leaf such at trichome bases, stomatal openings, hydathodes (structures that allow the exudation of water from leaves), and beneath or in openings in the cuticle (Corpe & Rheem, 1989; Pfeifer, 1997; reviewed by Beattie & Lindow, 1999). In addition, the relative abundance of pigmented bacteria on the phyllosphere increases as radiation increases, and these pigments allow bacteria to withstand greater UV exposure by absorbing radiation and quenching oxygen free radicals (Corpe & Rheem, 1989; Sundin & Murillo, 1999; Kim & Sundin, 2000, 2001; Jacobs *et al.*, 2005; Gunasekera & Sundin, 2006). Poplawsky *et al.* (2000) discovered that survival of *X. campestris*, the most destructive pathogen attacking Brassicaceae worldwide, decreases 1,000 fold in the absence of its naturally-produced xanthomonadin pigments. In general, we predict bacterial abundance and diversity in tropical forests to increase from the canopy to the forest floor where UV penetration can diminish to less than 1% (Bjorkman &

Ludlow, 1972; Chazdon & Fletcher, 1984). Moreover, bacteria in high light habitats (canopy, early successional) will likely be restricted to subsets with traits that confer UV tolerance. To our knowledge, the degree to which these adaptations are costly for phyllobacteria remains unexplored.

2.4 TROPICAL LEAF ARCHITECTURE LIKELY SUPPORTS GREATER PHYLLOBACTERIAL COLONIZATION AND ABUNDANCE COMPARED TO TEMPERATE LEAVES

The architecture of leaves in tropical forests likely enhance abundance and diversity of phyllobacteria compared to their temperate counterparts. These traits include longer leaf life span, larger leaf surface area, lower degree of deciduousness, and higher hydathode density (Table 1). Together, these traits promote a larger and more stable substrate for bacteria to colonize or persist on and provide more microsites that afford protection. Moreover, some traits that confer defense from arthropod enemies may make them more vulnerable to bacterial enemies. For instance, 30% of vascular plants have glandular trichomes, or specialized hair tissues with glands that secrete chemicals to ward off herbivores (reviewed by Levin, 1973; Wagner, 1991; Wagner *et al.*, 2004; Tissier, 2012). Glandular as well as non-glandular trichomes (simple hairs) can be constitutive and even induced in response to herbivore attack (Traw & Dawson, 2003; Traw, 2002; Traw & Bergelson, 2003; Shepard *et al.*, 2005; Gonzales *et*

al., 2008). However, bacteria aggregate and are protected around the bases of glandular trichomes (Huang, 1986; Monier & Lindow, 2003, 2005), and in addition may benefit from secondary metabolites produced at the base of these trichomes (Karamanoli *et al.*, 2012; but *cf.* Reisberg *et al.*, 2012). If these bacteria are pathogenic, then a trait that deters herbivores may simultaneously enhance disease. This can potentially be a costly trade-off and suggests that the benefits of producing glandular trichomes must be particularly beneficial for plants if in these trichomes harbor and protect bacterial enemies.

Overall, we predict that colonization and abundance of bacteria on leaves in the tropics will be greater than in temperate zones. Out of 15 leaf traits, 11 show a sharp contrast between temperate and tropical forests (Table 1). Out of these 11 traits, seven will likely favor higher bacterial colonization, abundance, and survivorship among tropical leaves while 4 traits will favor an increase in these metrics among temperate leaves. Although simplified, we feel that these traits may be used to predict which systems, as well as which species and growth forms, will likely harbor more abundant and diverse communities of foliar bacteria. In fact, we argue that phyllobacterial communities should be classified as an independent leaf functional trait, which would classify phyllobacteria as a major axis of plant ecological strategy variation (see also Kembel *et al.*, in press).

2.5 INTERACTIONS AMONG BACTERIA ON THE LEAF SURFACE

2.5.1 Competition and niche partitioning

Carbon sources required for microbial growth are often limited on the leaf surface (Bashi & Fokkema, 1977; Fokkema *et al.*, 1979; Dik & Vampelt, 1992; Wilson & Lindow, 1994a; Mercier & Lindow, 2000). Studies to date demonstrate that phyllobacterial populations increase as carbon resources increase and epiphytic bacteria rapidly consume these resources. For example, Mercier and Lindow (2000) quantified the total amount of mono- and polysaccharide sugars on leaves of 6 different temperate crop species. Next, they inoculated the leaf surface of all species with the bacterial epiphyte *Pseudomonas fluorescens*. Bacteria reached higher densities on plant species with higher sugar content. Moreover, in less than 24 hours *P. fluorescens* depleted sugar levels on bean leaves by as much as 80 percent (Mercier & Lindow, 2000). Experiments have also confirmed that interspecific competition among bacterial species occurred for sugars suggesting these bacteria occupied similar resource niches (Ji & Wilson, 2002; Innerebner *et al.*, 2011). In laboratory experiments, Innerebner *et al.* (2011) demonstrated that gram-negative *Sphingomonas* spp. decreased the population size of *P. syringae*, a pathogen with a similar carbon use profile, by up to 340-fold on the phyllosphere of *Arabidopsis*. Thus, we predict similar competitive interactions among phyllobacterial species in the wild but this remains speculative, especially the degree to which there is niche specialization or other processes that mediate or promote coexistence (see below).

Some epiphytic bacteria appear to specialize on distinct carbon sources (e.g., amino acids, organic acids, and carbohydrates) thereby allowing some degree of niche partitioning. In one of the only studies of its kind, Wilson & Lindow (1994a) directly evaluated the relationship

between species coexistence of epiphytic bacteria and their degree of ecological niche overlap. They inoculated potato leaves with five different bacterial species representing four different genera, and found that coexistence was promoted and competitive interactions mitigated when overlap in resource use was the least. Wilson & Lindow (1994a) suggested bacteria could be placed within contrasting guilds (*sensu* Root, 1967) based solely on whether the bacteria specialized on amino acids versus organic acids versus carbohydrates. The variability of these compounds on the phyllosphere is likely to vary widely among plant species suggesting a critical basis for host specialization or affinity as well as coexistence.

Niche differentiation via habitat specialization likely occurs on the phyllosphere based upon fine scale leaf-surface heterogeneity, which is akin to the pit and mound topography that occurs on the forest floor (e.g., Putz, 1983; Peterson *et al.*, 1990). Leaf surface landscapes are complex because of the presence of stomates, trichomes, and veins, as well as wide spatial variation in waxy cuticle layers and epiphyllous lichens in tropical systems (Mechaber *et al.*, 1996; Lucking, 2001; Mechaber *et al.,* 2002; Vorholt, 2012). In fact, Andrews (1992) noted that the distance to the top of an 800μm trichome for a bacterium on the cuticle is four times greater than the distance from a person on the sidewalk to the top of the Sears Tower in Chicago. The extent to which phyllobacteria specialize on contrasting microhabitats isn't clear, but evidence to date suggests that pigmented bacterial species are UV-tolerant may occur more readily across the leaf surface while other bacteria require "nooks and crannies" that shield them from harsh environmental conditions (see reviews by Beattie & Lindow, 1999; Lindow & Brandl, 2003; reviewed by Andrews & Harris, 2000). Mechaber *et al.* (1996) used atomic force microscopy to document the upper leaf landscape of cranberry. They found that young leaves contained a more homogeneous regular pattern of broad expanses or plateaus while older leaves were less regular

where heights changed more rapidly over short distances. Thus, we predict that the more irregular and sharper topographical contrasts that are occur on older leaves will enhance bacterial diversity and coexistence on the phyllosphere (Ricklefs, 1977; Comins & Noble, 1985; Tilman, 1994). This also might suggest the existence of repeatable patterns of bacterial succession as leaves age (see Redford & Fierer, 2009).

2.5.2 Bacterial aggregation

Highly dense patches of bacteria enable individuals to communicate and even exchange genetic material with each other, which may explain how and when bacteria become pathogenic. The crowding of bacteria allows for quorum sensing (Table 5), a process by which individuals communicate intraspecifically so that certain traits are expressed when bacterial density reaches a minimum threshold (reviewed by von Bodman *et al.*, 2003). Pathogens use quorum sensing to coordinate certain behaviors such as biofilm formation and exopolysaccharide production to enhance survival (Table 2). Additionally, quorum sensing allows pathogens to mount attacks together against plant hosts by triggering certain bacterial behaviors such as the production of chemicals that may be used to breach plant cell walls (von Bodman *et al.*, 2003). Conversely, bacterial mutualists enhance plant performance by using quorum sensing to produce plant hormones and inducing plant resistance to pathogens (discussed below; reviewed by Hartmann *et al.*, 2014). Perhaps more importantly, aggregating bacterial cells may spur the transfer of virulence or symbiosis-related factors among each other via horizontal gene transfer (the swapping of genetic material among neighboring bacteria; Bailey *et al.*, 1996; van Elsas *et al.*, 2003; Sorensen *et al.*, 2005). Indeed, plasmid gene transfer among *P. putida* strains on bean leaves occurred at frequencies as high as 33 percent in one experiment and as high as 40 percent

among *P. syringae* cells in another (Normander *et al.*, 1998; Bjorklof *et al.*, 2000). In all, aggregates of phyllosphere bacteria particularly among protected microsites (e.g., trichome bases, stomates, hydathodes) will likely enhance bacterial survival and even increase the pathogenicity among different species.

2.6 BACTERIAL-FUNGAL INTERACTIONS ON THE PHYLLOSPHERE

Bacteria can dramatically reduce fungal pathogen disease severity on the phyllosphere (see Table 3), and this may be particularly important in controlling fungal pathogens in tropical agroecosystems. For example, in Colombia, the fungal pathogen black Sigatoka (*Mycosphaerella fijiensis*) causes leaf spot disease in banana plantations that reduce banana yields by nearly 40 percent over vast regions pantropically (Marin *et al.*, 2003). Ceballos *et al.* (2012) recently discovered that two widespread bacterial epiphytes, gram-positive *Bacillus subtilis* and *B. amyloliquefaciens*, isolated from banana leaves in Colombia caused dramatic reductions (>90 percent) of black sigatoka (Ceballos *et al.*, 2012) by interfering with fungal hyphae formation and inhibiting the germination of ascospores. The ability of these bacteria to form microbial biofilms appeared necessary for these bacteria to suppress the fungus. These results, though narrow and simplified in scope, suggest that bacteria may commonly mitigate or mediate fungal pathogens in natural systems. This area is ripe for additional research.

There are a few documented cases where phyllobacteria induce systemic host resistance to the entire plant from attack by fungal pathogens and other enemies (Bargabus *et al.*, 2002, 2004; Tran *et al.*, 2007; Verhagen *et al.*, 2010; Brotman *et al.*, 2012; Desoignies *et al.*, 2013). This is similar to when exposure to a fungal pathogen triggers induced systemic host resistance;

however, here the bacteria act as an early warning system and "alert" their host plants to the presence of pathogenic fungi (van Loon *et al.*, 1998; Pieterse *et al.*, 1998). This mutualism appears common for rhizosphere bacteria and confers plant resistance to soil pathogens, nematodes, and insects (Van Loon *et al.*, 1998; Van Loon *et al.*, 2006a; van Wees *et al.*, 2008; Pineda *et al.*, 2010). Unfortunately the degree to which phyllosphere bacteria are mutualists and suppress disease *in situ* is poorly understood. However, when they do, Jacobsen (2006) argued that these bacteria would benefit plant hosts most likely by triggering systemic resistance. In greenhouse and field experiments on sugar beet leaves, Bargabus *et al.* (2002, 2004) demonstrated that nonpathogenic bacteria (*P. fluorescens*, *Bacillus mycoides* and *B. pumilus*, respectively) produced compounds to induce resistance to pathogenic fungi (*Heterodera schachtii* and *Cercospora beticola*, respectively), thereby reducing fungal abundance by up to 90 percent. Similarly, Tran *et al.* (2007) and Desoignies *et al.* (2013) found that non-pathogenic bacteria could also significantly suppress fungal pathogens in the laboratory on both tomato and beet leaves. Further, *Pseudomonas* spp., one of the most abundant genera in phyllosphere communities among agricultural crops as well as tropical trees, commonly induce systemic resistance to fungal infection (reviewed by Jankiewicz & Koltonowicz 2012; Vorholt 2012, Kembel *et al.* in press). These findings are important because fungal pathogens are major agents of mortality for numerous species of tropical tree seedlings, particularly those in shaded understories (Augspurger, 1984; Augspurger & Kelly, 1984; Wenny, 2000). Though induced systemic host resistance may be common in the wild, to our knowledge the degree to which phyllobacterial mutualists induce enemy resistance for plant hosts has never been evaluated outside of an agricultural context.

2.7 BACTERIA IN THE INTERIOR PORTIONS OF LEAVES

2.7.1 Gaining access to the interior of the leaf

Phyllobacteria use multiple pathways to gain access into the leaf interior where they can then act as mutualists or pathogens. Phyllobacteria enter leaves at leaf openings such as trichome bases, stomata, or hydathodes (reviewed by Beattie & Lindow, 1995), or wounds created by insects (Agrios, 2005). Additionally, some insects vector bacteria that passively disseminate bacteria onto or into preferred plant hosts (see above). Pathogenic bacteria also gain access to leaf interiors with extracellular virulence factors (Table 6). For example, *P. syringae* produces coronatine, a jasmonic acid mimic that suppresses the tomato defense to pathogens and induces stomatal opening to help gain access to the apoplast (Zhao *et al.*, 2003; Melotto *et al.*, 2006; Melotto *et al.*, 2008). More recently, Schellenberg *et al.* (2010) discovered that *P. syringae* produces syringolin to open stomates on bean and *Arabidopsis* leaves. Once in the apoplast, bacteria typically have much higher growth rates (reviewed by Beattie & Lindow, 1999) where they can act as mutualistic endophytes or become pathogenic. Yu *et al.* (2013) recently found that once inside the apoplast of bean leaves, the pathogen *P. syringae* alters its gene expression from genes that code for exploration to those that produce enzymes and phytotoxins. This suggests that after entry pathogens adapt quickly and can immediately switch their resource allocation to evading the plant immune system (see Table 6).

2.7.2 Bacterial endophytes significantly promote plant growth: lessons from root endophytes

Much of what we know about the ecology of bacterial endophytes comes from root-associated bacteria (see reviews by Anand *et al.*, 2006; Hardoim *et al.*, 2008; Berg, 2009; Compant *et al.*, 2010). Bacterial endophytes in roots protect plant hosts from pathogens and pests. Like epiphytes, endophytes colonize an ecological niche similar to phytopathogens and may simply compete for similar niches or carbon resources thereby reducing the abundance of bacterial pathogens (Hallmann *et al.*, 1997). For example, up to 35 percent of root-associated bacteria inhibit pathogen growth *in vitro* (e.g., Berg *et al.*, 2002, 2005; Berg & Hallmann, 2006). Additionally, root bacterial endophytes may induce plant host systemic resistance to pathogens (discussed above), which can significantly decrease the severity of bacterial or fungal pathogens. Further, root endophytes produce or alter plant hormonal levels to enhance plant growth (see reviews by Rosenblueth & Martinez-Romero, 2006; Kloepper & Ryu, 2006; Hardoim *et al.*, 2008). For example, root endophytes produce the plant growth regulator auxin**,** which controls root and meristem cell elongation and aid in regenerating wounded tissues (Davis 1995, Schmelz *et al.*, 2003; Spaepen *et al.*, 2007; but *cf* Silverstone *et al.*, 1993; Brandl & Lindow, 1998). In fact, more than 80 percent of bacteria in the rhizosphere produce auxins, however the prevalence of this among foliar endophytes remains unexplored (Ramos-Solano *et al.*, 2008; but *cf* Hoffman *et al.*, 2013 for endohyphal bacteria). Additionally, root endophytes produce gibberellin (Gutierrez-Manero *et al.*, 2001) and cytokinins (Bhore *et al.*, 2010), which control diverse aspects of plant growth and development including root and stem elongation, leaf expansion and senescence (reviewed by Santer *et al.*, 2009). Root endophytes also break down the plant hormone ethylene to alleviate its adverse effects on plant growth (Glick, 2005; Saleem *et al.*,

2007). Though we recognize that conditions in the rhizosphere and phyllosphere are different in many ways (e.g., different stressors, O_2 levels, moisture regimes, etc.) and make direct comparisons somewhat tricky, we ultimately predict that foliar endophytes in the wild likely use similar mechanisms to enhance plant performance.

Additionally, bacterial endophytes isolated from all plant tissues including leaves inhibit pathogens by synthesizing antifungal compounds (reviewed by Strobel *et al.*, 2004; Brader *et al.*, 2014). For example, pseudomonads comprise one of the most common and ubiquitous genera of bacterial endophytes and produce a group of antifungal peptides called pseudomycins (Strobel *et al.*, 2004; Berdy, 2005). These peptides decrease the fungal pathogens that cause Dutch elm disease (*Ceratocystis ulmi*) and banana's black Sigatoka *in vitro* (*Mycosphaerella fijiensis*; Harrison *et al.*, 1991; Ballio *et al.*, 1994). While all of the above work was done *in vitro*, we predict that foliar endophytes in the wild may synthesize antifungal properties to ward off pathogens in leaves and suggest that this should be a research priority in the future.

2.8 FOLIAR PATHOGENS: WHO THEY ARE, HOW THEY OVERCOME PLANT DEFENSES, AND THEIR DELETERIOUS POTENTIAL TO TROPICAL PLANTS

2.8.1 The main players

Over 100 species of foliar bacteria are pathogenic and once in the apoplast cause hundreds of diseases to crops worldwide, though none are more "scientifically and economically important" than *Pseudomonas syringae* (Jackson, 2009; Mansfield *et al.*, 2012). Pathogens may

either be necrotrophs that first destroy host cells and later feed on the contents or biotrophs that derive nutrients from host cells without disrupting them (Glazebrook 2005). Many bacterial pathogens, including *P. syringae*, display both lifestyles (Glazebrook 2005). *P. syringae* is by far the most extensively studied and possibly the most ubiquitous foliar pathogen in the world (Morris *et al.*, 2013). At least 57 pathovars (strains or set of strains) of *P. syringae* exist, which are often highly specialized to particular host species (Bull *et al.,* 2010; Hirano & Upper, 2000; Lindeberg *et al.*, 2012). Further *P. syringae* strains inhabit a variety of environments and interact with a wide range of plants in most regions of the world (Silby *et al.*, 2011). *P. syringae* causes disease in the families Sapinadaceae, Amaranthaceae, Meliaceae, Rosaceae, Fabaceae, and Actinidaceae (Horst, 1990; Sarkar & Guttman, 2004). All of these families are well represented in the tropics, particularly Fabaceae (the third largest angiosperm family), whose woody species are mostly confined to tropical and subtropical habitats (Rundel 1989).

Xanthomonas is a prominent and largely pathogenic bacterial genus that commonly plagues tropical crop systems. *Xanthomonas* comprises almost 30 species, which typically have mixed biotrophic-necrotrophic lifestyles and collectively cause disease in nearly 400 plant species (van Loon *et al.*, 2006b; Ryan *et al.*, 2011). Three *Xanthomonas* species (*X. oryzae*, *X. campestris*, and *X. axonopodis*) afflict pantropical hosts and are among the top ten most "scientifically and economically important" plant pathogenic bacteria in the world (Mansfield *et al.*, 2012). Eighteen sequenced *Xanthomonas* genomes have been described, 12 of which cause disease among tropical crop species, including sugarcane, banana, cassava, citrus crops, and rice (Ryan *et al.*, 2011). These species cause major crop losses. For example, in just three years *X. campetris* caused a decrease in Ugandan banana and plantain yields by 30-52 percent (Karamura *et al.*, 2006). This pathogen is expected to spread throughout East and Central Africa at a rate of

8 percent per year in banana populations and reduce yield by over 50 percent by 2015 (Kayobyo *et al.,* 2005; Abele & Pillay, 2007). In a 50 ha tropical forest in Panama, Kembel *et al.* (in press) found that Xanthomonadaceae is one of the most common families on the phyllosphere and xanthomonads alone made up almost 9 percent of the core microbiome. If these bacteria are pathogenic and tree host species are differentially vulnerable, then these pathogens may be major agents of forest turnover particularly in the small size classes and in areas around parent trees.

2.8.2 How they overcome plant defenses

After entering the leaf, pathogens attempt to suppress the complex plant immune system. Pathogens inject effector proteins into plant cells, which help in evading the plant's first line of defense (Table 6) and aid in nutrient acquisition and dispersal (reviewed by Dodds & Rathjen, 2010). During the second line of defense (Table 6), plants use the jasmonic acid (JA) and salicylic acid (SA) hormone pathways to activate defenses to necrotrophs and biotrophs, respectively (Glazebrook, 2005). These two pathways are mutually inhibitory, and certain bacterial pathogens exploit this negative cross-talk to evade detection (Traw *et al.*, 2004; Cipollini *et al.,* 2004; reviewed by Thaler *et al.*, 2012). For example, *P. syringae* produces jasmonic acid mimics to suppress the salicylic-acid-mediated defense in plant hosts (Zheng *et al.*, 2012). These interactions frequently culminate in the induction of a hypersensitive response (HR) from plant hosts, which involves deliberate cell suicide localized at the infection site to limit pathogen spread (reviewed by Lam *et al.*, 2001; Coll *et al.*, 2011). Successful pathogens develop effectors to evade the last line of defense and invade host cells to cause disease.

2.8.3 Bacterial pathogens in tropical systems

Pathogens are particularly detrimental to tropical crops where disease losses may be 50 to 100 percent higher than in temperate systems, though it remains uncertain the degree to which bacterial pathogens are inimical in hyper-diverse tropical forests (Hill & Waller, 1982; Thurston, 1998). The tropics are ideal for bacterial survival and persistence (see above), thus bacterial pathogens are likely to be more abundant and damaging. In fact, Wellman (1968, 1972) documented the known diseases (fungal and bacterial) among crops with ranges in both temperate and tropical zones. He concluded that for every disease that infected a given crop in temperate areas, there were 10 in the tropics (see also Gilbert, 2005). Clearly, a new focus on tropical bacteria is needed because papers studying pathogens in temperate systems have outnumbered tropical studies by over 25:1 (Lodge *et al.*, 1996).

2.9 FOLIAR BACTERIA IN THE TROPICS

2.9.1 Phyllobacteria in tropical habitats

Phyllobacteria in the tropics are diverse, significantly contribute to forest nutrient cycling, and are likely fairly host-specific (Abril *et al.,* 2005; Furnkranz *et al.*, 2008; Kembel *et al.*, in press). To our knowledge, Lambais *et al.* (2006) were the first to use culture-independent methods to identify phyllosphere bacteria of tree species in the tropics. They identified up to 671 bacterial OTUs on each of 9 phylogenetically diverse canopy tree species, and estimated that the

phyllosphere of Brazilian Atlantic forest alone harbors as many as 10 million bacterial OTUs. Clone libraries generated for three of these tree species (*Trichilia catigua*, *T. clausenni*, and *Campomanesia xanthocarpa*) suggested that some of these phyllobacterial taxa may be to some degree host-specific. For example, Proteobacteria were twice as common on the phyllosphere of *Trichilia* spp. versus *C. xanthocarpa*. Further, cyanobacteria made up almost 15 percent of the total sequences on *C. xanthocarpa*, however there was not a single cyanobacterial sequence found on either *Trichilia* species. Furnkranz *et al.* (2008) quantified nitrogen-fixation among phyllosphere bacteria of 13 herb, shrub, and tree species in a lowland Costa Rican forest. The bacteria associated with three of these plant species (*G. cauliflora*, *P. wendlandii*, and *C. drudei*) fixed up to 6 μmol of N_2 per m^2 per day, enough to provide significant nitrogen input to the forest. Although the bacterial communities did not differ on the two high nitrogen fixing plant species (*G. cauliflora* and *C. drudei*) versus the low one (*C. laevis*), nitrogen fixation was highly variable among the 13 plant species sampled. Though preliminary, these data suggest that nitrogen fixation will be quite patchy among tropical plants because they occur on host species that may be wildly scattered across the landscape and similar bacterial communities may fix very different amounts of nitrogen depending on the host species.

Evidence from two recent studies suggests that host phylogenetic relationships are critical for structuring bacterial communities on the phyllosphere of tropical trees. Kim *et al.* (2012) characterized bacterial communities on leaves of six tree species in an arboretum in Malaysia. The relative abundance of bacterial taxa differed significantly among tree species. These differences were particularly prominent for Gammaproteobacteria, which is the second most common bacterial phylum among tree species in Panama (Kembel *et al.*, in press). More recently, Kembel *et al.* (in press) quantified bacterial communities on the phyllosphere of 57

mid-canopy tree species in a moist tropical forest. Bacterial taxa exhibited high host affinity, with plant host taxonomy explaining 47 percent of the variation of bacterial communities. A suite of plant host traits dealing primarily with growth and mortality rates, nutrient concentrations, and leaf characteristics was also important in explaining variation (26%) among bacterial communities. Overall, these findings suggest that bacterial communities on the phyllosphere of tropical trees are associated with particular plant hosts and are structured by key plant traits.

2.9.2 Bacterial endophytes in tropical habitats

Though a handful of studies have characterized foliar bacterial endophytes among tropical trees, few generalizations are possible about these bacterial communities or their ecology. Bacterial endophytes have been studied primarily among crop species and have only been characterized in very few gymnosperm and angiosperm species (see reviews from Hallmann *et al.*, 1997; Hardoim *et al.*, 2008; Berg, 2009; Compant *et al.*, 2010; Izumi, 2011; Carrell & Frank, 2014). Coffee, cacao, *Citrus*, and *Eucalyptus*, mangroves are the only tropical trees for which foliar endophytes have been characterized (Araujo *et al.*, 2001; Vega *et al.*, 2005; Shiomi *et al.*, 2006; Hu *et al.*, 2010; Melnick *et al.*, 2011; Paz *et al.*, 2012). The endophytic strains isolated were predominantly *Bacillus* spp. in all studies, and the authors suggested these strains could be used as biocontrol agents against fungal as well as bacterial diseases. In a recent study using plant stems, Bascom-Slack *et al.* (2009) isolated 14 endophytic actinomycete bacterial species from 12 shrub and tree species from 10 plant families in a Peruvian rainforest. Because these studies relied solely on culture dependent methods that fail to detect as much as

99% of resident bacteria (reviewed by Muller & Reppel, 2014), it is difficult to draw conclusions about bacterial endophytic communities in tropical systems. With the exception of these few studies, the identity and ecology of foliar endophyte communities among tree hosts remain poorly explored globally. Bacterial endophytes have not been characterized from a single tree among some of the most common plant genera in the world, including *Abies*, *Acacia*, *Alnus*, *Carpinus*, *Fagus*, *Fraxinus*, and *Shorea*, many of whose species have tropical distributions (Izumi 2011). This should be a research priority because Strobel (2012) argued that every plant on earth hosts both bacterial and fungal endophytes. Without knowing which bacteria species are present and in what abundance, it is impossible to understand anything about their impact on their hosts let alone anything about their function in the ecosystem.

A subset of gram-negative *Burkholderia* spp. reside in leaf galls of tropical angiosperms and act as lifelong obligate endosymbionts to plant hosts (reviewed by Compant *et al.*, 2008). Unlike endophytes that colonize internal leaf tissue between mesophyll cells (Stone *et al.*, 2000), *Burkholderia* colonize intracellularly and are surrounded by a host membrane (Reinhold-Hurek & Hurek, 2011). This association occurs in about 500 species in the families Primulaceae and Rubiaceae, particularly in the genera *Pavetta*, *Sericanthe*, and *Psychotria* of the Rubiaceae family (Miller, 1990). Despite the predominantly pantropical distribution of both families, leaf nodulated plants are restricted to tropical parts of Asia and Africa (Miller, 1990). Because *Psychotria* plants grown without *Burkholderia* resulted in distorted leaves, stunted growth, and eventual death of plant hosts (Gordon, 1963), van Oevelen *et al.* (2003) suggested that these two organisms have an obligate association with one another. Recently, Carlier and Eberl (2012) sequenced the genome of *B. kirkii* and discovered a collection of genes responsible for secondary metabolite synthesis on the *B. kirkii* plasmid. They hypothesized that these bacteria produce

compounds to ward off pathogens and herbivores, however future studies are needed to test this hypothesis.

2.10 WHAT ARE THE IMPACTS OF BACTERIAL PATHOGENS FOR PLANT COMMUNITIES IN TROPICAL HABITATS: TWO PERSPECTIVES

One on hand, the detrimental impact of foliar bacterial pathogens may increase as the abundance of single host tree species increases, which can promote and maintain plant diversity in the tropics (Gillett, 1962; Janzen, 1970; Connell, 1971). Frequency-dependent reduction of conspecifics is the cornerstone for the Janzen-Connell hypothesis, which hypothesizes that density-dependent enemies regulate plant populations (Janzen, 1970; Connell, 1971). This phenomenon occurs via specialist pests, who cause a reduction in the competitive ability of key plant species and make room for other plant species (Janzen, 1970; Connell, 1971). Frequency-dependent tree mortality has been observed numerous times in the tropics (most recently by Bagchi *et al.*, 2014; see reviews by Carson *et al.*, 2008; Mordecai, 2011; Comita *et al.*, 2014), though not a single study determined whether this pattern exists for foliar bacteria. Griffin *et al.* (unpublished data) recently demonstrated that seedlings of 5 tree species grew more after reducing their foliar bacteria for three years in a Panamanian rain forest, suggesting that these bacteria are primarily pathogenic. In addition, studies to date indicate that prominent foliar pathogens in agroecosystems are relatively host specific (e.g., Leyns *et al.*, 1984; Ryan *et al.*, 2011; Lindeberg *et al.*, 2012; see above). Moreover, wind, rain and insects all spread pathogens to new hosts (Butterworth & McCartney, 1991; Pruvost *et al.*, 2002; Bock *et al.*, 2005;

Nadarasah & Stavrinides, 2011), suggesting that conspecific aggregations of tree species will facilitate bacterial colonization among nearby conspecifics. If this is true, the implications for the maintenance of species diversity in tropical forests are clear: enemies will build up around conspecifics and reduce their performance and dominance.

Conversely, bacterial pathogens may not be host specific "enough" and therefore not act to maintain plant community diversity in tropical forests. It's possible that the degree to which foliar pathogens specialize to plant hosts in the tropics is less than in temperate systems. Kembel *et al.* (in press) found that a large portion of bacterial communities co-occur among 57 tree species in a tropical forest, though they did not determine whether or not these bacteria were pathogens. If these bacteria are generalists, they may simply spill over to other plant species in close proximity (Dobson 2004, Power & Mitchell 2004). Second, foliar pathogens in the tropics may be widespread and cause disease for plants everywhere. For example, Morris *et al.* (2008) hypothesized that the dispersal of the bacterial pathogen *P. syringae* is widespread, which increases the pathogen's exposure to reservoir plant species and even other susceptible plant species (Brown & Hovmoller, 2002; Keesing *et al.*, 2006; Morris *et al.*, 2008). Additionally, highly competitive or common species may be tolerant of a bacterial pathogen, thus causing the pathogen to spillover and harm rarer species (Dobson, 2004; Power & Mitchell, 2004). Pathogen spillover may cause positive feedbacks and lead to single-species dominance as the exposure to susceptible plant species increases as tolerant species become more abundant in a community (reviewed by Mordecai, 2011). Although more studies are needed, it's possible that bacterial pathogens are generalists and thus are less likely to maintain high plant diversity.

It is clear that we cannot resolve this question at this time because no studies have measured the ability of foliar pathogens to enhance plant diversity. Studies to date suggest that

fungal endophytes (Arnold *et al.*, 2003) and insect herbivores (Dyer *et al.*, 2007) are host specific in tropical forests, though this topic remains contentious (Cannon & Simmons, 2002; Suryanarayanan *et al.*, 2002; Novotny *et al.*, 2002; Novotny & Basset, 2005; Novotny *et al.*, 2006). Though the jury is still out, particularly for foliar bacteria, we predict that bacterial pathogens will turn out to be host specific enough and thus play an important role in the maintenance of hyper-diversity in tropical forests.

2.11 CONCLUSIONS AND FUTURE DIRECTIONS

Our goal was to review the "the great unseen majority" of the plant phyllosphere, and it is clear that many unanswered questions remain. Though we make predictions as to the origins of foliar communities, much work needs to be done to fully understand how contributors disperse bacteria to the phyllosphere. Additionally, the degree to which bacterial communities and their impacts on plant hosts vary among environmental gradients, as well as whether these interactions drive plant biogeographic patterns, should be studied. Further, many questions remain about the crosstalk between bacteria and plant hosts (e.g., among bacterial mutualists and hosts to confer reciprocal fitness benefits). Moreover, we know very little about the evolutionary dynamics and origin of plant-bacterial symbioses, particularly among leaf-associated bacteria. That being said, Meyer & Leveau (2012) argue that the phyllosphere is an ideal system to test important concepts of ecological theory (e.g., top-down versus bottom-up regulation, island biogeography, species-area relationships, and landscape ecology), particularly in the tropics where leaf areas are larger and conditions are ideal for bacteria.

The recent development of new molecular technologies will lead to novel phylogenetic and functional insight of foliar bacterial communities. Researchers recently published the first phyllospheric metagenomes, -proteomes, and –transcriptomes for crop and model plant species under agricultural and natural conditions (Delmotte *et al.*, 2009: soybean, clover, and *Arabidopsis*; Knief *et al.*, 2012: *Arabidopsis* and *Medicago truncatula*). For example, Delmotte *et al.* (2009) found that on average over 30 percent of proteins identified on the phyllosphere on soybean, clover, and *Arabidopsis* had never been previously described. Additionally, we can begin to gain new insights on tritrophic interactions among plants, bacteria, and fungi. The "omics" approaches enable us to explore species interactions, communication, development, and diversity, and even reveal the contribution of each partner to these interactions. This will be critical for our understanding of community ecology on the phyllosphere, where plants, bacterial, and fungal communities interact (*sensu* Bonfante & Anca, 2009 for tritrophic interactions in the rhizosphere). Additionally, future studies should include systematic assessments of bacterial and fungal community members that simultaneously sample the rhizosphere, root endosphere, and the phyllosphere (inside leaf and the leaf surface) on the same plant host. Ultimately, the increasing pace and cost effectiveness of molecular technology development will lead to system-level and even global understanding of the composition, physiology, and ecology of bacterial communities on the phyllosphere.

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2.14 TABLES

Table 1. Leaf traits among temperate and tropical systems

A comparison of leaf traits between temperate and tropical systems and their predicted effects on bacterial communities. We (1) list leaf traits that increase habitat suitability for foliar bacteria; (2) compare the prevalence of these traits between temperate and tropical systems; and (3) predict how these traits structure foliar bacteria. Here we define colonization as arrival per unit time; attachment as ability to stick to the leaf surface; and entry as the ability to gain access to internal leaf tissue. Though not a plant trait, we include damage in the table because it provides conduits for bacteria to enter leaf tissue and insects can vector bacteria among individuals.

Leaf Trait that improves suitability for bacteria	***Temperate habitats***	***Tropical Habitats***	***Effect on foliar bacteria***
Leaf lifespan	lower	higher[1, 2]	Increases time for colonization, decrease diversity [3,4,5,6, 42] but *cf.* [7, 46, 47] Cuticle erodes, greater wettability[43]
Lower deciduousness (wet forests)	lower	higher[2, 8]	Increases time for colonization, decrease diversity [3,4,5,6, 42] but *cf.* [7]
Higher degree of leaf venation	lower	higher[9]	Increases bacterial entry and movement [10]
Higher hydathode density	lower	higher[11]	Increases entry [12]
Higher SLA (evergreen wet forests)	lower	higher[13 but *cf.* 14]	Increases colonization [15]
Higher insect damage	lower	higher[1, 2]	Increases colonization and entry [16, 17]
Higher leaf wettability	lower	higher[18]	Increases entry [19, 20]
Lower toughness (fiber)	higher[1, 2, 21]	lower	Increases bacterial attachment and entry, increases intercellular movement [20, 22, 23, 24]
Lower chemical defenses	higher[1, 2, 25, 26]	lower	Increases abundance [27]
Higher trichome density	unknown	unknown	Increases colonization, attachment, and entry [29, 30, 31] ; increases spatial heterogeneity and enhances microbial diversity[39]

Lower drip tip prevalence	lower	higher[32, 35]	Increases attachment and splash dispersal[32, 33, 34, 35]
Lower degree of cuticular waxes	lower	higher in young leaves and abaxial surface[28]	Increases attachment and entry and nutrient acquisition [36, 43]
Higher stomatal density	No difference	No difference; however, lower stomatal density in understory than in canopy in tropics [40 cited in 37]	Increases entry [12] but *cf.* [38, 41]
Higher surface heterogeneity	unknown	unknown [1, 2]	Increases species coexistence [44, 45]
Higher degree of dissection	unknown	unknown	Decreases attachment and colonization [15]

Sources by corresponding numbers: (1) Coley & Aide 1991; (2) Coley & Barone 1996; (3) Ercolani 1991; (4) Redford & Fierer 2009; (5) Friesen *et al.* 2011; (6) Yadav *et al.* 2011; (7) Jackson & Denney 2011; (8) Aerts 1995; (9) Roth-Nebelsick *et al.* 2001; (10) Thorne *et al.* 2006; (11) Tukey 1970; (12) Beattie & Lindow 1999; (13) Murphy & Lugo 1986; (14) Asner *et al.* 2003; (15) Delmotte *et al.* 2009; (16) Stavrinides *et al.* 2009; (17) Nadarasah & Stavrinides 2011; (18) Aryal & Nuener 2010; (19) Evans *et al.* 1992; (20) Lindow & Brandl 2003; (21) Hallam & Read 2006; (22) Choong *et al.* 1992; (23) Yadav *et al.* 2005; (24) Alfano & Collmer 1996; (25) Levin 1976; (26) Levin & York 1978; (27) Joosten & van Veen 2011; (28) Neinhuis & Barthlott 1997; (29) Huang 1986; (30) Monier & Lindow 2003; (31) Monier & Lindow 2005; (32) Richards 1996; (33) Ivey & DeSilva 2001; (34) Burd 2007; (35) Malhado *et al.* 2012; (36) Marcell & Beattie 2002; (37) Bazzaz & Pickett 1980; (38) Melotto *et al.* 2006; (39) Vokou *et al.* 2012; (40) McLean 1919; (41) Melotto *et al.* 2008; (42) Kinkel 1997; (43) Beattie 2002; (44) Comins & Noble 1985; (45) Tilman 1994; (46) Thompson *et al.* 1993; (47) Penuelas *et al.* 2012.

Table 2. The traits associated with bacterial pathogens on the phyllosphere

We list the traits that enhance pathogen survival, who the pathogens are, and whether the strains resided on plant host leaves or if studies were conducted *in vitro*. Additionally, we note the mechanisms involved for each trait as well as the effect of these traits on pathogen survival and persistence.

Trait	***Bacterial species***	***Host species***	***Mode of Action***	***Effect***	***Sources***
Low susceptibility to desiccation	*Pseudomonas syringae, Pantoea stewartii, Xanthomonas campestris, X. axonopodis*	Bean, ivy, grand fir, walnut, cherry laurel	Exopolysacch-aride (EPS) production; biosurfactant production	Maintains hydrated level surrounding bacteria and increases survival	1-3, 30-35, reviewed by 39
Low susceptibility to UV radiation	*P. syringae, P. aeruginosa, Pantoea stewartii, X. campestris*	Bean	Pigment production	Absorbs radiation and quenches free radicals	22-27,
High motility	*P. syringae*; *Xanthomonas* spp.	Bean, *In vitro*	Enhanced by quorum sensing, flagellin production, "riding" other motile bacteria	Enables cells to locate resources and to gain access to protected sites	3, 4, 5, 28
Efflux pump expression	*X. oryzae, Erwinia* spp., *Pseudomonas* spp.	*Arabidopsis*, bean, *in vitro*	Evade plant immune system by enabling bacterial effectors safe passage into plant host cells	Plant antimicrobial compound resistance	41-47
Resistance to heat and oxidative stress	*P. syringae*	Bean	EPS (aliginate) production	Reduces susceptibility to reactive oxygen intermediates	1-3, 6
Coenzyme production	*P. aeruginosa, Erwinia carotovora* (now *Pectobacterium carotovorum*)	*Arabidopsis*, corn	Facilitates quorum sensing	Increases ability to macerate plant tissue	7-10, 36
Virulence	*P. syringae, P. stewartii*	Bean, sweet corn, maize	Controlled by *vir* genes	Enables rapid invasion of internal leaf tissue; causes more host disease symptoms and dehydration	3, 11

Bacterial cell adhesion	*P. stewartii*	*In vitro*	Docking an locking through a series of physiochemical interactions	Enhances biofilm formation	11, 37
Biofilm formation	*P. stewartii, P. syringae, Xylella fastidiosa, Clavibacter michiganensis, X. campestris*	Corn; grape vine; potato	After initial colonization on surface, controlled via quorum sensing, cell division, and recruitment	Enhances microbial resistance to antibiotic compounds, enhances communication among cells	Reviewed by 12-15, 21, 29, 40
Ability to transfer DNA	*Agrobacterium tumefaciens*	*In vitro*, used for GMOs of alfalfa, corn, cotton, creeping bentgrass, rapeseed, rice, soybean, sugar beet, wheat	Conjugation system controlled by quorum sensing that transfers plasmids from donor to recipient via a complex secretion system	Allows cells to obtain tumor-inducing plasmids	16-18, reviewed by 19; 20

Sources by corresponding numbers: (1) Leigh & Choplin, 1992; (2) Ophir & Gutnick, 1994; (3) Quinones *et al.*, 2005; (4) Haefele & Lindow, 1987; (5) Lindow *et al.*, 1993; (6) Keith & Bender, 1999; (7) Jones *et al.*, 1993; (8) Rahme *et al.*, 2000; (9) Whitehead *et al.*, 2002; (10) Von Bodman *et al.*, 2003; (11) Koutsoudis *et al.*, 2006; (12) Watnick & Kolter, 2000; (13) O'Toole *et al.*, 2000; (14) Morris & Monier, 2003; (15) Flemming & Wingender, 2010; (16) Piper *et al.*, 1993; (17) Ellis *et al.*, 1982; (18) Zhang *et al.*, 1993; (19) Pitzschke & Hirt, 2010; (20) FDA, 2013; (21) Mann & Wozniak, 2012; (22) Corpe & Rheem, 1989; (23) Sundin & Murillo, 1999; (24) Kim & Sundin, 2000; (25) Kim & Sundin, 2001; (26) Jacobs *et al.*, 2005; (27) Gunasekera & Sundin, 2006; (28) Hagai *et al.*, 2014; (29) Mah & O'Toole, 2001; (30) Schreiber, 1996; (31) Knoll & Schreiber, 1998; (32) Knoll & Schreiber, 2000; (33) Schreiber *et al.*, 2005; (34) Chang *et al.*, 2007; (35) Rigano *et al.*, 2007; (36) Dong *et al.*, 2000; (37) Dunne, 2002; (38) Danhorn & Fuqua, 2007; (39) Beattie, 2011; (40) Rudrappa *et al.,* 2008; (41) Goel *et*

al., 2002; (42) Burse *et al*., 2004a; (43) Burse *et al*., 2004b; (44) Kang & Gross, 2005; (45) Maggiorani Valecillos *et al*., 2006; (46) Stoitsova *et al*., 2008; (47) Fan *et al*., 2011.

Table 3. Mutualist bacterial strains on the phyllosphere

A review of bacterial strains that significantly reduce the severity of fungal and oomycete pathogens on the phyllosphere. While bacteria have been characterized as biocontrol agents against other pathogens on crop fruits and other tissues (reviewed by Janisiewicz & Korsten, 2002; Sharma *et al.*, 2009), we exclusively focus on the phyllosphere here. We list the mutualistic bacterial strains and the pathogenic strains they excluded, as well as the plant host and whether the studies were conducted in the field, the lab, or *in vitro*. Last, we note the proposed mechanism for pathogen reduction.

Plant Host	***Bacterium***	***Location***	***Pathogen***	***Origin of Biocontrol Strain***	***Mechanism***	***Sources***
Alfalfa	*Bacillus cereus*	In lab	*Phytophthora medicaginis*	Cultured, but strain source not specified	Antibiosis	1
Rice	*Bacillus megaterium*, *Aspergillus niger*	*In vitro* and *in vivo*	*Rhizoctonia solani*	Isolated strains from the field	Not determined	2
Cacao	*Bacillus* spp. (endophytes)	Greenhouse, in lab	*Phytophthora capsici, Moniliophthora roreri, M. perniciosa*	Isolates from sugar beet, tomato, and potato; endophytes isolated from cacao in Ecuador	Competitive exclusion, suggestive of induced systemic resistance	3, 4
Cucumber	*B. pumilus, B. subtilis, Curtobacterium flaccumfaciens*	Greenhouse and in the field in Alabama	*Colletotrichum orbiculare*	*Bacillus subtilis*: product from Texas, others unspecified		
Banana	*Bacillus subtilis*, *B. amyloliquefaciens*	*In vitro*	*Mycosphaerella fijiensis*	Isolated from crops in Colombia	>80 percent inhibition of ascospore germination, hydrolytic enzyme production	5, 12
Mangrove	*B. amyloliquefaciens* (endophytes)	*In vitro,* pots, in field in China	*Ralstonia solanacearum*	Isolated from mangrove leaves	Anti microbial substances	6

Chickpea	*B. cereus*	In lab and in field in India	*Botrytis cinera*	Isolated from chickpea rhizosphere in India	Inhibited fungal germination, lysed conidia	15
Groundnut	*B. circulans/ Serratia marcescens* (+ colloidal chitin)	Greenhouse and in the field in India	*Phaeoisariopsis personata*	Selected from collection of peanut-associated strains	Inhibited fungal germination, lysed conidia, activating defense enzymes	13, 14
Grapevine	*Pseudomonas* spp.	*In vitro*	*B. cinerea*	Cultured from rhizosphere	Elicits host systemic resistance	16
Sugar Beet	*Bacillus mycoides*, *Bacillus pumilis*	Glasshouse and in the field	*Cercospora beticola*	Isolated from sugar beet leaves in Montana	Elicits host systemic resistance to pathogen	7, 8
Tomato	*Bacillus* spp., *Pantoea* spp.	Greenhouse and field conditions	*B. cinerea, Fulvia fulva, Alternaria solani*	Randomly selected bacterial strains	Plant hormone production, quorum sensing capabilities	9
Centella asiatica	*Bacillus subtilis, Pseudomonas fluorescens* (endophytes)	*In vitro*, in lab	*Colletotrichum*	Isolated from inside *Centella* leaves from Madagascar	Allelochem-icals, induced plant defense	10
Various crops, primarily tomato, grape vine, strawberry	*Bacillus* spp., *Pantoea* spp. *Pseudomonas* spp., *Bacillus* spp.	Greenhouse and field conditions *in vitro*	*B. cinera*	Review	Competition, antibiosis	11
Cucumber	*B. mycodies, B. mojavensis*	Greenhouse	*Glomerella cingulate*	Isolated from sugar beet leaves; sugar beet seed emryos	Induced systemic acquired resistance	17

Sources by corresponding numbers: (1) Silo-Suh *et al.*, 1994; (2) De Costa *et al.*, 2008; (3) Melnick *et al.*, 2008; (4) Melnick *et al.*, 2011; (5) Ceballos *et al.*, 2012; (6) Hu *et al.*, 2010; (7) Bargabus *et al.*, 2002; (8) Bargabus *et al.*, 2004; (9) Enya *et al.*, 2007; (10) Rakotoniriana *et al.*, 2013; (11) Elad, 1996; (12) Collins & Jacobsen, 2003; (13) Kishore *et al.*, 2005a; (14)

Kishore *et al.*, 2005b; (15) Kishore & Pande, 2006; (16) Verhagen *et al.*, 2010; (17) Neher *et al.*, 2009.

Table 4. What is an endophyte?

The term "endophyte" has been extant in the literature for almost 140 years. De Bary (1866) was the first to define "endophyte" as "any organisms occurring within plant tissues." Over time, however, many definitions for endophyte have been used (see review by Hyde & Soytong 2008), though the most commonly used is Petrini's definition (1991). Petrini (1991) defined endophytes as "all organisms inhabiting plant organs that at some time in their life, can colonize internal plant tissues without causing apparent harm to the host." Bacteria (as well as fungi), though, may have dormant or latent phases inside leaf tissue before causing disease to plant hosts, which Wilson (1995) characterized as the "continuum of infection patterns." Thus, under this definition bacteria that are clearly pathogens may be considered mutualistic endophytes (Schulz & Boyle, 2005). For example, Bashan & Okon (1981) demonstrated that tomato plants grown in *P. syringae* infested soil were symptomless but produced up to 30% less foliage than plants in sterile soil! We therefore side with De Bary (1866) and more recently Henis & Bashan (1986) and define foliar bacterial endophytes in this review as bacteria that have colonized the interior portions of leaf tissue.

Table 5. Quorum sensing

Bacteria can monitor their own population density through the production and release of small, diffusible signals that enable them to synchronize the expression of specialized gene systems (Waters & Bassler, 2005). This process is called quorum sensing, which simply put means that bacteria can count their own numbers and alter their behavior accordingly (reviewed by von Bodman *et al.*, 2003). Thus, individual bacteria can in essence "gang up" on their hosts, which may be particularly beneficial for pathogenic bacteria that aggregate at protected sites on the leaf surface (reviewed by Beattie & Lindow, 1999). In fact, quorum sensing might even camouflage bacterial pathogen populations by preventing or delaying host plant response until density is high enough to mount a formidable attack (Abramovitch *et al.*, 2006). Additionally, bacteria can use quorum sensing to simultaneously produce compounds that can enhance stress tolerance to heat, UV radiation, or drought (Quinones *et al.*, 2005; see Table 2). Other compounds produced via quorum sensing increase virulence by breaking down plant cell walls or aiding motility thereby promoting infection (Whitehead *et al.*, 2002; Shepherd & Lindow, 2009). Quorum sensing molecules have been identified among many bacterial species that commonly associate with plants (see reviews by Cha *et al.*, 1998; Loh *et al.*, 2002; Von Bodman *et al.*, 2003; Ahmad *et al.*, 2008; Hartmann *et al.*, 2014). In fact, Elasri *et al.* (2001) identified quorum-sensing molecules from a pool of 340 bacterial strains isolated from tissues of over 60 temperate crop species as well as in soil. They discovered that a larger portion of foliar and stem associated strains contained quorum-sensing molecules (49 percent) than root-associated strains (28 percent) and free-living strains from soil (0 percent). Although the degree to which phyllobacteria among tropical

plant hosts rely on quorum sensing to coordinate group behavior is unknown, these findings suggest that a large portion of leaf-associated bacteria rely on this phenomenon for survival and function.

Table 6. Bacterial pathogens and the plant immune system

Bacterial pathogens and the plant immune system: an evolutionary arms race
Plants have evolved two main lines of defense to detect bacterial pathogens, and pathogens have developed mechanisms to manipulate defense responses by secreting virulence effector molecules. The first line of plant defense is called the MAMP-triggered immunity (MTI), where plant extracellular pattern recognition receptors (PPRs) attempt to identify microbe-associated molecular patterns (MAMPs, also called PAMPs for pathogen-associated molecular patterns; reviewed by Dangl *et al.*, 2013; Newman *et al.*, 2013). Bacterial pathogens use one of six highly evolved secretion systems, most commonly types III and IV, to interfere with MTI by delivering effector proteins into plant cells (reviewed in Wooldridge, 2009). These effector proteins either inhibit plant cellular functions or mimic plant hormones (discussed in "Foliar Pathogens" section). In Type IV secretion, pathogens use conjugation to deliver effector molecules into host cells (reviewed by Zechner *et al.*, 2012; Christie *et al.*, 2014; Low *et al.*, 2014). *Agrobacterium tumefaciens*, for example, uses type IV secretion to induce tumors in many agricultural crop species (reviewed by Pitzschke & Hirt, 2010). The foremost system for pathogens, though, is the type III secretion system (reviewed by Jones & Dangl, 2006). Here, bacteria use a flagellar body like a syringe to inject a conglomerate of 20-30 proteins directly into the plant tissue cells (see recent reviews by Izore *et al.*, 2011; Buttner, 2012). Some of the most ubiquitous and deleterious bacterial pathogens, such as *P. syringae*, *Erwinia amylovora*, *Ralstonia solanacearum*, and *Xanthomonas* spp., use the Type III secretion system (Buttner, 2012). If bacterial pathogens successfully enter the host cell, they meet the plant's highly

specialized second line of defense called effector-triggered immunity (ETI, formerly known as gene-for-gene resistance; reviewed by Jones & Dangl, 2006). Plant ETI hinges on pathogen recognition by a class of receptor proteins that contain nucleotide-binding (NB) and leucine-rich repeat (LRR) domains. It is here where pathogens are "specifically recognized" by plant receptors, upon which the plant can resist disease and launch a hypersensitive response (HR, see "Foliar Pathogens.") This step in particular has led to co-evolutionary dynamics between bacteria and plant hosts, where pathogen effectors and plant receptors are notably diverse, variable, and frequently change (Boller & He, 2009; Dodds & Rathjen, 2010). While successful pathogen detection results in various defense responses, a successful pathogen suppresses or evades detection and is thereby able to cause disease.

Table 7. Scientific names

Common name	Scientific name
Alfalfa	*Medicago sativa*
Banana and plantain	*Musa* spp.
Bean	*Phaseolus vulgaris*
Cacao	*Theobroma cacao*
Cassava	*Manihot esculenta*
Cherry laurel	*Prunus laurocerasus*
Citrus	*Citrus* spp.
Clover	*Trifolium* spp.
Coffee	*Coffea* spp.
Cotton	*Gossypium hirsutum*
Cranberry	*Vaccinium macrocarpon*
Creeping bentgrass	*Agrostis stolonifera*
Cucumber	*Cucumis sativus*
Grand fir	*Albies grandis*
Grape vine	*Vitis* spp.
Grapefruit	*Citrus * paradisi*
Groundnut	*Arachis hypogaea*
Ivy	*Hedera helix*
Mangrove	*Bruguiera gymnorrhiza*
Potato	*Solanum tuberosum*
Rapeseed	*Brassica napus*
Rice	*Oryza* spp.
Soybean	*Glycine max*
Strawberry	*Fragaria ananassa*
Sugar beet	*Beta vulgaris*
Sugar cane	*Saccharum* spp.
Tomato	*Solanum lycopersicum* (formerly *Lycopersicum esculentum*)
Walnut (common walnut)	*Juglans regia*
Wheat	*Triticum* spp.

3.0 SOIL FERTILITY MEDIATES SEEDLING RESPONSES TO FOLIAR BACTERIA IN A TROPICL FOREST: EXPERIMENTAL EVIDENCE AND ECOLOGICAL IMPLICATIONS

3.1 INTRODUCTION

The phyllosphere (leaf surface) is perhaps the world's largest terrestrial microbial habitat (Vorholt 2012), yet we know little about the impacts of foliar bacteria on plant performance in nature. This is striking because bacteria colonize leaves in densities of up to 10 million cells/cm^2 and the global leaf surface area is over 1 billion km^2, which is more than double the earth's surface area (Lindow & Brandl 2003, Delmotte *et al.* 2009, Vorholt 2012). In particular, tropical forests comprise nearly half of the world's leaf area (Perry *et al.* 2008), and these habitats are likely ideal for bacteria because temperature and humidity are high and UV radiation is low (reviewed by Griffin & Carson 2015). Understory plants in deeply shaded habitats are likely particularly vulnerable to microbial pathogens or conversely rely on mutualistic bacteria to defend themselves against pathogens (Gilbert 2002, Griffin & Carson 2015). Very few studies have characterized foliar bacterial *communities* among tropical trees (Lambais *et al.* 2006, Furnkranz *et al.* 2008, Kim *et al.* 2012, Kembel *et al.* 2014), let alone assessed their impacts. In

the only empirical field study to assess the net impact of foliar bacteria on natural plant hosts, Traw *et al.* (2007) demonstrated that foliar bacteria at ambient levels decreased *Arabidopsis thaliana* seed production by over 55%. Whether foliar bacteria have similar deleterious impacts elsewhere is unknown.

The degree to which the impact of foliar bacteria varies among coexisting plant species is predicated on the degree to which bacterial communities are host specific. On one hand, large overlaps of bacterial phyllosphere communities suggest that the net impact of bacteria may be similar among host species. For example, recent studies have demonstrated that a large majority of bacteria on the phyllosphere make up a "core microbiome," meaning that a large subset of taxa are commonly shared among individuals in the same habitat or region (Shade & Handelsman 2012, Rastogi *et al*. 2012, Kembel *et al*. 2014, reviewed by Griffin & Carson 2015). Moreover, because bacteria are capable of dispersing widely over large distances via insect vectors or the water cycle, they may commonly colonize many plant species across plant assemblages (e.g., Morris *et al*. 2008, Chapman *et al*. 2015). Indeed, Kembel *et al*. (2014) demonstrated that while only 1.4% of phyllosphere bacterial diversity was present on over 90% of all trees sampled in Panama (57 species), this small subset of bacteria made up 73% of the total sequences. This means that a very small group of bacteria occur repeatedly among numerous tree species. On the other hand, phyllosphere communities may, like insect herbivores, be host specific and therefore differentially impact plant hosts (e.g., Dyer *et al*. 2007). For example, Redford *et al*. (2010) demonstrated that no bacterial sequences co-occurred among 56 temperate tree species in Colorado. Moreover, the most common bacterial taxon represented less than 5% of the total sequences, suggesting that phyllosphere communities are, to a large degree, host specific. Kembel *et al*. (2014) also found that host taxonomy and host traits

explained 51% of the variation in bacterial communities among tree species in Panama. Thus, the jury is still out on the degree to which the impact of bacterial communities will or will not be highly host specific.

Soil resource supply rates might mediate bacterial impacts to plant hosts, even in the shaded understory. It has only recently been demonstrated that woody seedlings in tropical forests are co-limited by soil nutrients (Wright *et al.* 2011, Pasquini & Santiago 2012, Santiago *et al.* 2012, Pasquini *et al.* 2015), though it is not clear why this is so. Foliar bacteria may mediate the degree of this soil nutrient limitation but empirical data are non-existent. Results from agricultural systems and grasslands suggest that interactions between soil resource availability and foliar microbes are common, but these studies focus mainly on fungal pathogens (e.g., Mitchell *et al.* 2003, Amtmann *et al.* 2008). Several studies have demonstrated that soil nutrient enrichment mediates outcomes between plants and microbes, typically by mitigating or exacerbating the impact of pathogens (reviewed by Dordas 2008, Johnson *et al.* 2010). For example, potassium reduces fungal and bacterial pathogen severity in agricultural systems whereas nitrogen tends to increase obligate pathogen severity and decrease facultative pathogen severity (reviewed by Dordas 2008, Amtmann *et al.* 2008, Johnson *et al.* 2010). The effects of phosphorus addition on disease severity are inconsistent and equivocal (reviewed by Dordas 2008, Johnson *et al*. 2010). The degree to which plant responses to foliar bacteria are mediated by soil resource supply and the degree to which results from agricultural systems apply to more natural systems are unknown.

Overall, it is possible that foliar bacteria provide an important yet cryptic dimension for niche differentiation, particularly if their impacts vary among plant species, as well as along key resource gradients. Gradients in soil resources and light availability have been associated with

species-specific traits and trade-offs that are necessary for niche partitioning (Clark *et al*. 1998, Harms *et al*. 2001, Condit *et al*. 2002, reviewed by Wright 2002, Silvertown 2004, and Kitajima & Poorter 2008). Still, it remains unclear how these resources can facilitate the coexistence of hundreds of tree species in hyper diverse tropical forests (e.g., Hubbell 1999, 2001, Chave 2004). However, though speculative, frequent interactions between soil resources and plant-bacterial associations could significantly facilitate coexistence by narrowing species potential niches down to finer realized niches, thereby opening niche space for other community members (Chase & Leibold 2003, Silvertown 2004). Specifically, foliar bacteria may regulate species-specific plant responses along key niche axes (e.g., water, light, soil nutrient availability). In this manner, foliar bacteria may reduce species niche breadths by further determining species performance differences across resource supply levels.

To address the issues raised above, we tested the following mutually compatible hypotheses:

1) *The Host Tree Hypothesis*: The magnitude of the impact of foliar bacteria varies significantly among coexisting plant species.

2) *The Limiting Nutrient Hypothesis*: The magnitude of the impact of foliar bacteria varies significantly with soil nutrient availability (e.g., N vs. P vs. K).

3) *The Interaction Hypothesis*: There are frequent interactions between soil nutrient supplies and the impact of foliar bacteria among host plant species

To address these hypotheses, we experimentally reduced foliar bacteria for 29 months and measured growth responses for seedlings of 5 woody species nested within a fully factorial experiment where nitrogen, phosphorus, and potassium and all of their combinations were added to large replicated tropical forest plots for 15 years. We focused on growth rates because size differences among coexisting seedlings and saplings in the shaded understory determine which

individuals will reach the canopy (e.g., Brown & Whitmore 1992, Boot 1996, Zagt & Werger 1998).

3.2 METHODS

3.2.1 Study site and species

We conducted this study on the Gigante Peninsula in a mature (~ 200 yr.) secondary tropical forest in Panama (9˚06'31''N, 79˚50'37''W; Fig. S4). Annual precipitation averages 2,600 mm, of which less than 10% falls during the 4-month dry season between January and April. The soils consist of endogleyic cambisols and acric nitisols (Koehler *et al*. 2009). We selected five common woody species from five different families located throughout the site (hereafter referred to by genus name): *Alseis blackiana* (Rubiaceae), *Desmopsis panamensis* (Annonaceae), *Heisteria concinna* (Olacaceae), *Sorocea affinis* (Moraceae) and *Tetragastris panamensis* (Burseraceae). Nomenclature follows Garwood (2009) and Croat (1978). All five species are relatively shade tolerant as seedlings, vary in life history traits, and span a wide range of maximum adult heights (Wright *et al.* 2003, Gilbert *et al.* 2006). *Sorocea* is a shrub, *Desmopsis* and *Heisteria* are understory treelets, *Alseis* is a mid-canopy tree, and *Tetragastris* is a canopy tree (for additional life history and taxonomic details see Croat 1978, Wright *et al*. 2010).

3.2.2 Design of the fertilization experiment

We employed a 2 × 2 × 2 factorial design with nitrogen (N), phosphorus (P), and potassium (K) and replicated the eight treatments four times (Fig. S4). The 32 experimental plots each measured 40 x 40 m. All plots but two were separated by at least 40 m, and those two were separated by 20 m and a 3-m deep streambed. Beginning in 1998, we added fertilizer by hand four times a year at approximately six-week intervals between June and November within the wet season (May-December). Each year, we applied 125kg N ha^{-1} $year^{-1}$ as urea, 50 kg P ha^{-1} $year^{-1}$ as triple super-phosphate, and 50 kg K ha^{-1} $year^{-1}$ as KCl.

3.2.3 Seedling measurements and antibiotics

In January 2010, we selected six relatively healthy individuals (minimal signs of necrosis or insect damage) of each species (~20 – 30cm tall) within the inner 30 x 30 m of each plot. We randomly assigned antibiotic and control (sterile water) treatments to three individuals of each species in each plot. For 29 months, we sprayed seedling leaves every 10-15 days with antibiotics or sterile water to saturation. We placed a plastic sheet around the base of each seedling before application to prevent exposure of soil microbes to either treatment. The plastic sheet extended beyond the crown of each individual and was left in place until no liquid was visibly dripping off the plant. We alternated the antibiotic treatment every other application between 100 ppm of Agri-mycin 17 (a commercial formulation of streptomycin, Hummert International #02-0150; Earth City, MO) and 1752 ppm of Agry-Gent Plus 800 (a commercial cocktail formulation of gentamicin and oxytetracycline, Química Agronómica de México, Chihuahua, México). These are two of the most commonly used broad-spectrum antibiotics in

temperate and tropical agriculture (McManus *et al.* 2002). These antibiotics inhibit protein synthesis for both Gram-positive and Gram-negative bacteria and are highly effective under field conditions (McManus *et al.* 2002, Traw *et al.* 2007).

We measured seedling height and survival after 29 months of treatments. Data were collected blindly with respect to treatments. Personnel did not know whether seedlings were treated with antibiotics or sterile water or the nutrient treatment applied to the plot. We measured the height of each seedling to the nearest 1 mm. Additionally, we measured percent canopy openness above each seedling using a concave densiometer at breast height (Forest Densiometers, Bartlesville, Oklahoma, USA); however, canopy openness had no effect on the models detailed below and we present results without canopy openness.

3.2.4 Antibiotic effectiveness

To quantify the degree that the antibiotics decreased foliar bacteria in the field, we cultured and quantified bacterial colony abundance and morphotype richness on King's Broth media (N = 316; see Traw *et al.* 2007 for details). We are well aware that these culturing methods sample only a small proportion of the total microbial diversity (e.g., Amman *et al.* 1995); thus we used culture-dependent protocols only to confirm that antibiotics decreased absolute abundance and morphotype richness over time (see Traw *et al.* 2007). We do not make assertions about bacterial community structure and composition.

We tested the effectiveness of each antibiotic separately versus control sterile water applications before the experiment began (0 months) and after 14 and 23 months of applications to evaluate whether bacteria became resistant to the alternating antibiotic regime (our data showed this was unlikely, see below and Figs. S9-S10). Briefly, we removed leaf tissue via a

sterile hole punch (6.35mm diameter) from a randomly selected leaf before and two days after antibiotic or water application. We placed each leaf disk in 200µl sterile 10mM $MgSO_4$ buffer in 1.5ml centrifuge tubes and immediately took them to the lab to be cultured at room temperature (23°C) for 72 hours. To assess epiphytic (leaf surface) bacteria, we placed each tube on a vortex mixer for 10-15 seconds in order to slough off bacteria into the $MgSO_4$ buffer and then plated 30ul of the buffer solution onto King's Broth plates (Kniskern *et al.* 2007). To culture endophytic bacteria, which we define as bacteria occupying the interior portions of leaf tissue (Griffin & Carson 2015), we sterilized leaf surfaces following Arnold and Lutzoni (2007). Briefly, we immersed leaf disks in 95% ethanol (10s), 10% chlorine bleach (2 min), and 70% ethanol (2 min). We then ground the leaf disk in an eppendorf tube with a sterile pestle. Last, we diluted all samples to a 1:100 solution with sterile 10 mM $MgSO_4$ before plating 30 µl of each sample onto plates. To quantify morphotype richness, we identified over 50 distinct colony morphologies that we classified using the characteristics described by Zinniel *et al.* (2002) and Traw *et al.* (2007).

To ensure that plastic sheets we placed around seedlings were effective and prevented the antibiotics from reducing soil bacteria, we cultured soil samples taken from seedlings treated with antibiotics outside of the experimental array. We selected eight seedlings from each species (N = 40) and treated four randomly selected individuals with antibiotics and the other four with sterile water, using plastic sheets to prevent antibiotics from interacting with soil microbe communities. We then cultured bacterial communities from soil samples collected before application and two days after application. We took 5 grams of topsoil (A horizon) from a randomly selected area underneath each seedling and cultured bacterial communities in the lab (for details see Wiggins & Kinkel 2005). Briefly, we dried soil samples overnight under two

layers of cheesecloth at room temperature to prevent bacterial colonization from the laboratory air. To homogenize the mixture, we placed samples in 50 ml of sterile water and shook them on an orbital shaker at 175 rpm for one hour at room temperature. We then cultured bacteria following the techniques described above.

3.2.5 Statistical analyses

We performed a MANOVA to evaluate mean relative growth rate differences among antibiotic- and control-treated seedlings among soil nutrient treatments. We calculated relative growth rate of height (cm cm^{-1} $month^{-1}$) for each seedling as

$$G = (\ln H_1 - \ln H_0)/(t_1 - t_0)$$

where H_0 and H_1 were initial and final seedling heights (cm) and t_1-t_0 was the time period in months (Santiago *et al*. 2012). The average ($\bar{G}$) for each species in a plot was simply the mean of the values of G over the three conspecific plants in each antibiotic treatment (treated or control). We then calculated the difference in average relative growth rate for each species (i) with or without antibiotic application in each plot as:

$\delta\ \bar{G}(i) = \bar{G}$ (i, antibiotic) **-** $\bar{G}$ (i, control).

Because all 5 species were nested (and non-independent) within each plot, our response vector for plot j was:

$$\delta\ \bar{G}_j = (\delta\ \bar{G}(1), \delta\ \bar{G}(2), \delta\ \bar{G}(3), \delta\ \bar{G}(4), \delta\ \bar{G}(5))_j$$

where the numbers 1 through 5 refer to the five plant species. A MANOVA of this response vector tests whether growth differences between control and antibiotic treated plants differed across nutrient treatments and adjusts for correlated response variables. Post hoc Tukey

studentized range tests with corrected significance values ($\alpha = 0.05$ and corrected for the number of means being compared) on the individual elements of the vector then provide insights into which species differed in their responses to the antibiotics across nutrient treatments. We chose this approach to avoid pseudofactorialism, which is a problem in many studies using nested factorial designs (Hurlbert 2013).

We performed identical MANOVAs to evaluate responses of bacterial abundance and morphotype richness to antibiotic treatments during three time points throughout the experiment. Colony abundance and morphotype richness were log transformed to meet the assumption of univariate normality. We used post hoc t-tests to determine if differences in colony abundance and richness after treatment differed significantly from 0. We used SAS 9.4 for statistical analyses and SigmaPlot 11 for graphing.

3.3 RESULTS

3.3.1 Antibiotic effectiveness

Colony abundance. Agri-mycin and Agry-gent each significantly decreased mean abundance of epiphytic bacteria (compared to pre-treatment) by 55% and nearly 50%, respectively (Figs. S5A and B; $T_{1,315} = -10.17$, $P < 0.0001$; $T_{1,315} = -10.80$, $P < 0.0001$). The sterile water treatments had no effect on epiphyte abundance compared to pre-treatment (Figs. S5A and B; Agri-mycin: $T_{1, 315} = 0.48$, $P = 0.63$; Agry-gent: $T_{1, 316} = 1.79$, $P = 0.07$). Further, both Agri-mycin and Agry-gent decreased mean abundance of endophytic bacteria in surface-sterilized leaves (compared to pre-treatment) by over 50% and almost 50%, respectively (Figs.

S5C and D; $T_{1, 314} = -11.64$, $P < 0.0001$; $T_{1, 315} = -10.33$, $P < 0.0001$). The sterile water treatment had no effect on endophyte abundance compared to pre-treatment (Figs. S5C and D; Agri-mycin: $T_{1, 315} = -0.91$, $P = 0.37$; Agry-gent: $T_{1, 314} = -1.35$, $P = 0.18$). There were almost no differences (with one exception) in the degree to which either Agri-mycin or Agry-gent reduced bacterial abundance among nutrient treatments and plant species (see Tables S9-S12). Our findings for both antibiotics suggest that the effectiveness of the antibiotics did not vary among resource supply treatments, among species, or through time.

Morphotype richness. Agri-mycin and Agry-gent significantly decreased mean epiphyte morphotype richness (compared to pre-treatment) by 15% and 20%, respectively (Figs. S6A and B; $T_{1, 315} = -5.35$, $P < 0.0001$; $T_{1, 315} = -8.30$, $P < 0.0001$). The sterile water treatment had no effect on morphotype richness compared to pre-treatment (Figs. S6A and B; Agri-mycin: $T_{1, 316} = -0.58$, $P = 0.56$; Agry-gent: $T_{1, 316} = 1.04$, $P = 0.30$). Further, Agri-mycin and Agry-gent decreased mean endophyte richness (compared to pre-treatment) by 35% and almost 40%, respectively (Figs. S6C and D; $T_{1, 313} = -11.42$, $P <0.0001$; $T_{1, 316} = -10.33$, $P < 0.0001$). And, the sterile water treatment had no effect on endophyte morphotype richness compared to pre-treatment (Figs. S6C and D; Agri-mycin: $T_{1, 315} = 0.72$, $P = 0.47$; Agry-gent: $T_{1, 313} = 1.36$, $P = 0.18$). There were no differences in the degree to which either Agri-mycin or Agry-gent reduced bacterial richness among nutrient treatments and plant species (see Tables S13-S16). Thus, our findings suggest that the effectiveness of the antibiotics did not vary among resource supply treatments, among species, or through time.

3.3.2 Antibiotic effects on soil bacterial communities

When applied to leaves and shielded from the soil by a plastic sheet, neither Agri-mycin nor Agry-gent affected *soil* colony abundance or *soil* morphotype richness (Figs. S7A-D; Agri-mycin abundance: $T_{1, 19} = -0.4604$, $P = 0.65$; Agry-gent abundance: $T_{1, 19} = 0.31$, $P = 0.76$; Agri-mycin richness: $T_{1, 19} = 0.2457$, $P = 0.81$; Agry-gent richness: $T_{1, 19} = 1.10$, $P = 0.29$). Additionally, sterile water had no effect on soil colony abundance or soil morphotype richness in either experiment (Figs. S7 A-D; Agri-mycin control abundance: $T_{1, 19} = -0.75$, $P = 0.46$; Agry-gent control abundance: $T_{1, 19} = 0.20$, $P = 0.62$; Agri-mycin control richness: $T_{1, 19} = 0.14$, $P = 0.89$; Agry-gent control richness: $T_{1, 19} = 0.42$, $P = 0.68$). Thus, there is no evidence that our antibiotic or water treatment applied to leaves caused any changes in soil bacterial abundance or richness.

3.3.3 Seedling growth responses to foliar bacteria

Applying antibiotics for 29 months caused a net increase in relative growth rate for seedlings of *Heisteria*, *Alseis*, and *Tetragastris* by 36%, 47%, and 49%, respectively. These responses differed significantly from *Sorocea*, whose net growth decreased by 17% after 29 months of antibiotic applications ($F_{4, 159} = 3.92$, $P = 0.0046$, Fig. 1, Tukey test: minimum significant difference: $P < 0.0045$). Antibiotic applications had no net effect on *Desmopsis* growth.

3.3.4 Soil nutrients mediate seedling growth responses to foliar bacteria among woody species

Overall, K enrichment mitigated the negative impact of foliar bacteria on seedling growth after 29 months of application (Table 1, $F_{5, 20} = 3.19$, $P = 0.0282$). Specifically, across all species, applying antibiotics increased growth rate by almost 40% in the absence of K but had no effect on plant growth responses in +K treatments (Fig. 2). K addition caused significant changes in the effect of antibiotics on growth for both *Desmopsis* and *Heisteria* (Fig. 2, Tukey tests: $P < 0.0043$ and $P < 0.0051$, respectively). For *Desmopsis*, applying antibiotics increased growth by 43% in –K treatments and decreased growth by 32% in +K treatments. For *Heisteria*, applying antibiotics increased growth by 85% in –K treatments but had no effect in +K treatments. K enrichment also caused substantial reductions of the effect of antibiotics on seedling growth for *Sorocea* and *Tetragastris*, although the effect was not significant for these species (Fig. 2).

In contrast to K, P enrichment generally exacerbated the negative impacts of foliar bacteria after 29 months of antibiotic applications (Table 1, $F_{5, 20} = 3.01$, $P = 0.0349$), though this effect differed among species (Fig. S8). There was also a significant P x N interaction (Table 1, $P = 0.0164$), thus the effect of P on performance often depended on the presence or absence of N addition (Fig. 3). Specifically, when we reduced bacteria for *Alseis*, –P treatments caused a decrease in plant growth by over 67% but P addition caused a 34% increase in growth (thus bacteria shifted from harmful to beneficial); however these stark contrasts for the presence or absence of P only occurred when we added N (Fig. 3A). Conversely, when we reduced bacteria for *Heisteria*, –P treatments caused an increase in growth by 37% but P addition caused a 80%

decrease in growth (thus bacteria shifted from beneficial to harmful); however, this only occurred when we did *not* add N (Fig. 3B).

3.4 DISCUSSION

We demonstrated that the net impact of foliar bacteria was highly host specific and caused substantial decreases (36 - 49%) in growth for seedlings of three of five tree species and caused sharply contrasting growth responses in the two remaining tree species (Fig. 1). Potassium enrichment consistently mitigated the negative impact of bacteria (Fig. 2). P and N enrichment caused an interaction so that P enrichment either mitigated or exacerbated the negative impact of foliar bacteria depending on N enrichment and tree species (Fig. 3). Thus, plant responses to foliar bacteria depended on soil resource supply in deeply shaded habitats. In addition, our data demonstrate that antibiotic applications decreased bacterial abundance and morphotype richness throughout the entire experiment, though only by ~50% (Figs. S9 and S10). Thus, our results are likely conservative estimates of the impact that bacteria have on plant performance. Overall, we found strong support for all three of our hypotheses (see introduction); specifically, the impact of reducing bacteria on plant growth rates 1. varied substantially among host species (Fig. 1), 2. varied substantially with the supply of K (Fig. 2 and S10), and 3. varied among combinations of N supply, P supply, and plant species (Fig. 3). To our knowledge, this is the first empirical study to experimentally evaluate the impact of foliar bacteria *in situ* among multiple coexisting species. Overall, foliar bacteria were major determinants of seedling performance in contrasting resource environments and may have the potential to alter the rank-

order performance of coexisting plant species. These results suggest that foliar bacteria may interact with soil fertility to comprise another important, yet cryptic dimension of niche differentiation. Below we discuss these findings and suggest potential mechanisms.

3.4.1 Soil resource supply mediated plant responses to foliar bacteria

The effects of foliar bacteria on host performance varied strongly with soil nutrient supply. Potassium enrichment mitigated the negative impact of bacterial communities and even caused bacterial communities to switch from causing a net decrease in growth to causing a slight increase in growth (Fig. 2). This outcome is consistent with results in agricultural systems, where potassium typically decreases host plant susceptibility to pathogens (reviewed by Dordas 2008). Though the underlying mechanisms for how potassium mitigates pathogen virulence remain speculative, Dordas (2008) proposed that adding potassium decreases pathogen entry into cells because it promotes the development of thicker outer cell walls, thereby potentially enhancing plant defenses against bacterial and possibly fungal pathogens.

We found sharp contrasts regarding the degree to which phosphorous and nitrogen either mitigated or exacerbated the impact of bacterial communities for particular species. These findings are also consistent with findings in agricultural systems, where the effects of phosphorus and nitrogen on plant susceptibility to disease are variable and context dependent (Dordas 2008). We discovered that the impact that applying phosphorus had on plant species responses to bacterial reductions depended on the presence or absence of nitrogen addition (Fig. 3). For one species (*Alseis*), P addition caused an increase in plant performance when we reduced bacteria (thus bacteria were harmful), but only in the presence of N. For a second species (*Heisteria*), P addition caused a decrease in plant performance when we reduced bacteria (thus bacteria were

beneficial), but only in the absence of N. We do not want this complexity to get in the way of a key take home message, specifically, variation in the impact of foliar bacteria depended upon interactions with soil resource supply and this in turn varied by species (Fig. 3). These findings *suggest* that foliar bacterial-plant interactions might contribute to a much greater degree of fine-scale habitat heterogeneity than previously recognized. Silvertown (2004) suggested this exact possibility, however to date there has been no evidence to support this contention. While further studies are needed to explore the physiological mechanisms underlying our results, it is clear that underlying soil nutrient resources mediated foliar bacteria-plant interactions. Ultimately, we suggest the interactions between plants and foliar bacteria create fine-scale habitat heterogeneity which may be relevant to plant species coexistence among patches of forest soil that vary in fertility and among host plant species.

The mechanisms whereby bacteria and their interactions with macronutrients mediate plant performance are unknown, though our results call for studies that evaluate a suite of alternatives. Our findings are consistent with a tradeoff between plant allocation to defense and growth, when removing a plant's bacterial burden allows the plant host to allocate more resources to growth (Coley *et al.* 1985, Bazzaz *et al.* 1987). Under these circumstances, endophytic bacteria may usurp limiting resources, interfere or co-opt host physiology by commandeering the plant immune system, produce enzymes that macerate plant host tissues, or any combination of these mechanisms (reviewed by Griffin & Carson 2015). One parsimonious mechanism may be that bacteria build up on the leaf surface in particular microhabitats (up to 10 million cells/cm^2) particularly around stomata to such an extent that they interfere with gas exchange and photosynthesis (Lindow & Brandl 2003). While this may at first seem unlikely, it is well known that up to 80% of bacteria on leaf surfaces form dense biofilms at protected sites

on and inside leaves (reviewed by Beattie & Lindow 1999, Morris & Monier 2003). Conversely, in some cases and under varying levels of macronutrients, bacterial reductions reduced plant growth rates. Under these circumstances, bacteria may function to some degree as mutualists by competitively excluding pathogenic fungi and possibly even inducing systemic resistance to fungal pathogens (reviewed by Griffin & Carson 2015). Ultimately, our study demonstrates the *net* effects of bacterial communities on plant performance, and future culture-independent methods can assess how bacterial communities respond to antibiotic applications and link particular bacterial taxa to plant performance.

3.4.2 Implications for niche assembly and the maintenance of diversity

Patchiness and gradients in soil fertility are widely recognized as a key niche dimension or niche axis for plants, which can promote the maintenance of diversity (Tilman 1982). The mechanism underlying this niche dimension is often assumed to be direct resource competition, where different plant species are better competitors along resource gradients. Here, we propose a new dimension for niche differentiation at a fine scale whereby interactions between plants and their foliar bacteria are mediated by soil resource availability. This in turn mediates plant growth and survivorship of seedlings and saplings for numerous species, favoring some but not others, over small spatial scales as N, P and K vary spatially throughout the forest. Thus our results suggest a mechanism that could lead to a much finer partitioning of forest habitats. Consequently, the interactions between plants and their foliar bacteria along resource gradients provide an additional and novel dynamic (plant-bacteria-resource interactions) on a long recognized resource-based niche axis.

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3.7 TABLES AND FIGURES

Table 8. MANOVA results for the effects of N, P, and K on relative growth rates

MANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) on mean relative growth rate differences between antibiotic- and control-treated seedlings over five species (*Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*) and four replicates of all factorial combinations of N, P and K (N = 32 plots). We calculated relative growth rate as $G = (\ln H_1 - \ln H_0)/(t_1-t_0)$ for each seedling where H_0 and H_1 were initial and final seedling heights (cm) and t_1-t_0 was the time period in months, averaged growth rates over three seedlings for each antibiotic treatment in each plot, and analyzed the difference between mean growth rates with and without antibiotics ($\delta\ \bar{G} = \bar{G}$ (antibiotic**) -** $\bar{G}$ (control)).

Factor	Df value	F	P value
N	5, 20	0.62	0.6843
P	5, 20	**3.01**	**0.0349**
K	5, 20	**3.19**	**0.0282**
N × P	5, 20	**3.66**	**0.0164**
N × K	5, 20	1.59	0.2088
P × K	5, 20	1.67	0.1879
N × P × K	5, 20	0.59	0.7070

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Figure 1. Species differences in the magnitude of bacterial impact on growth rates

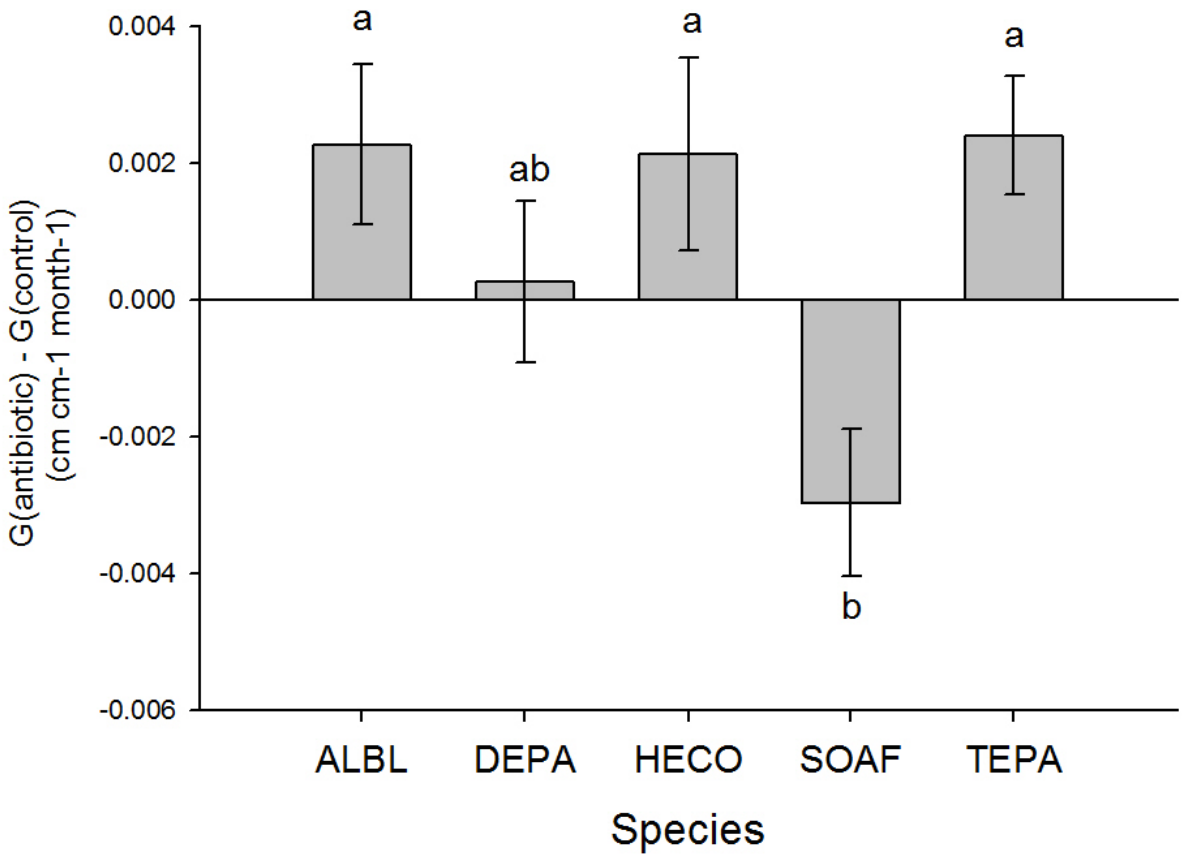

Figure 1. The difference in mean relative growth rates of control and antibiotic treated seedlings after 29 months of applications (letters represent significant species differences determined by Tukey tests; minimum significant difference: $P < 0.0045$). When bars are above the line antibiotic applications increased plant relative growth rates and when below the line, antibiotic applications decreased growth rates. All soil nutrient treatments are pooled ($N = 32$ plots). Letters correspond to differences determined by post-hoc Tukey studentized range tests among species. Bars represent mean values (± 1 SE).

Figure 2. K enrichment effects on bacterial impacts on growth among plant species

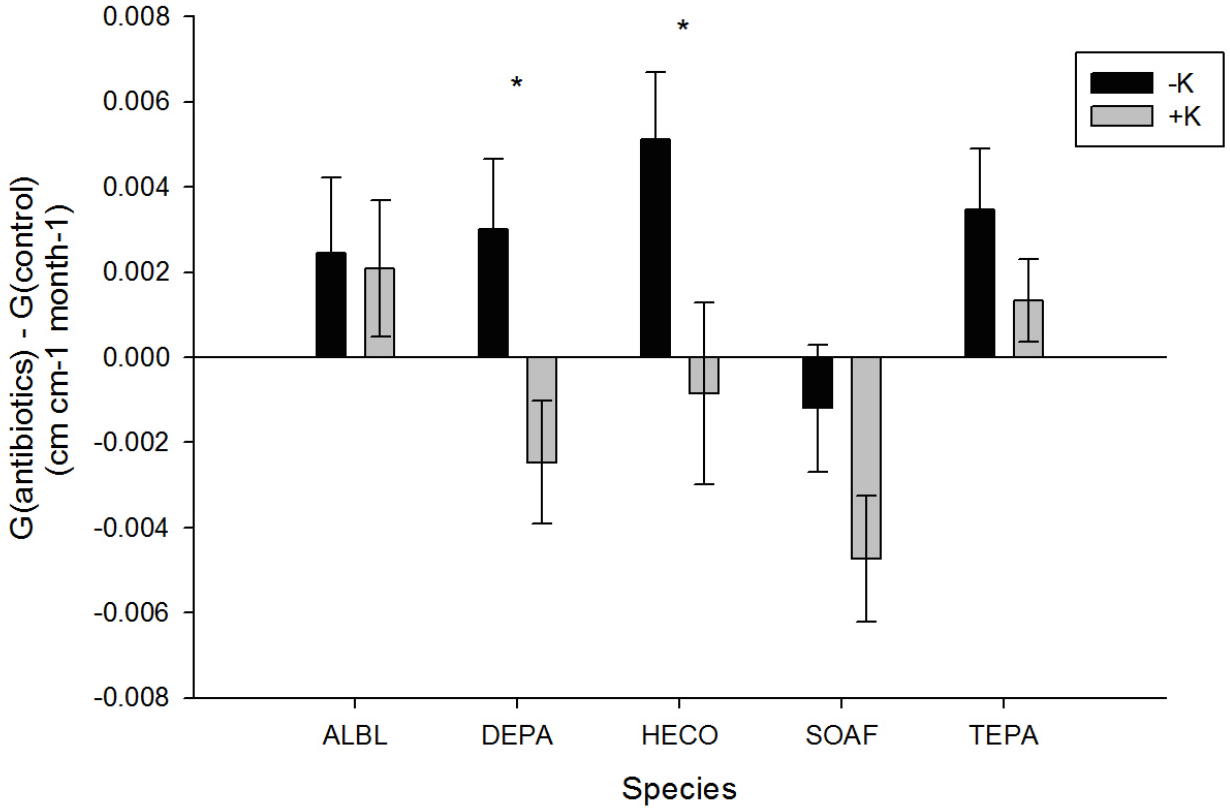

Figure 2. Significant effects of potassium (K) enrichment on mean relative growth rates of control and antibiotic treated seedlings after 29 months among *Alseis blackiana* (ALBL), *Desmopsis panamensis* (DEPA), *Heisteria concinna* (HECO), *Sorocea affinis* (SOAF), and *Tetragastris panamensis* (TEPA) (* represents significant Tukey test differences between potassium treatments for single species: Tukey tests: $P < 0.0043$ for *Desmopsis* and $P < 0.0051$ for *Heisteria*). When bars are above the line antibiotic applications increased plant relative growth rates and when below the line, antibiotic applications decreased growth rates. Light bars represent values for plots where K was added (K, K + N, K + P, N + P + K, $N = 16$ plots). Dark bars represent values for plots where K was not added (C, N, P, N + P, $N = 16$ plots). Bars represent mean values per plot (± 1 SE).

Figure 3. N*P effects on bacterial impacts on seedling growth among plant species

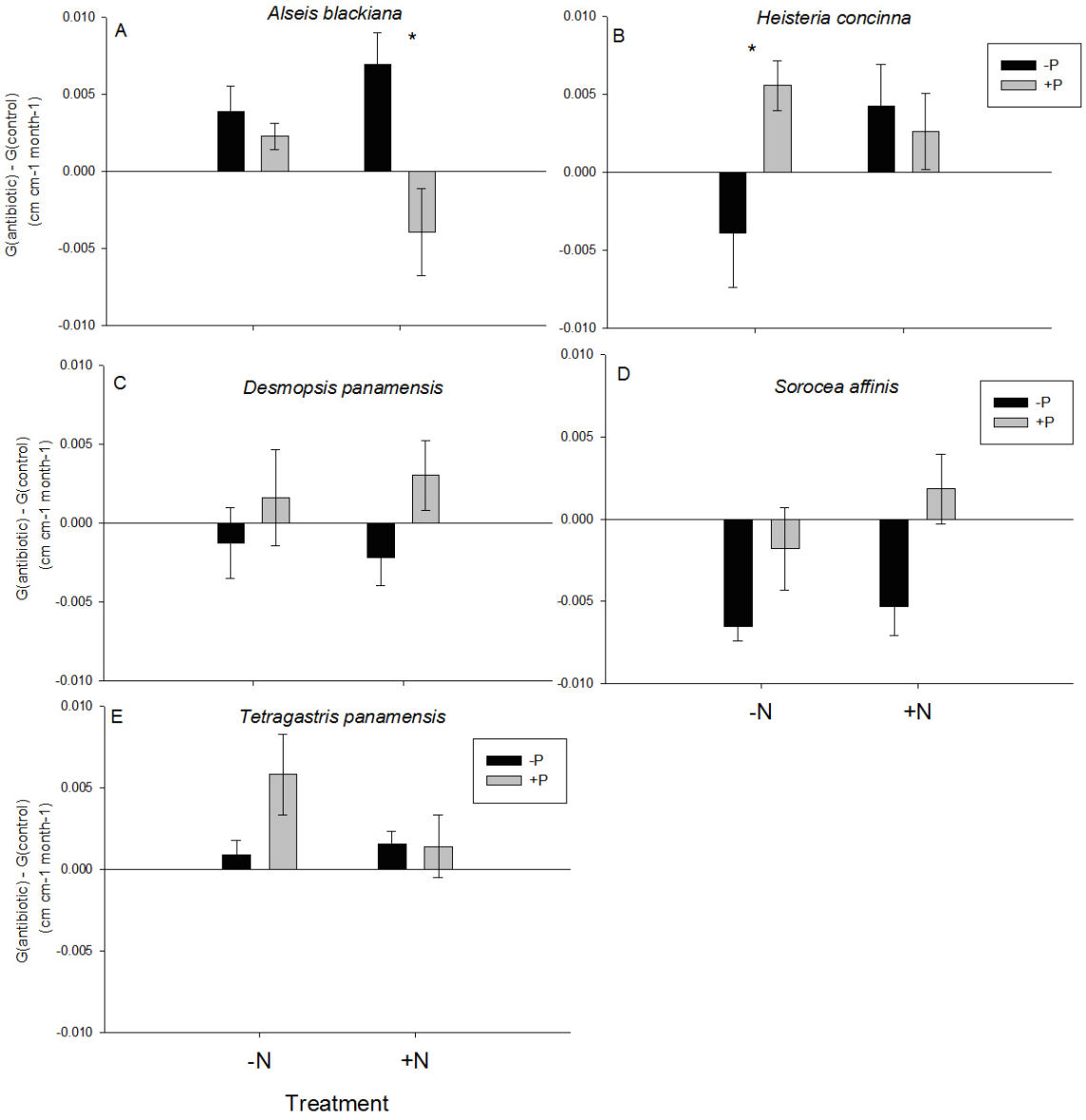

Figure 3. Growth responses of the 5 species to the antibiotic treatment in the presence and absence of P addition (dark vs. grey bars) and the presence vs. absence of N (x axis). When bars are above the line antibiotic applications increased plant relative growth rates and when below the line, antibiotic applications decreased growth rates. These interaction plots illustrate the significant nitrogen-phosphorus interaction ($F_{5,\ 20}$ = 3.66, P = 0.0164, N= 32 plots) on the difference in mean relative growth rates of control and antibiotic treated seedlings after 29 months of applications for A) *Alseis blackiana*, B) *Heisteria concinna*, C) *Desmopsis panamensis*, D) *Sorocea affinis,* and E) *Tetragastris panamensis*. Bars represent mean values (± 1 SE). *Alseis* and *Sorocea* respond in the opposite direction to N addition (species effects) whereas *Alseis* and *Heisteria* illustrate the significant N x P interaction.

3.8 SUPPLEMENTARY MATERIALS

Figure S4. The Gigante Fertilization Experiment

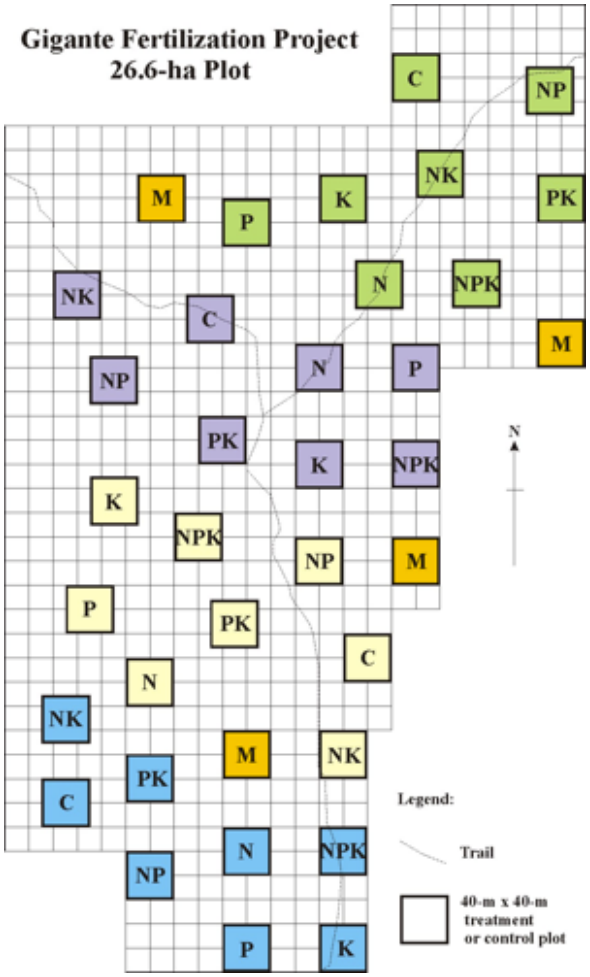

Figure S4. Map of study area showing the placement of nutrient treatments within the site. Treatments are represented by the combination of added nutrients: nitrogen (N), phosphorus (P), and potassium (K). Control plots (C) and micronutrient plots (M) are also shown. Colors represent replicates (N =4). We did not use the micronutrient treatment plots for this study.

Figure S5. Effects of antibiotics on epiphyte and endophyte abundance at month 0

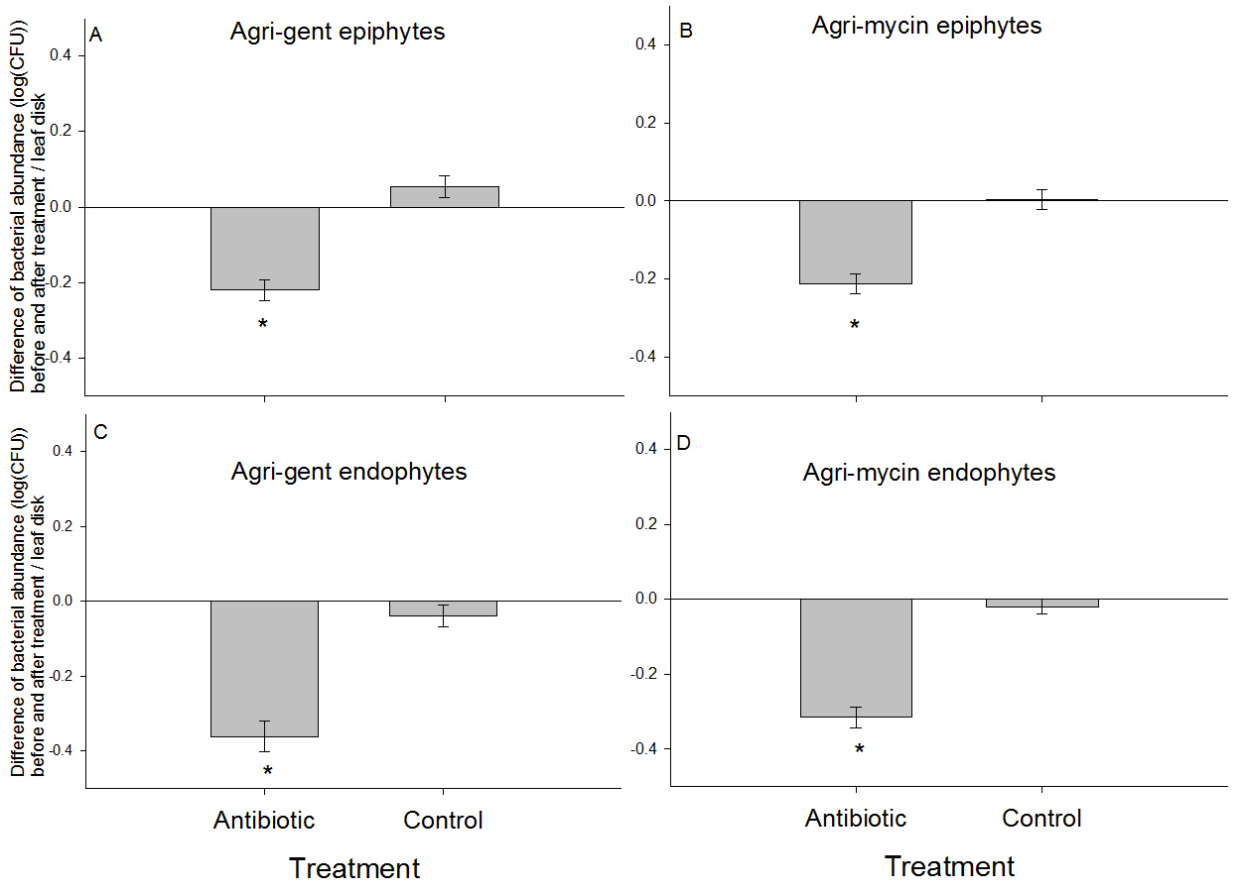

Figure S5. Effects of antibiotics or sterile water on epiphyte and endophyte bacterial abundance at month 0. When bars are above the line the treatment (antibiotic or control) increased plant relative growth rates, and decreased growth rates when the bars are below the line. We cultured bacterial communities on and inside leaves (endophytes) before antibiotic or control water application. We then cultured bacteria from the same leaves 2 days after applications. Panel A shows effects of Agry-gent and water control on epiphyte bacterial abundance (N = 315). Panel B shows effects of Agri-mycin and water control on epiphyte bacterial abundance (N = 315). Panel C shows effects of Agry-gent and water control on bacterial abundance inside leaves (N = 314). Panel D shows the effects of Agri-mycin on bacterial endophyte abundance (N = 315). Disparities among sample sizes reflect cultured plates that were contaminated and could not be used. Bars represent mean values (± 1 SE). All tree species and nutrient treatments are pooled because there were no soil nutrient or species differences in the degree to which antibiotics decreased abundance and diversity (with one exception, see Tables 1-8).

Figure S6. Effects of antibiotics on epiphyte and endophyte richness at month 0

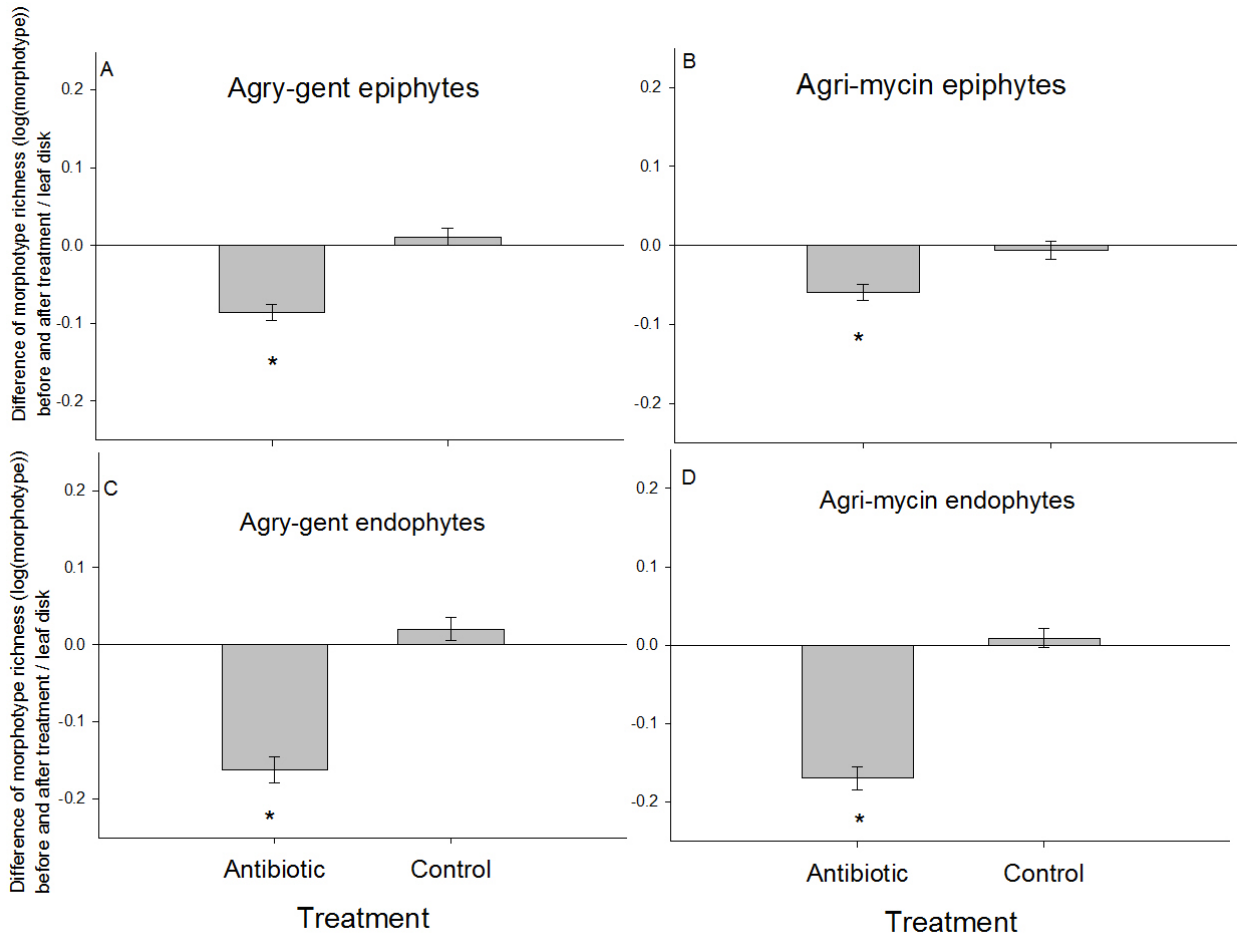

Figure S6. Effects of antibiotics or sterile water on epiphyte and endophyte morphotype richness at month 0. When bars are above the line the treatment (antibiotic or control) increased plant relative growth rates, and decreased growth rates when the bars are below the line. We cultured bacterial communities on and inside leaves before antibiotic or control water application and then resampled 2 days after applications. Panel A shows effects of Agry-gent and water control on epiphyte morphotype richness (N = 315). Panel B shows effects of Agri-mycin and water control on epiphyte morphotype richness (N = 315). Panel C shows effect of Agry-gent and water control on endophytic richness (N = 313). Panel D shows the effects of Agri-mycin on endophyte richness (N = 316). Disparities among sample sizes reflect cultured plates that were contaminated and could not be used. Bars represent mean values (± 1 SE). All tree species and nutrient treatments are pooled because there were no soil nutrient or species differences in the degree to which antibiotics decreased abundance and diversity (see Tables 1-8).

Figure S7. Effects of antibiotics on soil bacterial abundance and morphotype richness

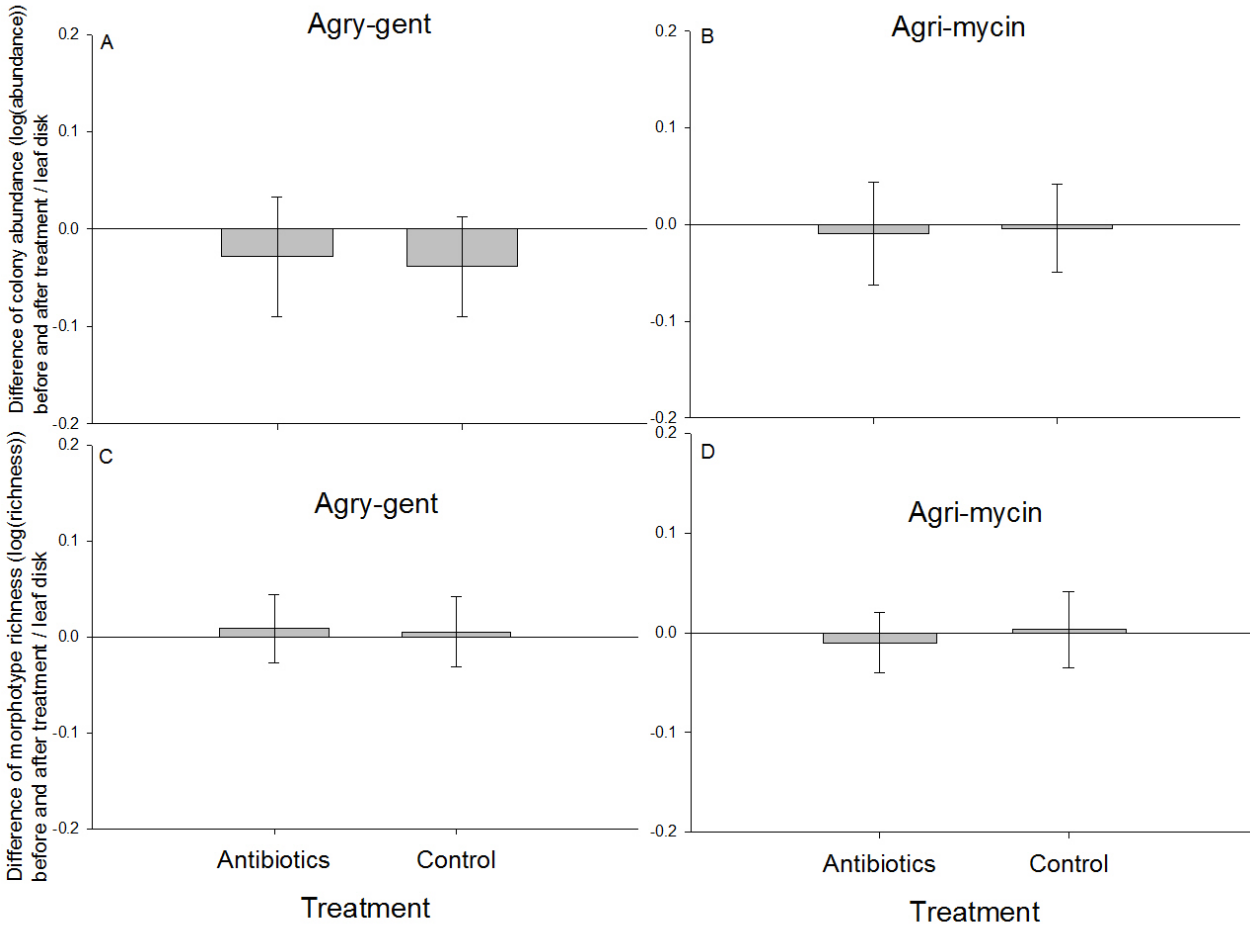

Figure S7. Effects of antibiotics or sterile water on soil bacterial abundance and morphotype richness. When bars are above the line the treatment (antibiotic or control) increased plant relative growth rates, and decreased growth rates when the bars are below the line. Panel A shows effects of Agry-gent and control water on soil colony abundance (N = 20). Panel B shows the effects of Agri-mycin and control water on colony abundance (N = 20). Panel C shows the effects of Agry-gent and control water on morphotype richness of soil bacteria (N = 20). Panel D shows the effects of Agri-mycin and control water on morphotype richness (N = 20). Bars represent mean values (± 1 SE). Soil samples from all tree species are pooled because there were no species differences in the degree to which antibiotics decreased abundance and diversity (see Tables 1-8).

Figure S8. Significant effects of P enrichment on relative growth rate

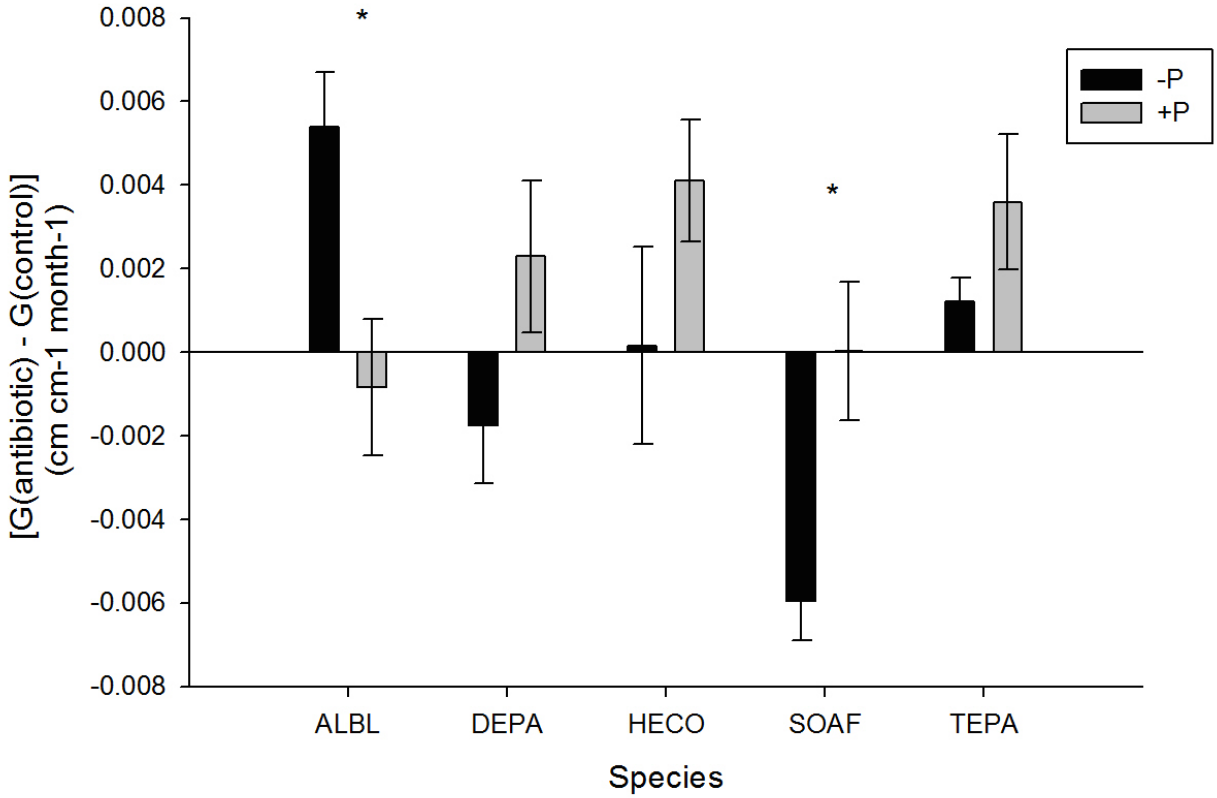

Figure S8. Significant effects of phosphorus (P) enrichment on mean relative growth rates of control and antibiotic treated seedlings after 29 months among *Alseis blackiana, Desmopsis panamensis*, *Heisteria concinna, Sorocea affinis*, and *Tetragastris panamensis* (* represents significant Tukey test species differences: Tukey tests: $P < 0.0041$ for *Alseis* and $P < 0.0037$ for *Sorocea*). Bars represent mean values per plot (± 1 SE). When bars are above the line antibiotic application increased plant relative growth rates and when below the line, antibiotic application decreased growth rates. Light bars represent values for plots where P was added (P, P + K, P + N, N + P + K, $N = 16$ plots). Dark bars represent values for plots where P was not added (C, N, K, N + K, $N = 16$ plots).

Figure S9. Effects of antibiotics on bacterial abundance and richness on and inside leaves at month 14

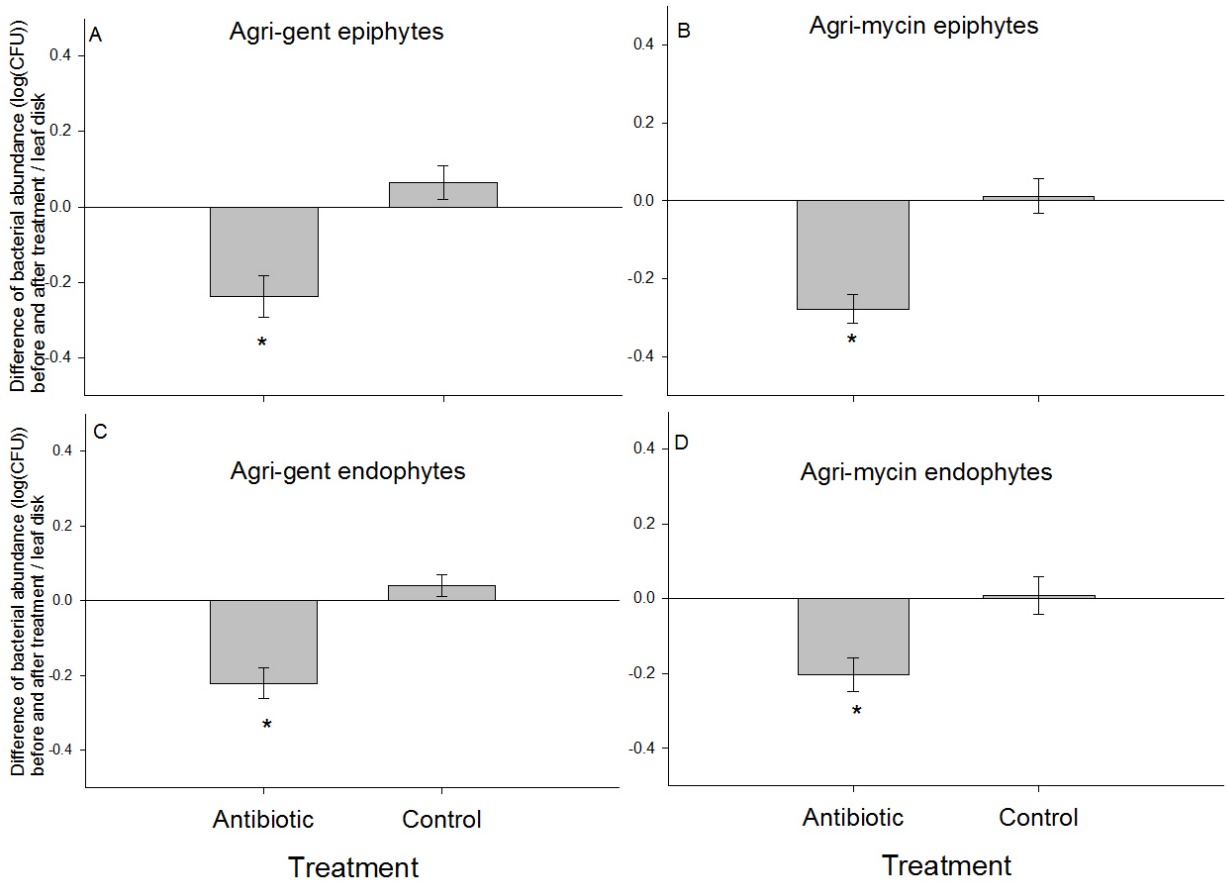

Figure S9. Effects of antibiotic applications or sterile water on bacterial abundance and morphotype richness on and inside leaves at month 14. When bars are above the line the treatment (antibiotic or control) increased plant relative growth rates, and decreased growth rates when the bars are below the line. We cultured bacterial communities on and inside leaves (endophytes) before antibiotic or control water application. We then cultured bacteria from the same leaves 2 days after applications. Panel A shows effects of antibiotics on epiphyte bacterial abundance (N = 39). Panel B shows effects of antibiotics on bacterial abundance inside leaves (N = 39). Panel C shows effects of antibiotics on morphotype richness on leaves (N = 39). Panel D shows the effects of antibiotics on morphotype richness inside leaves (N = 39). Bars represent mean values (± 1 SE). All tree species are pooled because there were no species differences in the degree to which antibiotics decreased abundance and diversity (see Tables 1-8).

Figure S10. Effects of antibiotics on bacterial abundance and richness on and inside leaves at month 23

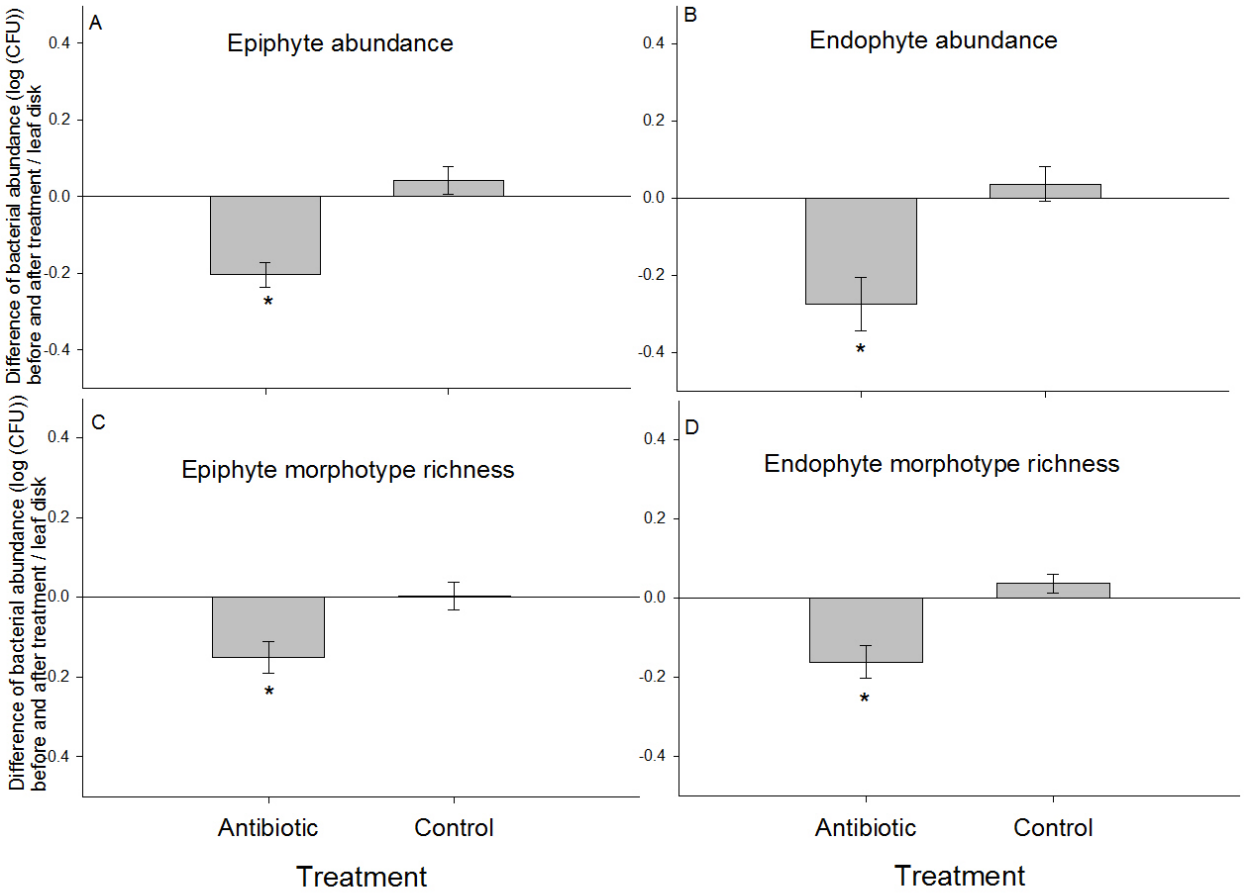

Figure S10. Effects of antibiotic applications or sterile water on bacterial abundance and morphotype richness on and inside leaves at month 23. When bars are above the line the treatment (antibiotic or control) increased plant relative growth rates, and decreased growth rates when the bars are below the line. We cultured bacterial communities on and inside leaves (endophytes) before antibiotic or control water application. We then cultured bacteria from the same leaves 2 days after applications. Panel A shows effects of antibiotics on epiphyte abundance (N = 39). Panel B shows effects of antibiotics on bacterial abundance inside leaves (N = 39). Panel C shows effects of antibiotics on morphotype richness on leaves (N = 39). Panel D shows the effects of antibiotics on morphotype richness inside leaves (N = 39). Bars represent mean values (± 1 SE). All tree species are pooled because there were no species differences in the degree to which antibiotics decreased abundance and diversity (see Tables 1-8).

Table S9. MANOVA results of Agry-gent effectiveness on epiphyte abundance

Table S9. MANOVA results of Agry-gent effectiveness on bacterial epiphyte abundance among nitrogen (N), phosphorus (P), and potassium (K) enrichments (log(abundance) before and after treatment; $N = 32$ plots).

Factor	Df value	F	P value
N	5, 20	0.45	0.8063
P	5, 20	1.07	0.4067
K	5, 20	1.21	0.3423
N × P	5, 20	1.91	0.1371
N × K	5, 20	1.89	0.1407
P × K	5, 20	1.16	0.3628
N × P × K*	5, 20	**3.16**	**0.0291**

Data presented are *P*-values for fixed effects. Bolded values are significant ($P < 0.05$).
*Tukey test: *Heisteria*: P = 0.0093.

Table S10. MANOVA results of Agri-mycin effectiveness on epiphyte abundance

Table S10. MANOVA results of Agri-mycin effectiveness on bacterial epiphyte abundance among nitrogen (N), phosphorus (P), and potassium (K) enrichments (log(abundance) before and after treatment; $N = 32$ plots).

Factor	Df value	F	P value
N	5, 20	1.09	0.3974
P	5, 20	0.63	0.6799
K	5, 20	0.53	0.7509
N × P	5, 20	0.70	0.6320
N × K	5, 20	0.94	0.4760
P × K	5, 20	0.33	0.8878
N × P × K	5, 20	0.25	0.9368

Data presented are *P*-values for fixed effects. Bolded values are significant ($P < 0.05$).

Table S11. MANOVA results of Agry-gent effectiveness on endophyte abundance

Table S11. MANOVA results of Agry-gent effectiveness on endophyte bacterial abundance among nitrogen (N), phosphorus (P), and potassium (K) enrichments (log(abundance) before and after treatment; $N = 32$ plots).

Factor	Df value	F	P value
N	5, 20	1.63	0.1979
P	5, 20	1.39	0.2702
K	5, 20	0.85	0.5294
N × P	5, 20	2.03	0.1181
N × K	5, 20	1.39	0.2709
P × K	5, 20	0.82	0.5498
N × P × K	5, 20	0.86	0.5218

Data presented are *P*-values for fixed effects. Bolded values are significant ($P < 0.05$).

Table S12. MANOVA results of Agri-mycin effectiveness on endophyte abundance

Table S12. MANOVA results of Agri-mycin effectiveness on endophyte bacterial abundance among nitrogen (N), phosphorus (P), and potassium (K) enrichments (log(abundance) before and after treatment; $N = 32$ plots).

Factor	Df value	F	P value
N	5, 20	2.47	0.0672
P	5, 20	0.80	0.5628
K	5, 20	1.53	0.2259
N × P	5, 20	1.64	0.1953
N × K	5, 20	0.39	0.8490
P × K	5, 20	0.39	0.8513
N × P × K	5, 20	1.49	0.2388

Data presented are *P*-values for fixed effects. Bolded values are significant ($P < 0.05$).

Table S13. MANOVA results of Agry-gent effectiveness on epiphyte richness

Table S13. MANOVA results of Agry-gent effectiveness on epiphyte morphotype richness among nitrogen (N), phosphorus (P), and potassium (K) enrichments (log(richness) before and after treatment; $N = 32$ plots).

Factor	Df value	F	P value
N	5, 20	0.38	0.8560
P	5, 20	0.69	0.6385
K	5, 20	0.79	0.5708
N × P	5, 20	1.16	0.3615
N × K	5, 20	0.32	0.8935
P × K	5, 20	0.68	0.6413
N × P × K	5, 20	0.13	0.9830

Data presented are *P*-values for fixed effects. Bolded values are significant ($P < 0.05$).

Table S14. MANOVA results of Agri-mycin effectiveness on epiphyte richness

Table S14. MANOVA results of Agri-mycin effectiveness on epiphyte morphotype richness among nitrogen (N), phosphorus (P), and potassium (K) enrichments (log(richness) before and after treatment; $N = 32$ plots).

Factor	Df value	F	P value
N	5, 20	1.59	0.2095
P	5, 20	2.46	0.0683
K	5, 20	0.64	0.6686
N × P	5, 20	0.46	0.8024
N × K	5, 20	0.70	0.6310
P × K	5, 20	0.48	0.7904
N × P × K	5, 20	1.45	0.2492

Data presented are *P*-values for fixed effects. Bolded values are significant ($P < 0.05$).

Table S15. MANOVA results of Agry-gent effectiveness on endophyte richness

Table S15. MANOVA results of Agry-gent effectiveness on endophyte morphotype richness among nitrogen (N), phosphorus (P), and potassium (K) enrichments (log(richness) before and after treatment; $N = 32$ plots).

Factor	Df value	F	P value
N	5, 20	1.32	0.2938
P	5, 20	0.78	0.5749
K	5, 20	0.62	0.6855
N × P	5, 20	1.80	0.1582
N × K	5, 20	1.23	0.3330
P × K	5, 20	1.42	0.2598
N × P × K	5, 20	1.47	0.2442

Data presented are *P*-values for fixed effects. Bolded values are significant ($P < 0.05$).

Table S16. MANOVA results of Agri-mycin effectiveness on endophyte richness

Table S16. MANOVA results of Agri-mycin effectiveness on endophyte morphotype richness among nitrogen (N), phosphorus (P), and potassium (K) enrichments (log(richness) before and after treatment; $N = 32$ plots).

Factor	Df value	F	P value
N	5, 20	2.22	0.0925
P	5, 20	0.98	0.4514
K	5, 20	1.51	0.2301
N × P	5, 20	2.13	0.1036
N × K	5, 20	1.32	0.2966
P × K	5, 20	0.41	0.8335
N × P × K	5, 20	0.59	0.7070

Data presented are *P*-values for fixed effects. Bolded values are significant ($P < 0.05$).

4.0 CRYPTIC INTERACTIONS BETWEEN SOIL NUTRIENTS AND FOLIAR BACTERIA CAUSE SUBSTANTIAL IMPACTS ON LEAF TRAITS AND ENEMY DAMAGE FOR SEEDLINGS IN A TROPICAL FOREST

4.1 INTRODUCTION

Understanding the mechanisms that underlie performance among seedlings and saplings in the shaded understory is critical because small differences in performance determine survivorship and thus the individuals that eventually reach the canopy (e.g., Chazdon & Fletcher 1984, Brown & Whitmore 1992, Boot 1996, Zagt & Werger 1998, Valladares & Niimemets 2008). Two of the biggest determinants of seedling and sapling performance are insects and pathogens, which can cause substantial host mortality early in ontogeny in tropical habitats (Coley & Barone 1996, Gilbert 2002). For example, as little as 8% leaf damage can cause up to 100% mortality among tropical seedlings (e.g., Clark & Clark 1985, reviewed by Coley & Barone 1996). Though empirical studies from more natural systems are lacking, findings from agroecosystems suggest that pathogens are ten times more abundant and cause 50 to 100% more damage in tropical habitats compared to temperate habitats (Wellman 1968, 1972, Hill & Waller

1982, Thurston 1998, Gilbert 2005, Griffin & Carson 2015). Because of this pressure, seedlings invest heavily in structural and chemical defenses in their leaves (Coley 1983, Augspurger 1983, Augsburger 1984, Wright & Cannon 2001, Gilbert 2002**,** Wright *et al.* 2004, Lusk *et al.* 2008). Thus, for seedlings, leaf production, leaf retention, and leaf protection are critical, yet experimental studies designed to address the mechanisms that underlie leaf production and retention are lacking.

While light availability has garnered most of the attention in tropical forest understories (e.g., Chazdon & Fletcher 1984, Kobe *et al.* 1995, Denslow 1987, Wright & van Schaik 1994, Coomes & Grubb 2000, Poorter 2001), recent experiments make it clear that low soil nutrient availability can limit seedling growth rates, photosynthetic capacity, and constrain tissue nutrient concentrations (Wright *et al.* 2011, Pasquini & Santiago 2012, Santiago *et al.* 2012, Pasquini *et al.* 2014). Oddly, these increases in seedling performance did not translate into increases in leaf production or leaf retention (Santiago *et al.* 2012) for 5 species of tropical seedlings. This could be because increases in performance were not sufficient to increase production or retention or that variation among species (e.g., some species gained leaves while other lost them) or vulnerability to enemies masked any change among co-occurring species. Thus, when it comes to plant species that co-occur in the understory, we do not understand how soil resource availability and enemies determine the degree to which seedlings produce new leaves or keep their leaves. For example, Santiago et al. (2012) demonstrated that experimentally increasing both phosphorus and potassium together increased mean herbivore damage for seedlings of five woody species in the shaded understory of a forest in Panama. Unfortunately, this study, while an important step forward, could not evaluate the degree that damage varied among species or whether other plant enemies (e.g., pathogens) mediated plant response to soil nutrient

availability. Though it has been demonstrated that soil nutrients limit plant performance in the shaded understory, our understanding of the degree that nutrient availability mediates leaf production and enemy impacts among co-occurring species is in its formative stages (Wright *et al.* 2011**,** Pasquini & Santiago 2012, Santiago *et al.* 2012, Pasquini *et al.* 2015). Here, we build upon these previous studies and evaluate whether species with sharply contrasting life histories vary in their response to soil resource availability and, in addition, evaluate the degree that foliar bacteria mediate these responses.

Though plant-associated bacteria are important mediators of plant phenotypes, the degree to which foliar bacteria mediate leaf production and enemy impacts has never been assessed empirically (Friesen *et al.* 2010, Turner *et al.* 2013). On one hand, foliar bacterial communities may primarily be mutualists and increase plant performance. For example, mutualistic foliar bacteria competitively exclude pathogenic fungi, produce plant hormones, and even induce systemic resistance to fungal pathogens (reviewed by Griffin & Carson 2015). Thus, decreasing foliar bacterial loads may decrease leaf production and increase enemy damage. On the other hand, foliar bacteria may primarily be pathogens and decrease plant performance. For example, Traw *et al.* (2007) demonstrated that foliar bacterial reduction increased *Arabidopsis thaliana* seed production by 55% in a temperate field. Indeed, foliar pathogens can cause premature leaf senescence and abscission in crop species and in *Arabidopsis thaliana* (e.g., Smart 1994, Buchanan-Wollaston 1997, Bleecker & Patterson 1997, Guo & Gan 2005, Lim *et al.* 2007, Wingler & Roitsch 2008). Understanding whether foliar bacteria mediate metrics of plant performance is important because bacteria are the most abundance colonizers of leaves, occurring in densities of up to 10 million cells/cm^2 (Lindow & Brandl 2003, Delmotte *et al.* 2009). Moreover, the shaded understory of tropical forests is likely an ideal habitat for bacteria

because temperature and humidity are high and UV radiation is low (reviewed by Griffin & Carson 2015). For example, Kembel *et al.* (2014) recently demonstrated that a single tree, on average, hosts over 400 operational taxonomic units (OTUs), or as many bacterial taxa as there are tree species on a 16km^2 island in Panama. The degree to which these bacteria are differentially inimical or beneficial to co-occurring plant host species, however, is unknown.

It is possible that soil resource supply rates mediate or even mask bacterial impacts to plant hosts in the shaded understory. It has only recently been demonstrated that woody seedlings in tropical forests are co-limited by soil nutrients (Wright *et al.* 2011**,** Pasquini & Santiago 2012, Santiago *et al.* 2012, Pasquini *et al.* 2015). Foliar bacteria may act as an additional component of soil nutrient-plant interactions and increase or decrease plant performance at different resource supply levels. For example, leaf fungal endophytes in particular can increase or decrease herbivore damage in graminoids and even decrease pathogen damage in tropical trees (Arnold *et al.* 2003; reviewed by Schardl *et al.* 2004, Tanaka *et al.* 2012, Faeth & Saari 2012). It is possible that foliar bacteria act similarly to fungal endophytes and mediate leaf production and interactions with enemies. Alternatively, foliar bacteria, like herbivores, may mask seedling responses to nutrient enrichment (*sensu* Andersen *et al.* 2010, Santiago *et al.* 2012). In a recent study, Santiago *et al.* (2012) demonstrated that N, P, and K enrichment did not cause a change in leaf production among seedlings in a tropical forest. We plan to go one step further and empirically assess whether foliar bacteria interact with soil nutrients to increase or decrease leaf production and enemy impacts for seedlings in the shaded understory. Ultimately, foliar bacterial communities may function as an independent yet cryptic plant functional trait that more finely structure plant life history strategies and interactions with enemies (Friesen *et al.* 2011, Turner *et al.* 2013, Kembel *et al.* 2014).

In this study, we test the following mutually compatible hypotheses: 1) The degree to which soil nutrients mediate net leaf number and enemy damage varies substantially among co-occurring plant species; 2) The degree to which foliar bacteria substantially impact leaf number and enemy damage varies substantially with soil nutrient supply (N, P, K); and 3) There are frequent interactions between soil nutrients and foliar bacteria which substantially impact leaf number and enemy damage among co-occurring host plant species. To address these hypotheses, we experimentally reduced foliar bacteria for 29 months for seedlings of 5 common woody species in a tropical forest in Panama. We nested seedlings within a fully factorial experiment where nitrogen, phosphorus, and potassium and all of their combinations were added to large replicated tropical forest plots in Panama for 15 years.

4.2 METHODS

4.2.1 Study site and fertilization experiment

The Gigante Peninsula Fertilization consists of ~ 200 year old secondary tropical forest on the Barro Colorado Nature Monument in Panama (9˚06'31''N, 79˚50'37''W; Fig. S16). The soil at the site is composed of endogleyic cambisols and acric nitisols (Koehler *et al.* 2009). Annual precipitation averages 2,600 mm, of which less than 10% falls during the 4-month dry season between January and April.

We applied nitrogen (N), phosphorus (P), and potassium (K) in 40 × 40 m plots in a 2 × 2 × 2 factorial design (Fig. S16; see also Pasquini & Santiago 2012, Santiago *et al.* 2012, Pasquini *et al.* 2014). We replicated each soil treatment four times, and all but two plots were separated

by at least 40 m. We applied fertilizer by hand four times a year beginning in 1998 between June and November. We applied 125kg N ha^{-1} $year^{-1}$ as urea, 50 kg P ha^{-1} $year^{-1}$ as triple super-phosphate, and 50 kg K ha^{-1} $year^{-1}$ as KCl anually.

4.2.2 Study species

We selected five common woody species from five different families located throughout the site: *Alseis blackiana* (Rubiaceae), *Desmopsis panamensis* (Annonaceae), *Heisteria concinna* (Olacaceae), *Sorocea affinis* (Moraceae) and *Tetragastris panamensis* (Burseraceae). We will henceforth refer to each species by genus. Nomenclature follows Croat (1978) and Garwood (2009). Though *Alseis* is a pioneer species, all five species are relatively shade tolerant as seedlings, vary in life history traits, and span a wide range of maximum adult heights (Wright *et al.* 2003; Gilbert *et al.* 2006, Wright *et al.* 2010). Alseis is a mid-canopy tree, *Sorocea* is a shrub to small tree, *Desmopsis* and *Heisteria* are understory treelets, and *Tetragastris* is a canopy tree. For further details, see Croat (1978) and Wright *et al.* (2010).

4.2.3 Antibiotic applications

We randomly assigned three ~20-30cm tall seedlings of each species within the inner 30 x 30 m of each plot for antibiotic and three for control (sterile water) treatments. Beginning in 2010, we sprayed seedling leaves with antibiotics or sterile water to saturation every 10-15 days for 29 months. We placed a plastic sheet around the base of each seedling to prevent exposing soil microbes to either treatment and left the sheet to prevent liquid dripping to the ground. Even

so, we previously confirmed that neither antibiotic nor water applications caused a difference in soil bacterial abundance or richness (see Griffin *et al.* in review). We alternated the antibiotic treatments between streptomycin (100ppm of Agri-mycin 17, Hummert International #02-0150; Earth City, MO) and oxytetracycline (1752 ppm of Agry-Gent Plus 800, Química Agronómica de México, Chihuahua, México). Streptomycin and oxytetracycline inhibit protein synthesis for both Gram-positive and Gram-negative bacteria and are two of the most commonly used broad-spectrum antibiotics in temperate and tropical agriculture (MacManus *et al.* 2002, Vidaver 2002). Additionally, both products are highly effective in the field (McManus *et al.* 2002; Traw *et al.* 2007, Griffin *et al.* in review).

4.2.4 Seedling measurements

Leaf number and retention. We recorded the total number of leaves on each seedling at the beginning of the experiment and after 29 months of antibiotic or control treatments. In addition, we recorded leaf retention rate after 14 months for leaves used to estimate enemy damage (N = 2983, see below). We recorded leaf retention and damage after only 14 months because almost all leaves (~98%) had fallen before the end of the experiment (29 months).

Enemy damage. We followed protocols of Schnitzer *et al.* (2002) and Mangan *et al.* (2010) to estimate percent leaf area removed by leaf-chewing herbivores and percent affected by chlorosis or lesions (pathogen and scale damage). We randomly selected and marked four leaves from each seedling (or as many as were present) and estimated enemy damage at the beginning of the experiment and after 14 months of applications. We based percent loss estimations on a template of artificial (paper) leaves with 24 levels of damage: 0%, 1%, 2.5%, 5%, 7.5%, 10%, and in 5% increments up to 100% area removed (Carson & Root 2000, Schnitzer *et al*., 2002). It

is important to note that while some insect damage may cause lesions and chlorosis (Miller & Davidson 2005), the primary cause of this type of damage is due to fungi, bacteria, and viruses (e.g., Garcia-Guzman & Dirzo 2001, Myster 2002, Griffin, *personal observation*). Thus, hereafter we will refer to percent area loss by leaf-chewing insects as "herbivore damage" and chlorosis and lesions as "pathogen damage."

4.2.5 Statistical analyses

We performed a MANOVA to evaluate net leaf number differences among antibiotic- and control-treated seedlings among soil nutrient additions after 29 months of applications. We calculated net leaf number ($leaf^{-1}$ $month^{-1}$) for each seedling as

$$L = (\ln L_1 - \ln L_0)/(t_1 - t_0)$$

where L_0 and L_1 were initial and final leaf number and t_1-t_0 was the time period in months (Santiago *et al.* 2012). The average ($\bar{L}$) for each species in a plot was simply the mean of the values of L over the three conspecific plants (antibiotic treated or untreated). Next, we calculated the difference in average net leaf number for each species (i) with or without antibiotic application in each plot as:

$$\delta \bar{L}(i) = \bar{L}\ (i, \text{antibiotic}) - \bar{L}\ (i, \text{control}).$$

Because all 5 species were nested (and non-independent) within each replicate plot, our response vector for plot j was:

$$\delta \bar{L}_j = (\delta \bar{L}(1), \delta \bar{L}(2), \delta \bar{L}(3), \delta \bar{L}(4), \delta \bar{L}(5))_j$$

where the numbers 1 through 5 refer to the five plant species (or four depending on the performance metric). A MANOVA of this response vector tests whether net leaf number differences between control and antibiotic treated plants differed across nutrient treatments and

adjusts for correlated response variables (e.g., Myster 2002). Post-hoc Tukey studentized range tests with corrected significance values (α = 0.05) then provide insights into which species differed in their responses to the antibiotics across nutrient treatments. We chose this approach specifically to avoid pseudofactorialism, or misidentifying response variables as levels of an experimental variable (Hurlbert 2013).

For herbivory and pathogen damage analyses, we used the same equation above to calculate insect herbivory ($\bar{H}$) and pathogen (and insect) ($\bar{P}$) damage rates. Because *Alseis* seedlings drop and flush all of their leaves at the end of the dry season (Lovelock *et al.* 1998, Dalling *et al.* 2001), we did not use this species for either herbivore or pathogen analysis.

To determine whether the magnitude of the impact of foliar bacteria on net leaf number, herbivore damage, and pathogen damage varied substantially among co-occurring plant species, we ran independent ANOVAs for each variable followed up with post-hoc Tukey tests.

4.3 RESULTS

4.3.1 The impacts of nutrient enrichment on leaf number, retention, and enemy damage

Leaf number. The degree to which soil nutrients mediated leaf number varied substantially among species. For *Alseis*, K addition decreased leaf number by 88%; whereas for *Desmopsis*, K addition increased leaf number by 50% (Fig. 11A, $F_{1,31}$ = 23.58, P = < 0.0001; $F_{1,31}$ = 5.16, P = 0.0323). Moreover, for *Desmopsis*, P addition decreased leaf number by 52% in the absence of N addition but increased leaf number by 42% with N addition (N x K interaction, Fig. 1B, $F_{1,31}$ = 7.90, P = 0.0097). Finally, for *Alseis*, K additions decreased leaf

number by over 100% compared to when K was not added. This effect, however, only occurred when N was not added (N x K interaction, Fig. 11C, $F_{1,31} = 5.98$, $P = 0.022$). Overall, changes in leaf number were strongly dependent on soil resource supply.

Leaf retention. The degree to which soil nutrients impacted leaf retention varied among species. For *Sorocea*, N addition decreased retention by 38%, but this only occurred in the absence of P addition (N x P interaction, Fig. 12A, $F_{1,31} = 13.07$, $P = 0.0014$). For *Heisteria*, P addition and K addition each decreased leaf retention by 17%, however particular combinations of N, P, and K caused decreases and increases in retention by as much as 35% (P: $F_{1,31} = 7.07$, $P = 0.0137$; K: $F_{1,31} = 6.69$, $P = 0.0162$; N x P x K interaction, Fig. 12B, $F_{1,31} = 5.08$, $P = 0.0337$). For *Tetragastris*, N addition decreased leaf retention by 30%, however particular combinations of N, P, and K caused decreases and increases in retention by as much as 49% (N: $F_{1,31} = 13.78$, $P = 0.001$; N x P x K interaction, Fig. 12C, $F_{1,31} = 5.76$, $P = 0.0245$). Overall, rates of leaf retention were strongly dependent on soil resource supply.

Herbivore and pathogen damage. Although there were no main effects of nutrient addition on herbivore or pathogen damage rates, there were significant interactions between resource supply enemy damage for *Desmopsis* and *Tetragastris* (Supp. Tables S17-S18). Specifically, K addition increased herbivore damage for *Desmopsis* by 88% and P addition increased herbivore damage for *Tetragastris* by 46% (Figs. 13A and 3B; $F_{1,30} = 6.25$, $P = 0.020$; $F_{1,30} = 5.06$, $P = 0.0343$). In addition, when P was not added, K addition caused a 49% increase in pathogen damage rates for *Desmopsis*; however, K addition decreased pathogen rates by 33% when P was added (P x K interaction, Figure 13C; $F_{1,30} = 7.50$, $P = 0.0117$). Overall, rates of damage were strongly dependent on soil resource supply.

4.3.2 The impacts of antibiotic applications on leaf number, retention, and enemy damage

Leaf Number. Overall, antibiotic applications caused significant increases in leaf number but this varied substantially because of interactions among species and nutrient treatments. Specifically, for *Alseis,* applying antibiotics increased leaf number by 85% but this only occurred when N was not added (Fig. 14A, $F_{1,31} = 11.39$, $P = 0.0025$). Moreover, for *Alseis*, applying antibiotics decreased leaf number by 57% when K was not added but in sharp contrast increased leaf number by 47% when we added K (Fig. 14B, $F_{1,31} = 52.44$, $P < 0.0001$). For *Desmopsis*, antibiotic applications decreased leaf number by 67% without P addition but with P addition antibiotics increased leaf number by 90%. These impacts of antibiotics on P addition, however, only occurred when we did not add N (N x P interaction, Fig. 14C, $F_{1,31} = 12.80$, $P = 0.0015$). For *Tetragastris*, antibiotics decreased leaf number by 39% without P addition but with P addition antibiotics increased leaf number by 98%; however this only occurred when N was not added. Conversely, when N was added, antibiotics increased leaf number by 204% without P addition but with P addition antibiotics increased leaf number by only 21% (N x P interaction, Fig. 14D, $F_{1,31} = 13.76$, $P = 0.0011$). Finally, for *Sorocea*, antibiotics increased leaf number by 60% when K was added. The K effect, however, only occurred when we did not add N (N x K interaction, Fig. 14E, $F_{1,31} = 4.67$, $P = 0.0409$).

Overall, while these findings are complex, the take-home message is clear: the degree to which bacteria caused major changes in leaf number (as much as 204%) depended entirely on soil resource supplies and host species identity.

Leaf retention. Antibiotic applications caused significant decreases in leaf retention for *Tetragastris* but this varied substantially with nutrient additions. Specifically, antibiotic applications decreased leaf retention rates by 13% when N was added (Fig. 15A, $F_{1,31} = 5.37$, $P =$

0.0293). Moreover, antibiotic applications decreased leaf retention by 17% when K was added, but this only occurred when we did not add P. Conversely, antibiotic applications decreased leaf retention rates by 12% when P was added, but this only occurred when we did not add K (P x K interaction, Fig. 15B, $F_{1,31} = 5.25$, $P = 0.0311$).

Herbivore and pathogen damage. The effects of applying antibiotics on herbivore and pathogen rates depended on the presence or absence of N and K addition (Supp. Table S19, $F_{4,20} = 3.49$, $P = 0.0257$). For *Heisteria*, antibiotic applications decreased herbivore damage by 8% when N was not added but increased herbivore damage by 21% when N was added (Fig. 15C, $F_{1,30} = 5.29$, $P = 0.0309$). For *Tetragastris*, antibiotic applications decreased herbivore damage by 25% when K was not added but K addition increased herbivore damage by 38%; however the effects of K only occurred when we added N (N x K interaction, Fig. 15D, $F_{1,30} = 5.05$, $P = 0.0344$). In addition, for *Sorocea*, antibiotic applications increased pathogen damage by 29% in the absence of K but decreased pathogen damage by 35% when K was added (Fig. 15E, $F_{1,30} = 4.92$, $P = 0.0368$).

Overall, we demonstrate that combinations of N, P, and K cause substantial differences in leaf number and retention, and these effects differed among species. In addition, N, P, and K mediate herbivore and pathogen damage among plant species. Moreover, interactions between soil nutrients and antibiotic applications determine species-specific differences in leaf number, retention, and enemy impacts. For example, for *Tetragastris*, antibiotic applications decreased the magnitude of particular performance metrics by up to 15% but increased others by up to 82% depending on the presence or absence of N addition (Fig. S19). For Figures summarizing the impacts of interactions between soil nutrients and antibiotic applications on performance metrics for *Heisteria*, *Sorocea*, and *Tetragastris*, and *Desmopsis*, see Figs. S17-S20.

4.4 DISCUSSION

Here, for the first time, we demonstrate unequivocally that cryptic interactions among soil nutrients and foliar bacteria cause substantial changes in performance and enemy impacts among seedlings of co-occurring species in deep shade. Overall, we found strong support for all three of our hypotheses (see introduction). Specifically, soil nutrients caused substantial impacts on leaf number, retention and enemy damage among plant species (H1); interactions between soil nutrients and foliar bacteria caused substantial impacts on leaf number, retention and enemy damage (H2); and interactions between soil resources and foliar bacteria mediate leaf number, retention, and enemy damage among plant species (H3). Our results are contrary to those of Santiago *et al.* (2012), who demonstrated in the same system that soil nutrients had no effect on leaf number across the same five species. This is not meant to be a critique of Santiago *et al.* (2012) because our data suggest that microbial communities represent an entirely unexplored dimension of plant performance variation. Indeed, we found that interactions between soil nutrients and bacteria lead to sharp contrasts (by sometimes more than 200%) in leaf number, retention as well as enemy damage among co-occurring plant species. Thus, we suggest that without considering microbial communities, it is difficult to understand the causes of plant performance at larger scales. Overall, our data suggest that interactions among soil nutrients and foliar bacteria may have the potential to alter the rank-order performance of coexisting plant species in deeply shaded forests. Taken together, we *suggest* that foliar bacteria may interact with soil fertility to comprise another unexplored, yet important dimension of niche differentiation for coexisting woody species.

4.4.1 Bacterial-plant interactions: critical determinants of understory plant performance

We demonstrated that soil nutrients as well as their interactions with foliar bacteria mediated leaf number and enemy impacts for seedlings in deep shade. Surprisingly, Santiago *et al.* (2012) did not detect any impact of long-term nutrient enrichment on leaf number. Once we looked more closely at individual species and bacterial loads, we found large differences in leaf traits and enemy damage (by up to 200%). Recent studies have proposed that herbivore impacts can mask or exacerbate seedling responses to nutrient enrichment (e.g., Andersen *et al.* 2010, Santiago *et al.* 2012). He, we demonstrate unequivocally that foliar bacteria do just that. Specifically, our results demonstrate that it might be impossible to make conclusions about the factors that structure plant distributions at large scales without considering the impacts of microbial communities. Thus, our results suggest that foliar bacteria more finely mediate the degree to which soil resource availability impacts leaf traits among co-occurring species, which may have critical implications for species distributions at larger scales (see below). Ultimately, we argue that the foliar microbiome is a completely independent plant functional and represents a critical component in driving plant performance (e.g., Kembel *et al.* 2014, Griffin *et al.* in review).

4.4.2 Potential mechanisms and future directions

Though the mechanisms whereby foliar bacteria and their interactions with soil nutrients mediate plant performance *in situ* are largely unexplored, we suggest potential explanations and future work. For three out of five host species, we found that antibiotic applications increase leaf number. Foliar bacteria, like fungal pathogens, may cause leaf abscission, a process by which

plants "decide" to shed leaves after infection (Ostry 1987, Eyal *et al.* 1993, Patterson 2001, Davidson *et al.* 2011). Indeed, *Arabidopsis thaliana* upregulate genes associated with abscission in response to pathogen infection (Volko *et al.* 1998, Kubigsteltig *et al.* 1999). Recently, Busby *et al.* (2013) demonstrated in a greenhouse experiment that interactions between fungal endophytes and a known fungal pathogen caused a two-fold increase in leaf abscission rates in *Populus augustifolia*. Thus, reducing bacterial abundance may release the bacterial burden for seedlings and may result in substantially lower abscission rates. Conversely, for some species under and combinations of soil nutrient additions, bacterial reductions reduced leaf number. Here, bacteria may function to some degree as mutualists and thus decreasing their abundance and richness may constrain leaf production. For example, mutualistic foliar bacteria competitively exclude pathogenic fungi by producing plant hormones to increase plant host performance and can even induce systemic resistance to fungal pathogens (reviewed by Griffin & Carson 2015). Thus, decreasing the abundance of these bacteria may force plants to allocate more resources elsewhere (e.g., defense) instead of leaf production (e.g., Coley *et al.* 1985, Bazzaz *et al.* 1987). It is important to note that we demonstrates only the *net* effects of antibiotic applications, which reduce bacterial abundance and richness by ~50% in this forest (Griffin *et al.* in review). Moreover, we fully acknowledge that that our results may be due to changes in bacterial community structure rather than reduced abundance or diversity. Thus, future culture-independent methods can assess how bacterial communities respond to antibiotic applications and link particular bacterial taxa to mechanisms of leaf production. Either way, our findings still have the same implication: foliar bacteria are critical components that determine plant performance among seedlings in deeply shaded habitats.

We suggest that future studies address the mechanisms by which bacterial reduction enhances or diminishes enemy impacts. Plants can synthesize a broad range of secondary metabolites such as proteins, phenolics, and alkaloids that have deleterious effects on both pathogens and insects; thus, bacterial infection may also thwart insect enemies (Tierens *et al.* 2001, Wittstock & Halkier 2002, Thomma *et al.* 2002, Haq *et al.* 2004). On the other hand, pathogens and herbivores trigger mutually inhibitory jasmonic and salicylic acid pathways in *Arabidopsis* (e.g., Traw *et al.* 2003, reviewed by Stout *et al.* 2006, Pieterse *et al.* 2009). Thus, negative cross talks between the two pathways may result in trades-offs in resistance to microbial pathogens and insect herbivores (Glazebrook 2005, Koornneef & Pieterse 2008). Alternatively, because bacteria compete for limiting resources on the phyllosphere, reducing foliar bacteria may simply open up niche space and resources for other beneficial *or* pathogenic bacteria and fungi to colonize (e.g., Paine 1966, reviewed by Griffin & Carson 2015). Finally, because it is difficult to distinguish between bacterial and fungal damage on leaves in the field, studies to date have often categorized bacterial and fungal damage together when assessing foliar pathogen damage (e.g., Coley & Barone 1996, Garcia-Guzman & Dirzo 2001, Myster 2002, Mangan *et al.* 2010). Further studies should disentangle fungal and bacterial damage via high-throughput sequencing to determine the differential effects of bacteria and fungi.

4.4.3 Implications for plant diversity

Though studies have demonstrated that soil nutrient availability decreases realized niche space of co-occurring species, whether soil nutrients alone can maintain hyper-diversity of plant species in tropical forests remains uncertain (e.g., Hubbell 1999, 2001; reviewed by Wright

2002, Silvertown 2004, Kitajima & Poorter 2008). To date, the mechanism underlying this niche dimension is often assumed to be direct resource competition, where different plant species are better or worse competitors along resource gradients (Tilman 1982). Thus, variations in soil nutrient availability favor some species but not others as they contrast spatially throughout the ecosystem. Here, we propose an entirely new dimension for niche differentiation whereby interactions between plants and their foliar bacteria are mediated by soil resource availability. Thus, plant-bacterial interactions more finely partition niche space among coexisiting tree species in the forest along a familiar niche axis (e.g., soil nutrient availability), which functions to maintain plant diversity.

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4.7 TABLES AND FIGURES

Figure 11. The impacts of nutrient enrichment on leaf number

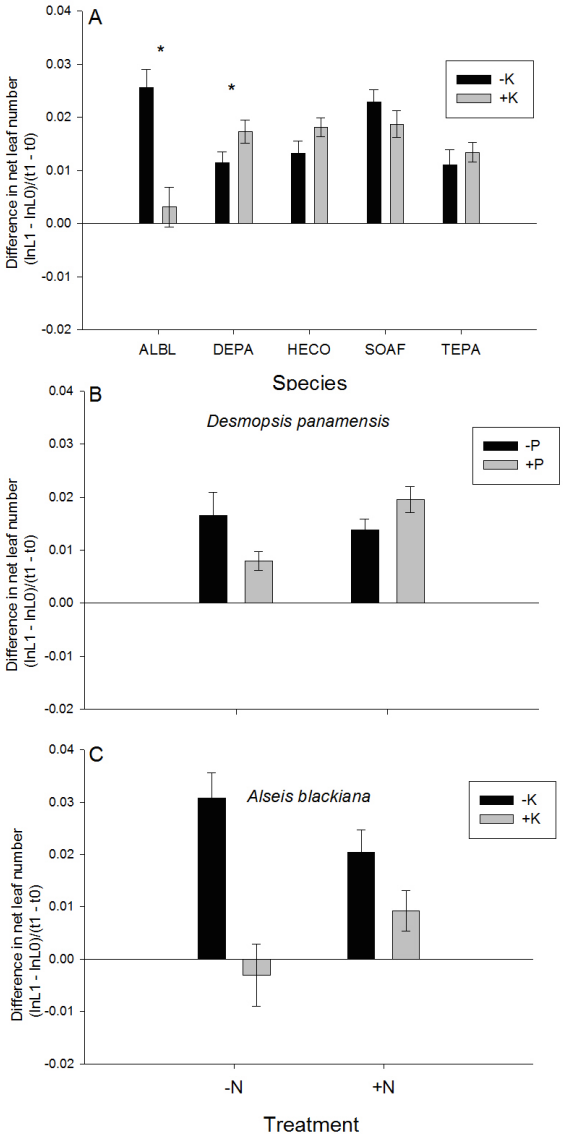

Figure 11. Significant effects of nutrient additions on net leaf number of seedlings after 29 months. When bars are above the line treatments increased leaf number and when below the line, treatments decreased leaf number. Panel A shows the significant effects of K addition on leaf number for *Alseis blackiana* and *Desmopsis panamensis* ($F_{1,31}$ = 23.58, P = < 0.0001; $F_{1,31}$ = 5.16, P = 0.0323). Panel B shows a significant N x P interaction on leaf production rates for *Desmopsis* ($F_{1,31}$ = 7.90, P = 0.0097). Panel C shows a significant N x K interaction on leaf production rates for *Alseis* ($F_{1,31}$ = 5.98, P = 0.022). Bars represent mean values (± 1 SE).
Figure 4.2.

Figure 12. The impacts of nutrient enrichment on leaf retention

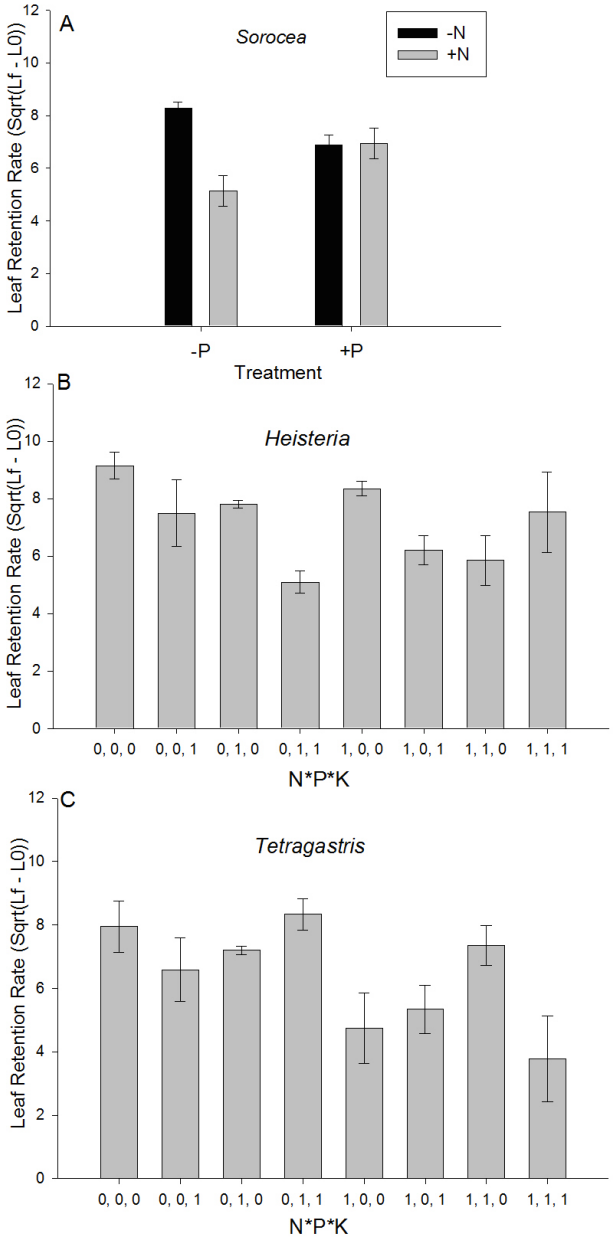

Figure 12. Significant effects of nutrient additions on leaf retention rates among seedlings after 14 months. Panel A shows the significant N x P interaction on leaf retention for *Sorocea affinis*, whereby N decreased retention only in absence of P addition ($F_{1,31} = 13.07$, $P = 0.0014$). Panel B shows a significant N x P x K interaction interaction on leaf retention for *Heisteria concinna* ($F_{1,31} = 5.08$, $P = 0.0337$). Panel C shows a significant N x P x K interaction on leaf retention for *Tetragastris panamensis* ($F_{1,31} = 5.76$, $P = 0.0245$). Bars represent mean values (± 1 SE).

Figure 13. The impacts of nutrient enrichment on enemy damage

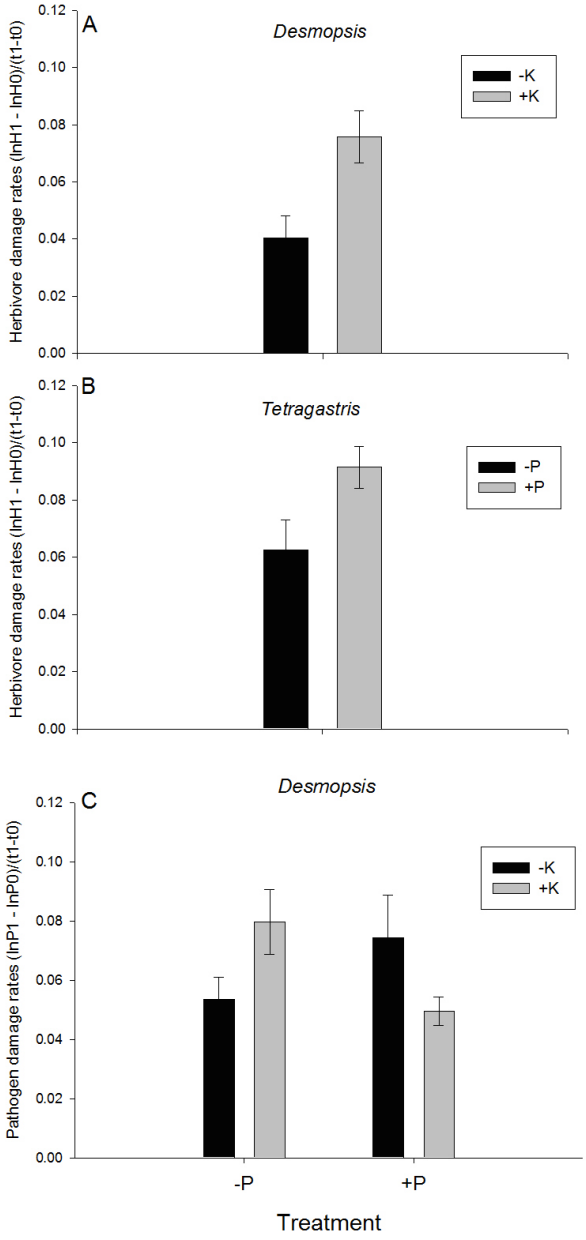

Figure 13. Significant effects of nutrient additions on herbivore and pathogen damage rates among seedlings after 14 months. Panel A shows the significant effects of K addition on herbivore damage for *Desmopsis panamensis* ($F_{1,30}$ = 6.25, P = 0.020). Panel B shows the significant effect of P addition on herbivore rates for *Tetragastris panamensis* ($F_{1,30}$ = 5.06, P = 0.0343). Panel C shows a significant P x K interaction on pathogen damage rates for *Desmopsis* ($F_{1,30}$ = 7.50, P = 0.0117). Bars represent mean values (± 1 SE).

Figure 14. The impacts of antibiotic applications on leaf number

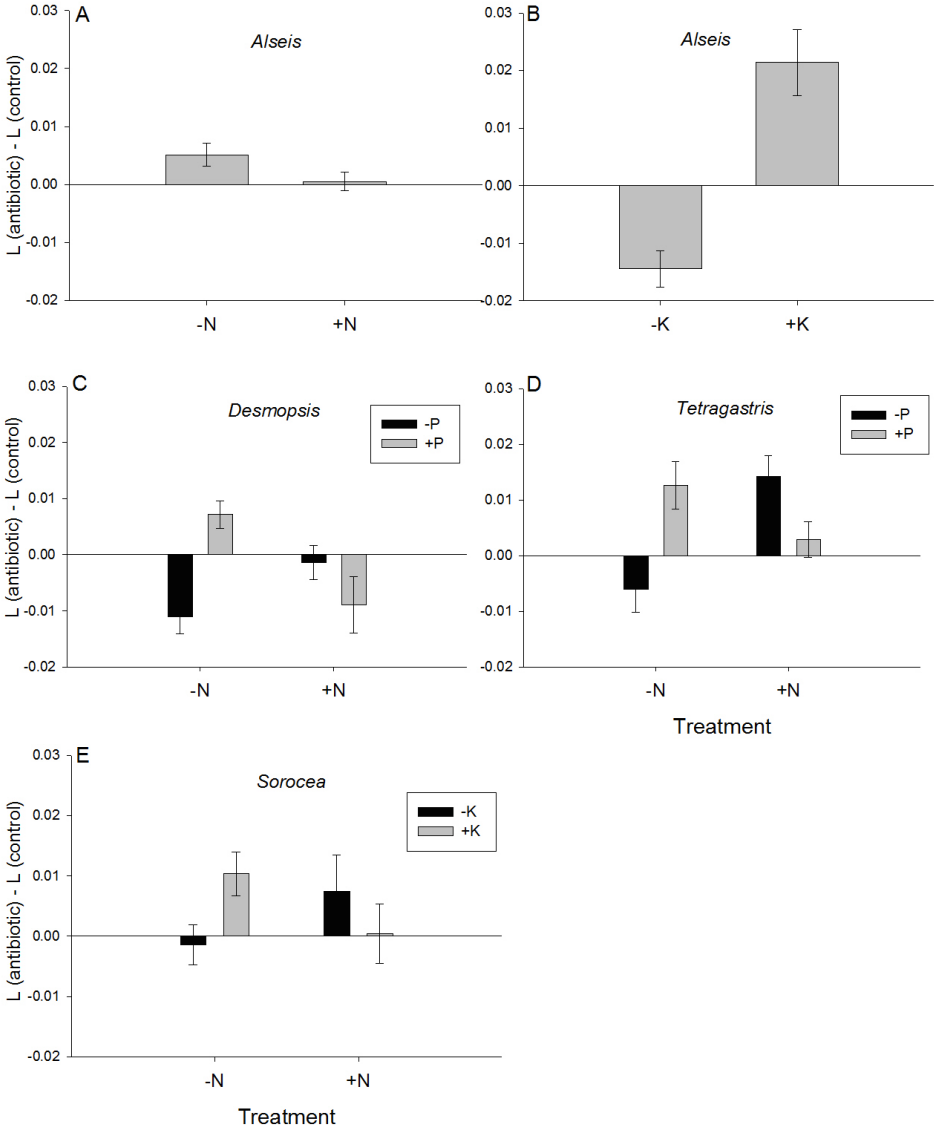

Figure 14. Significant effects of antibiotic applications on leaf number among species and nutrient treatments. When bars are above the line antibiotic applications increased leaf number and when below the line, antibiotic applications decreased leaf number. Panel A illustrates that, for *Alseis blackiana*, applying antibiotics increased leaf number when N was not added ($F_{1,31}$ = 11.39, P = 0.0025). Panel B illustrates that, for *Alseis*, applying antibiotics decreased leaf number when K was not added but increased leaf number when we K was added ($F_{1,31}$ = 52.44, P < 0.0001). Panel C illustrates a significant N x P interaction on the degree to which antibiotics impacted leaf number for *Desmopsis panamensis* ($F_{1,31}$ = 12.80, P = 0.0015). Panel C illustrates a significant N x K interaction on the degree to which antibiotics impacted leaf number for *Tetragastris panamensis* ($F_{1,31}$ = 13.76, P = 0.0011). Finally, Panel E illustrates a significant interaction on the degree to which antibiotics impacted leaf number for *Sorocea affinis* ($F_{1,31}$ = 4.67, P = 0.0409).

Figure 15. The impacts of antibiotic applications on leaf retention and enemy damage

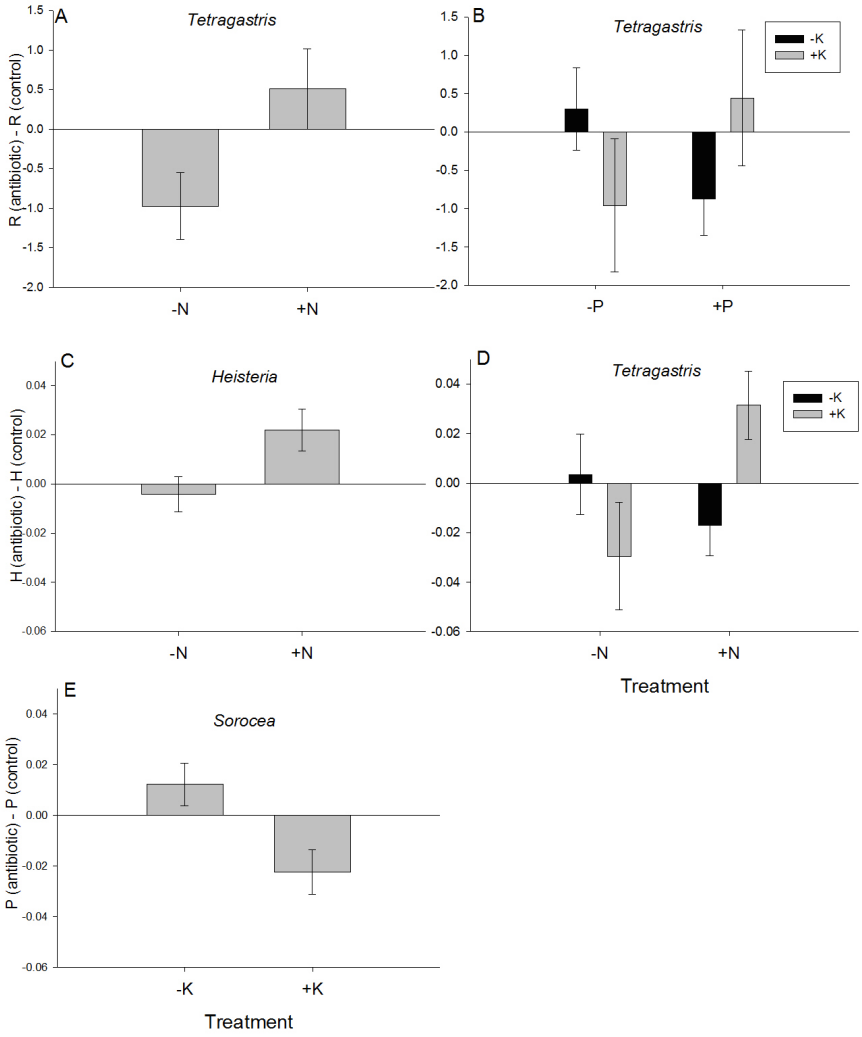

Figure 15. Significant effects of antibiotic applications on leaf retention and enemy damage among plant species and nutrient treatments after 14 months. When bars are above the line antibiotic applications increased leaf number, retention, or enemy damage. When below the line, antibiotic applications decreased leaf number, retention, or enemy damage. Panel A illustrates that N enrichment decreased leaf retention for *Tetragastris panamensis* ($F_{1,31}$ = 5.37, P = 0.0293). Panel B illustrates a significant P x K interaction on the degree to which antibiotics impacted leaf retention for *Tetragastris* ($F_{1,31}$ = 5.25, P = 0.0311). Panel C illustrates the significant effects of N enrichment on herbivore damage for *Heisteria concinna* ($F_{1,30}$ = 5.29, P = 0.0309). Panel D illustrates a significant N x P interaction on the degree to which antibiotics impacted herbivore damage for *Tetragastris* ($F_{1,30}$ = 5.05, P = 0.0344). Finally, Panel E illustrates the significant K effect on pathogen damage for *Sorocea affinis* (Fig. 5E, $F_{1,30}$ = 4.92, P = 0.0368). Bars represent mean values (± 1 SE).

4.8 SUPPLEMENTARY MATERIALS

Figure S16. The Gigante Fertilization Experiment

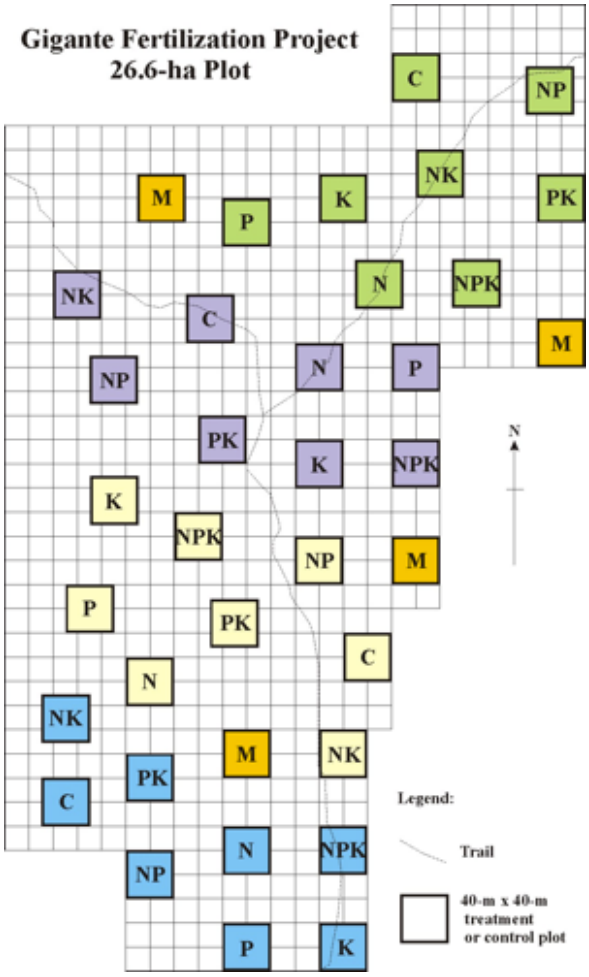

Figure S16. Map of study area showing the placement of nutrient treatments within the site. Treatments are represented by the combination of added nutrients: nitrogen (N), phosphorus (P), and potassium (K). Control plots (C) and micronutrient plots (M) are also shown. Colors represent replicates (N =4). We did not use the micronutrient treatment plots for this study.

Figure S17. Effects of antibiotics and N, P, K for *Heisteria concinna*

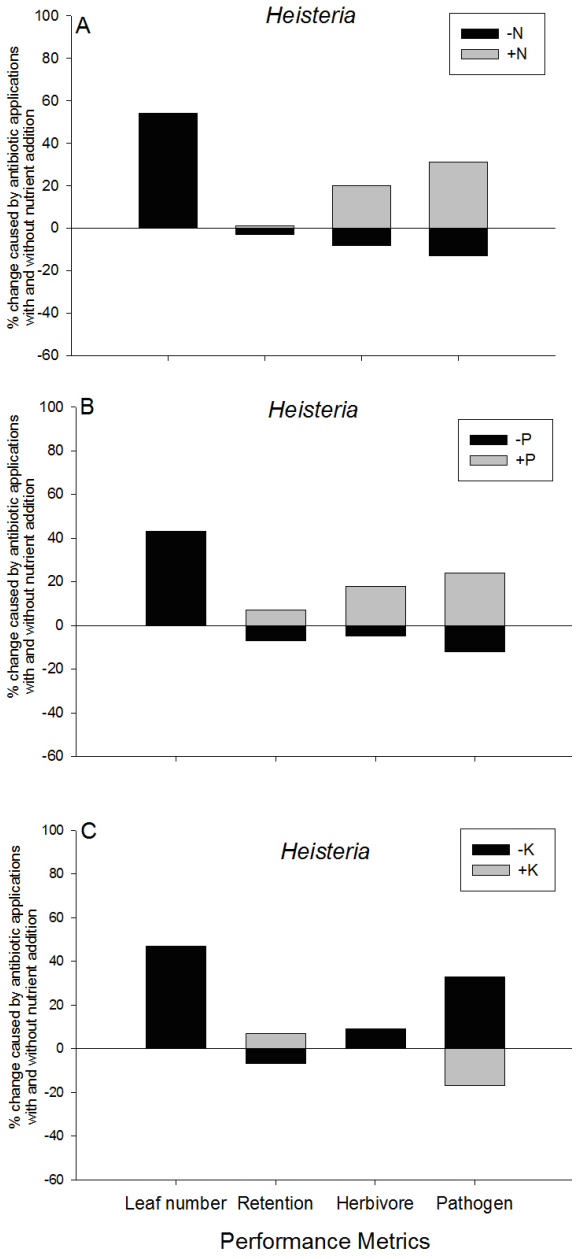

Figure S17. The effects of antibiotic applications on leaf number, retention, herbivore and pathogen damage after 14 months for *Heisteria concinna* in the presence or absence of A) nitrogen, B) phosphorus, or C) potassium addition. When bars are above the line, antibiotics increased the magnitude of the metric compared to the control (sterile water) in the presence (grey bars) or absence (black bars) of nutrient addition. When bars are below the line, antibiotics decreased the magnitude of the metric compared to the control in the presence or absence of nutrient addition. For example, antibiotic applications caused an increase in pathogen damage by 33% when K was not added but caused a decrease in pathogen damage by 17% when K was added.

Figure S18. Effects of antibiotics and N, P, K for *Sorocea affinis*

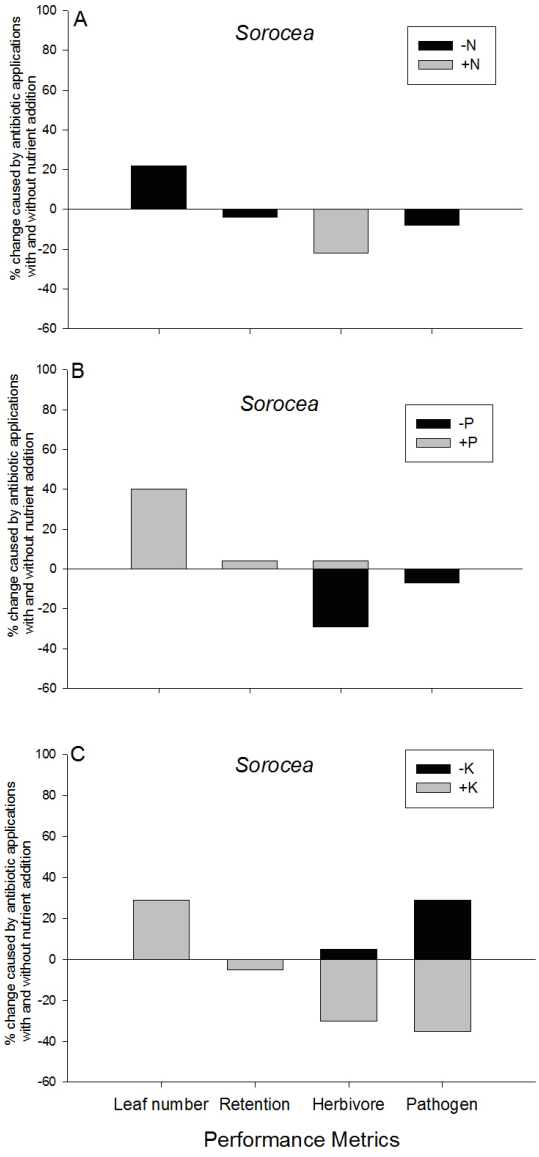

Figure S18. The effects of antibiotic applications on leaf number, retention, herbivore and pathogen damage after 14 months for *Sorocea affinis* in the presence or absence of A) nitrogen, B) phosphorus, or C) potassium addition. When bars are above the line, antibiotics increased the magnitude of the metric compared to the control (sterile water) in the presence (grey bars) or absence (black bars) of nutrient addition. When bars are below the line, antibiotics decreased the magnitude of the metric compared to the control in the presence or absence of nutrient addition. For example, antibiotic applications caused an increase in pathogen damage by 29% when K was not added but caused a decrease in pathogen damage by 35% when K was added.

Figure S19. Effects of antibiotics and N, P, K for *Tetragastris panamensis*

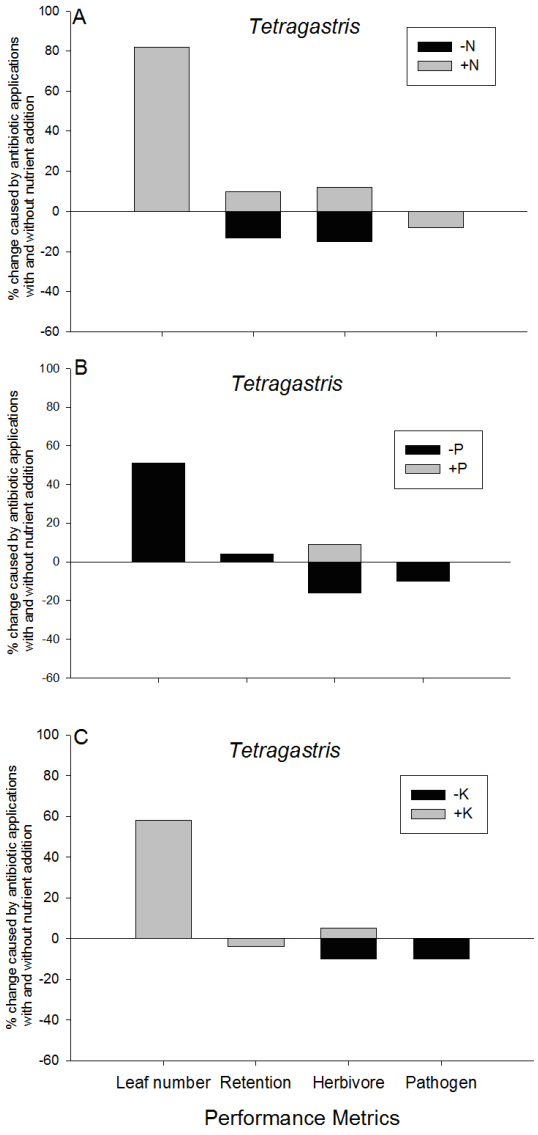

Figure S19. The effects of antibiotic applications on leaf number, retention, herbivore and pathogen damage after 14 months for *Tetragastris panamensis* in the presence or absence of A) nitrogen, B) phosphorus, or C) potassium addition. When bars are above the line, antibiotics increased the magnitude of the metric compared to the control (sterile water) in the presence (grey bars) or absence (black bars) of nutrient addition. When bars are below the line, antibiotics decreased the magnitude of the metric compared to the control in the presence or absence of nutrient addition. For example, antibiotic applications caused a decrease in herbivore damage by 12% when N was not added but caused an increase in herbivore damage by 15% when N was added.

Figure S20. Effects of antibiotics and N, P, K for *Desmopsis panamensis*

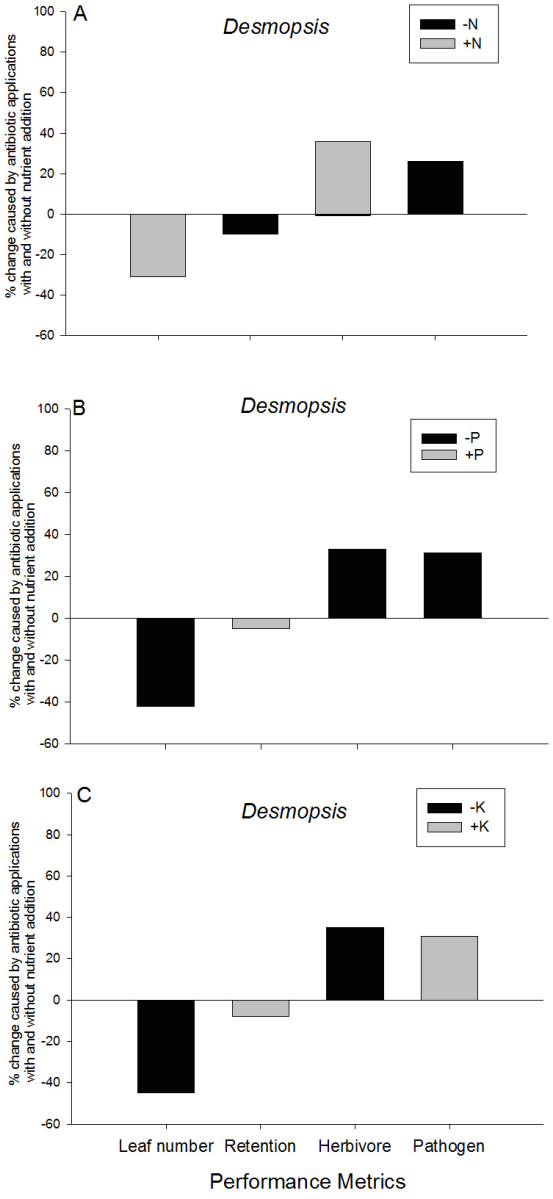

Figure S20. The effects of antibiotic applications on leaf number, retention, herbivore and pathogen damage after 14 months for *Desmopsis panamensis* in the presence or absence of A) nitrogen, B) phosphorus, or C) potassium addition. When bars are above the line, antibiotics increased the magnitude of the metric compared to the control (sterile water) in the presence (grey bars) or absence (black bars) of nutrient addition. When bars are below the line, antibiotics decreased the magnitude of the metric compared to the control in the presence or absence of nutrient addition. For example, antibiotic applications caused a decrease in herbivore damage by 1% when K was not added but caused an increase in herbivore damage by 36% when K was added.

Table S17. MANOVA results for the effects of N, P, and K on leaf herbivore damage

Table S17. MANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) on mean relative herbivore damage rates among control-seedlings over four species (*Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*) and four replicates of all factorial combinations of N, P and K (N = 32 plots). We calculate herbivore damage rate as $H = (\ln H_1 - \ln H_0)/(t_1-t_0)$ for each seedling where H_0 and H_1 were initial and final leaf count and t_1-t_0 was the time period in months, and mean production rates over three seedlings in each plot.

Factor	Df	F value	P value
N	4, 20	0.15	0.9620
P	4, 20	1.31	0.2997
K	4, 20	2.28	0.0962
N × P	4, 20	1.13	0.3701
N × K	4, 20	1.28	0.3103
P × K	4, 20	0.45	0.7708
N × P × K	4, 20	1.07	0.3984

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S18. MANOVA results for the effects of N, P, and K on leaf pathogen damage

Table S18. MANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) on mean pathogen damage rates among control-seedlings over four species (*Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*) and four replicates of all factorial combinations of N, P and K (N = 32 plots). We calculate pathogen damage rate as $P = (\ln P_1 - \ln P_0)/(t_1-t_0)$ for each seedling where H_0 and H_1 were initial and final leaf count and t_1-t_0 was the time period in months, and mean production rates over three seedlings in each plot.

Factor	Df	F value	P value
N	4, 20	0.45	0.7697
P	4, 20	1.30	0.3025
K	4, 20	0.77	0.5603
N × P	4, 20	1.24	0.3243
N × K	4, 20	0.41	0.7997
P × K	4, 20	1.76	0.1761
N × P × K	4, 20	0.74	0.5767

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S19. MANOVA results for the effects of antibiotic applications and N, P, and K on leaf herbivore damage

Table S19. MANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) on change in herbivore damage rates between antibiotic- and control-treated seedlings over four species (*Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*) and four replicates of all factorial combinations of N, P and K (N = 32 plots). We calculated herbivory rate as $H = (\ln H_1 - \ln H_0)/(t_1-t_0)$ for each seedling where H_0 and H_1 were initial and final percent leaf damage and t_1-t_0 was the time period in months, mean herbivory rates for four leaves over three seedlings for each antibiotic treatment in each plot, and the difference between mean herbivory rates with and without antibiotics ($\delta \overline{H} = \overline{H}$ (antibiotic**) -** $\overline{H}$ (control)).

Factor	Df	F value	P value
N	4, 20	2.06	0.1247
P	4, 20	1.19	0.3465
K	4, 20	0.28	0.8899
N × P	4, 20	0.23	0.9211
N × K	4, 20	**3.49**	**0.0257**
P × K	4, 20	0.09	0.9861
N × P × K	4, 20	0.62	0.6530

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S20. MANOVA results for the effects of antibiotic applications and N, P, and K on leaf pathogen damage

Table S20. MANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) on change in pathogen damage rates between antibiotic- and control-treated seedlings over four species (*Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*) and four replicates of all factorial combinations of N, P and K (N = 32 plots). We calculated herbivory rate as $P = (\ln P_1 - \ln P_0)/(t_1-t_0)$ for each seedling where P_0 and P_1 were initial and final percent leaf damage and t_1-t_0 was the time period in months, mean pathogen rates for four leaves over three seedlings for each antibiotic treatment in each plot, and the difference between mean pathogen rates with and without antibiotics ($\delta \bar{P} = \bar{P}$ (antibiotic) **-** $\bar{P}$ (control)).

Factor	Df	F value	P value
N	4, 20	0.70	0.5997
P	4, 20	0.51	0.7296
K	4, 20	1.36	0.2831
N × P	4, 20	0.41	0.8023
N × K	4, 20	0.81	0.5342
P × K	4, 20	1.09	0.3869
N × P × K	4, 20	0.94	0.4637

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

*Tukey test revealed that there was a significant SOAF * K interaction (minimum significant difference: $P < 0.0273$).

Table S21. MANOVA results for the effects of N, P, and K on net leaf number

Table S21. MANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) on net leaf number among control-treated seedlings over five species (*Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*) and four replicates of all factorial combinations of N, P and K (N = 32 plots). We calculated relative growth rate as $L = (\ln L_1 - \ln L_0)/(t_1-t_0)$ for each seedling where H_0 and H_1 were initial and final leaf count and t_1-t_0 was the time period in months, and net leaf number over three seedlings in each plot.

Factor	Df	F value	P value
N	5, 20	1.64	0.1959
P	5, 20	1.47	0.2434
K	5, 20	**6.90**	**0.0007**
N × P	5, 20	2.36	0.0772
N × K	5, 20	**3.38**	**0.0225**
P × K	5, 20	2.62	0.0560
N × P × K	5, 20	1.76	0.1676

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S22. MANOVA results for the effects of antibiotic applications and N, P, and K on net leaf number

Table S22. MANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) on net leaf number differences between antibiotic- and control-treated seedlings over five species (*Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*) and four replicates of all factorial combinations of N, P and K (N = 32 plots). We calculated relative growth rate as $L = (\ln L_1 - \ln L_0)/(t_1-t_0)$ for each seedling where H_0 and H_1 were initial and final leaf count and t_1-t_0 was the time period in months, mean production rates over three seedlings for each antibiotic treatment in each plot, and the difference between mean production rates with and without antibiotics ($\delta \bar{L} = \bar{L}$ (antibiotic) **-** $\bar{L}$ (control)).

Factor	Df value	F	P value
N	5, 20	**3.26**	**0.0259**
P	5, 20	1.50	0.2349
K	5, 20	**9.20**	**0.0001**
N × P	5, 20	**3.35**	**0.0233**
N × K	5, 20	**6.18**	**0.0013**
P × K	5, 20	0.51	0.7667
N × P × K	5, 20	1.51	0.2301

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

5.0 TREE SPECIES AND SOIL FERTILITY STRUCTURE BACTERIAL ENDOPHYTE COMMUNITIES AMONG TROPICAL TREE SEEDLINGS IN A LOWLAND PANAMANIAN FOREST

5.1 INTRODUCTION

The phyllosphere (surface and interior of leaves) is perhaps the world's largest terrestrial habitat, yet we know relatively little about the organisms that colonize these habitats (reviewed by Griffin & Carson 2015). Microbial endophytes, or those that live inside plant tissues, have been isolated from leaves of every plant species screened to date (Stone *et al.* 2000, Strobel *et al.* 2004, Rodriguez *et al.* 2009). The ubiquity of endophytes alone suggests that these cryptic organisms may be powerful drivers of ecological processes. Indeed, empirical studies have demonstrated that endophytes can increase or decrease plant performance, facilitate maintenance plant diversity, and even cause cascading effects across trophic levels (e.g., Clay & Holah 1999; reviewed by Rodriguez *et al.* 2009, Saikkonen *et al.* 2010). For example, some of these microbes are pathogenic and once in the apoplast cause up to 100% yield loss in agroecosystems, which has resulted in large-scale eradication efforts using fungicides and antibiotics (McManus

et al. 2002, Vidaver 2002, Yoon *et al.* 2013). Our understanding of the ecology of endophytes, however, is severely biased because studies primarily focus on fungal endophytes and not bacteria, which outnumber fungi in the phyllosphere by orders of magnitude (Lindow & Brandl 2003, Delmotte *et al.* 2009; but *cf* Griffin *et al.* in review, Chapter 3). Additionally, these studies used a single or few endophyte species or rely on culture-dependent techniques that sample as little as 0.1% of existing microbial communities (Aly *et al.* 2011, Amman *et al.* 1995, Rudgers *et al.* 2007, Hyde & Soytong 2008, Porras-Alfaro & Bayman 2011, Blumenstein *et al.* 2015, but *cf* Zimmerman & Vitousek 2012). Ultimately, bacterial endophyte community composition and their impacts to plant hosts *in situ* are unknown.

Endophyte communities may be particularly abundant and diverse among tropical trees in the shaded understory. High temperatures and humidity and low UV radiation in tropical habitats likely favor microbial survival and persistence (reviewed by Griffin & Carson 2015). Moreover, high water availability and insect abundance in tropical habitats likely facilitate or vector the spread of endophytes among plants (Griffin & Carson 2015). Indeed, Arnold & Lutzoni (2007) demonstrated that up to 99% of leaf segments sampled in a moist tropical forest in Panama were infected with fungal endophytes, compared to only 1% in arctic habitats. Further, endophyte diversity increased by almost 600% from arctic to tropical habitats (Arnold & Lutzoni 2007). Very few studies to date, however, have even characterized foliar bacterial community compositions among tropical trees, and those few have only identified bacteria on the leaf surface of adult trees (Lambais *et al.* 2006; Furnkranz *et al.* 2008; Kim *et al.* 2012; Kembel *et al.* 2014). Thus, even basic questions about the ecology, distribution, and drivers of bacterial endophyte diversity and community composition in tropical forests remain unknown.

The degree to which bacterial endophyte communities are host specific among co-occurring plants may have critical implications for plant community composition. On one hand, large overlaps of bacterial taxa among plant species suggest that the majority of bacterial taxa are widespread and interact with many plant hosts. For example, recent studies have demonstrated that a large majority of bacteria on the leaf surface make up a "core microbiome," meaning that a subset of taxa are commonly shared among individuals in the same habitat or region (Shade & Handelsman 2012, Rastogi *et al*. 2012, Kembel *et al*. 2014, reviewed by Griffin & Carson 2015). Kembel *et al*. (2014) demonstrated that while only 1.4% of phyllosphere bacterial diversity was present on over 90% of all trees sampled in Panama (57 species), this small subset of bacteria made up 73% of the total sequences. Thus, a very small group of bacteria reside on numerous tree species. On the other hand, endophyte communities may be host specific and thus impact plant hosts differently (*sensu* Dyer *et al*. 2007). In addition, Kembel *et al*. (2014) found that host taxonomy and host traits explained 51% of the variation in bacterial communities among tree species. Moreover, Griffin *et al.* (in review) recently demonstrated that there are species-specific interactions between plants and foliar bacteria. Specifically, bacteria decreased plant growth by up to 50% for some species and increased growth by up to 20% for others. The degree to which bacterial communities were host-specific, however, was not assessed. Ultimately, foliar bacteria may have the potential to alter rank-order performances for co-occurring tree species in shaded understories; thus, linking the composition of these communities to their impacts on plant performance is critical.

Recent studies have suggested that nutrient availability is a strong determinant of leaf-associated bacterial communities, which may have important implications for plant performance and trophic interactions (e.g., Ikeda *et al.* 2011, reviewed by Vorholt 2012, Griffin & Carson

2015). It has been known for years that bacterial strains are limited by nitrogen on the phyllosphere and specialize on particular nutrient resources, thereby facilitating niche partitioning (e.g., Wilson & Lindow 1994, Ji & Wilson 2002, Innerebner *et al.* 2011). In a recent study, Kembel *et al.* (2014) demonstrated that there were *correlations* between leaf nitrogen and phosphorus levels and bacterial community composition on the leaf surface of 57 tropical tree species. Experimental studies designed to address the degree to which soil nutrients impact endophyte community composition, however, are lacking. Nevertheless, one study demonstrated that soil nitrogen fertilization caused an increase in the relative abundance of some leaf-surface bacterial taxa by up to 96% and caused a decrease for others by up to 76% (Ikeda *et al.* 2011). Whether variation in nitrogen levels or other macronutrients such as phosphorus and potassium cause variations in endophyte community composition among plants in more natural systems is unknown. Identifying and quantifying the factors that contribute to bacterial community composition are critical because plant-associated microbes mediate plant functional traits and trophic interactions (Friesen *et al.* 2011, Turner *et al.* 2013). For example, fungal endophytes in grasses produce secondary metabolites that reduce insect and even mammalian herbivory by as much as 55% (Funk *et al.* 1983, Barker *et al.* 1984, Schardl *et al.* 2004, Tanaka *et al.* 2012, Faeth & Saari 2012). Ultimately, endophyte communities may mediate the impacts of nutrient enrichment on plant performance (see Chapter 4). Ultimately, foliar bacterial endophytes may function as an independent leaf functional trait, thus classifying endophytes as a major axis of plant ecological strategy (e.g., Kembel *et al.* 2014).

Though commercial antibiotics have been used for almost six decades to rid plants of bacterial pathogens in agricultural systems, the degree to which these applications impact bacterial community composition is unknown. The three most commonly used antibiotics in

temperate and tropical agriculture are streptomycin, gentamicin, and oxytetracycline (reviewed by McManus *et al.* 2002). While streptomycin and gentamicin are aminoglycosides and inhibit protein synthesis primarily among Gram-negative bacteria, oxytetracycline is a broad-spectrum antibiotic that inhibits a wide range of disease-causing bacteria (McManus *et al.* 2002, Vidaver 2002). These products have been extensively used among temperate and agricultural crops under various trade names to reduce pathogen abundance and damage caused primarily by members of Proteobacteria as well as water molds (reviewed by McManus *et al.* 2002). Indeed, these products decrease bacterial abundance on the surface and inside leaves by ~45-85% *in situ* (Traw *et al.* 2007, Griffin *et al*., in review, Chapter 3). The degree to which antibiotics impact community composition of bacteria, however, is poorly understood.

We aim to test the following hypotheses: 1) Bacterial endophyte richness, diversity, and community composition varies substantially among coexisting plant species; 2) Bacterial endophyte richness, diversity and community composition vary substantially with soil nutrient availability (N, P, K); 3) There are frequent interactions between soil nutrient supplies and endophyte community composition among host plant species; and 4) Antibiotic applications cause a decrease in bacterial richness and diversity and cause a substantial change in community composition. To address these hypotheses, we used high-throughput sequencing to quantify bacterial endophyte community composition for seedlings of 5 woody species nested within a large-scale factorial resource supply experiment (N, P, and K) in a tropical forest in Panama. In addition, we applied commercial antibiotics or sterile water to seedlings of a single species (*Tetragastris*) for 29 months before sequencing endophyte communities.

5.2 METHODS

5.2.1 Study Site

We conducted this study in a mature (~ 200 yr.) secondary tropical forest on the Barro Colorado Nature Monument in Panama (Fig. S27). The study site resides on Gigante Peninsula (9˚06'31''N, 79˚50'37''W), where annual precipitation averages 2,600 mm, of which less than 10% falls during the 4-month dry season between January and April. The soils consist of endogleyic cambisols and acric nitisols (Koehler *et al.* 2009).

5.2.2 Woody species

We selected five common woody species from five different families located throughout the site (hereafter referred to by genus name): *Alseis blackiana* (Rubiaceae), *Desmopsis panamensis* (Annonaceae), *Heisteria concinna* (Olacaceae), *Sorocea affinis* (Moraceae) and *Tetragastris panamensis* (Burseraceae). Nomenclature follows Croat (1978) and Garwood (2009). All five species are shade tolerant as seedlings, vary in life history traits, and span a wide range of maximum adult heights (Wright *et al.* 2003; Gilbert *et al.* 2006, Wright *et al.* 2010). *Alseis* is a mid-canopy pioneer species but can persist in the shaded understory (Dalling *et al.* 2001, Wright *et al.* 2010). *Sorocea* is a shrub or a small tree, *Desmopsis* and *Heisteria* are understory treelets, and *Tetragastris* is a canopy tree (Croat 1978 and Wright *et al.* 2010).

5.2.3 Fertilization experiment

We applied nitrogen (N), phosphorus (P), and potassium (K) in 40 × 40 m plots in a 2 × 2 × 2 factorial design, where we replicated all treatment combinations four times (Fig. S27). All plots but two were separated by at least 40 m, and those two were separated by 20 m and a 3-m deep streambed. Beginning in 1998, we added fertilizer by hand four times a year at approximately six-week intervals between June and November, which is well within the wet season (May-December). We applied 125kg N ha^{-1} $year^{-1}$ as urea, 50 kg P ha^{-1} $year^{-1}$ as triple super-phosphate, and 50 kg K ha^{-1} $year^{-1}$ as KCl.

5.2.4 Antibiotic applications

In January 2010, we selected six relatively healthy individuals (minimal signs of necrosis or insect damage) of each species (~20 – 30cm tall) within the inner 30 x 30 m of each plot. We randomly assigned antibiotic and control (sterile water) treatments to three individuals of each species in each plot. For 29 months, we sprayed seedling leaves every 10-15 days with antibiotics or sterile water to saturation. Two recent studies demonstrated that streptomycin applications to the canopy of apple orchards did not adversely affect bacterial community composition in soil below plant canopies (Walsh *et al.* 2013, Shade *et al.* 2013). Nevertheless, we placed a plastic sheet around the base of each seedling before application to prevent exposure of soil microbes to either treatment. The plastic sheet extended beyond the crown of each individual and was left in place until no liquid was visibly dripping off the plant. We alternated the antibiotic treatment every other application between 100 ppm of Agri-mycin 17 (a commercial formulation of streptomycin, Hummert International #02-0150; Earth City, MO) and

1752 ppm of Agry-Gent Plus 800 (a commercial cocktail formulation of gentamicin and oxytetracycline, Química Agronómica de México, Chihuahua, México). Streptomycin, gentamicin, and oxytetracycline are the three most commonly used broad-spectrum antibiotics in temperate and tropical agriculture (McManus *et al.* 2002).

5.2.5 Sample collection and DNA extractions

After 29 months of treatments, we sampled leaves from three control-treated seedlings from all five species per plot (N = 476). In addition, we sampled leaves of three antibiotic-treated seedlings of *Tetragastris* in each plot (N = 81). For each seedling, we placed hole-punched leaf samples from five randomly selected leaves in a sterile microcentrifuge tube to immediately take back to the lab for DNA extractions. In the lab, we sterilized leaf surfaces by using sequential immersion in 95% ethanol (10s), 10% chlorine bleach (0.525% NaOCl; 2 min), and 70% ethanol (2 min; adapted from Arnold & Lutzoni, 2007, Kaewkla & Franco 2013). We confirmed surface sterilization by aseptically rolling the leaf surfaces across King's Broth media (e.g., Kaewkla & Franco 2013, Griffin *et al.* in review). We then pulverized leaf samples with sterile pestles in sterile, RNA-ase free microcentrifuge tubes. We purified DNA using the Purelink Total Plant DNA Purification Kit (Invitrogen; using the product's protocol), and we stored the product in 10:1 Tris/EDTA buffer at -20C before library preparation.

5.2.6 Bacterial sequencing

We prepared amplicon libraries for Illumina sequencing using a two-stage PCR protocol. To exclude chloroplast DNA, we used cyanobacteria-excluding primers and targeted the V5–V6

region of the bacterial 16S rRNA gene. In addition, a 22-bp tail (CS1 and CS2, Fluidigm Corporation, California) was added to each 16S primer. (modified 799F: 5'-ACACTGACGACATGGTTCTACA-AACMGGATTAGATACCCKG; modified 1115R: 5'-TACGGTAGCAGAGACTTGGTCT-AGGGTTGCGCTCGTTG). Twenty-five-µl PCR reactions consisted of 2.5 µl 10× buffer (Roche Diagnostics) with MgCL2 at 18 nM, 0.5 µl dNTPs (10 µM each), 0.1 µl FastStart High Fidelity Taq (Roche Diagnostics), 1 µl each primer (10 µM), 8 µl of genomic DNA, and 11.9 µl molecular-grade water. PCR conditions were as follows 5 min initial denaturation at 95 °C, followed by 35 cycles of 45s at 94 °C, 60s at 55 °C, and 60s at 72 °C, with a final 10-min elongation at 72 °C. We performed a second PCR using1 µl of the first PCR product diluted 1/200 in pure grade water to add bar codes and Illumina adapters (PCRII_for: 5'-CAAGCAGAAGACGGCATACGAGATxxxxxxxxxxTACGGTAGCAGAGACTTGGTCT, where "x" represents barcode nucleotides; PCRII_rev: 5'-AATGATACGGCGACCACCGAGATCTACACTGACGACATGGTTCTACA). We performed single 20- µl reactions were performed for each sample for 15 cycles using 2 µl 10× buffer (Roche Diagnostics), 3.6 µl of MgCL2 (25 mM), 5% DMSO, 0.5 µl dNTPs (10 µM each), 0.2 µl FastStart High Fidelity Taq (Roche Diagnostics), 1 µl each primer (10 µM), 1 µl of first PCR product, and 9.8 µl molecular-grade water. PCR products were cleaned-up with AMPure beads (Beckman Coulter) and validated by agarose gel electrophoresis. We multiplexed 16S libraries by mixing equimolar concentrations of DNA from each sample. The resulting DNA library was sequenced using Illumina MiSeq 250-bp paired-end sequencing at Genome Quebec.

We used UPARSE to demultiplex raw sequences and remove paired end reads with low quality (<30) scores. We clustered operational taxonomic units (OTUs) at 97% similarity with

uclust with the Greengenes 16S rRNA in QIIME. We removed chimeric OTUs with BLAST algorithms.

5.2.7 Statistical analysis

We performed ANOVAs to evaluate differences in bacterial endophyte richness and diversity among tree species and the degree to which antibiotic applications caused changes in endophyte richness and diversity among *Tetragastris* seedlings. We used Shannon diversity ($e^{H'}$) because this measurement scales Shannon diversity index to units comparable to richness (Jost 2006, 2007). In addition, we performed MANOVAs to evaluate differences in bacterial endophyte richness and diversity differences among soil nutrient treatments. The average value $\bar{R}$ (or $\bar{D}$) for each species in a plot was simply the mean of the values over the three conspecific plants. Because all 5 species were nested (and non-independent) within each plot, our response vector for plot j was:

$$\delta\,\bar{R}_j = (\delta\,\bar{R}(1), \delta\,\bar{R}(2), \delta\,\bar{R}(3), \delta\,\bar{R}(4), \delta\,\bar{R}(5))_j$$

where the numbers 1 through 5 refer to the five plant species. A MANOVA of this response vector tests whether bacterial richness or diversity differed across nutrient treatments and adjusts for correlated response variables. Post hoc Tukey studentized range tests with corrected significance values ($\alpha = 0.05$ and corrected for number of species) on the individual elements of the vector allowed us to determine which species differed in their responses across nutrient treatments. We chose this approach to avoid pseudofactorialism, a problem in many studies using nested factorial designs (Hurlbert 2013).

5.2.8 Community analysis

To compare diversity levels among woody species for differences in sequencing depth between samples, we conducted rarefaction analyses with 5000 randomly selected sequences per sample (e.g., Carrell & Frank 2014).

To evaluate communities at an equal sequencing depth, we first rarified all samples to and inferred a maximum-likelihood phylogeny with FastTree (Price *et al.* 2009). We used weighted UniFrac distance matrices from the phylogenetic tree to analyze the degree of dissimilarity of communities among samples (Lozupne & Knight 2005, Lozupone *et al.* 2006). We ran PERMANOVAs to evaluate differences in bacterial community composition among tree species and nutrient additions (N, P, K and all combinations). In a separate analysis, we ran a PERMANOVA to evaluate the degree to which antibiotic applications caused a difference in community composition among *Tetragastris* seedlings. Afterwards, we ran Kruskal-Wallis tests to determine whether differences in the relative abundances of individual bacterial genera across host species, nutrient additions, and antibiotic applications were significant (Carrell & Frank 2015). We used False Discovery Rate (FDR) corrections as described by Pike (2011) to adjust significant *P* values to correct for multiple tests.

5.3 RESULTS

Overall, we identified 94,554 OTUs among control-treated seedlings. Rarefaction curves suggest that increased sampling would result in greater observed OTU differences among woody species (Fig. 21). Among all samples, the most abundance bacterial class was Actinobacteria

(~48%), followed by Alphaproteobacteria (~17%), Bacilli (~12%), Gammaproteobacteria (~9%), Betaproteobacteria (~4%), Thermoleophilia (~2%) and Deltaproteobacteria (~2%; Fig. 22). For control-treated individuals (N = 476), the mean OTU count was 29,440 (± 386). Additionally, we found that 10 bacterial OTUs belonging to 3 phyla and 9 families were present on 95% or more of all control-treated trees (Table S23). For *Tetragastris*, antibiotic applications reduced the mean OTU count from 29,949 OTUs (± 1005 SE) to 22,477 (± 800 SE), a 25% decrease.

5.3.1 Species differences

Diversity and richness. Overall, endophyte diversity but not richness differed among host species at every taxonomic level (Figs. 23 and 24, Tables S24 and S25). At the genus level, for example, endophyte diversity was ~ 40% lower for *Sorocea* and *Tetragastris* compared to *Desmopsis* and *Heisteria* (Fig. 23E). From phylum to genus, endophyte diversity was lowest for *Tetragastris* and highest for *Desmopsis*. Endophyte diversity was 46% lower for *Tetragastris* compared to *Desmopsis* at the genus level (compared to 18% lower at the phylum level). Endophyte richness, however, did not differ among tree species at any taxonomic level (Fig. 24, Table S25).

Community composition. Bacterial endophyte community compositions varied considerably among tree species (Table S26, $F_{4,471} = 5.81$, $P = 0.001$). Specifically, the relative abundances of 29 bacterial genera (from 11 bacterial families) substantially differed among tree species (Tables S27-S28). The abundance of the most common endophyte family, Mycobacteriaceae, varied among species by as much as 67%. Specifically, the abundance of Myobacteriaceae was highest among *Tetragastris* seedlings (46%) and was 67% higher than among *Heisteria* seedlings (28%). Moreover, in three cases we found specific endophytes on

some host species but not others (Table S27). For example, we only isolated Waddliceae endophytes from *Tetragastris* (T = 16.3; P = 0.003). Further, Rhodobacteraceae endophytes occurred on *Alseis*, *Sorocea*, and *Tetrgastris* but not *Desmopsis* and *Heisteria* (T = 9.70; P = 0.046). Finally, Rikenellaceae endophytes occurred on *Heisteria* and *Tetragastris* but not *Alseis*, *Desmopsis*, or *Sorocea* (T = 12.4; P = 0.015).

5.3.2 Nutrient enrichment

Diversity, richness, and composition. Although nutrient additions had no impacts on bacterial taxonomic richness or diversity, particular interactions among N, P, and K caused substantial changes in bacterial community composition (Tables S29-S38; Table S26). Specifically, nutrient enrichments either increased or decreased the relative abundances of 13 bacterial genera from 13 different families by up to 234% (See Table S39 for all significant nutrient x genus effects). For example, K enrichment caused anywhere from 69% decreases to 16% increases in the relative abundances of Haemophilus, Mycobacterium, Sphingomonas, and Fusobacterium (F = 17.39, P < 0.001; F = 17.32, P < 0.001; F = 15.04, P <0.001; F = 12.64, P <0.001). Moreover, N enrichment caused anywhere from 38% decreases to 12% increases in the relative abundances of Pseudomonas, Paenibacillus, and Actinomyces (F = 16.38, P < 0.001; F = 12.99, P < 0.001; F = 11.51, P < 0.001). In addition, the degree to which P enrichment decreased or increased the relative abundances of Actinomyces, Streptococcus, and Pseudonocardia differed substantially among plant species (P x Species interaction, Table S39, F = 5.59, P < 0.001; F = 4.98, P < 0.001; F = 4.77, P = 0.001). For example, for *Heisteria*, P addition decreased the relative abundance of Actinomyces by 21% and conversely, for *Desmopsis*, increased the relative abundance of Actinomyces by 234%.

Overall, relative abundances of bacterial endophytes were largely dependent on soil resource supply, and the degree to which P enrichment impacted particular endophytes differed among plant species.

5.3.3 The impacts of antibiotic applications on *Tetragastris* endophyte communities

Diversity and richness. While the mean OTU count was 25% lower among leaves treated with antibiotics, antibiotic applications caused an increase in endophyte richness and diversity at every taxonomic level for *Tetragastris* (Fig. 25, Tables S40-S49). Specifically, at the phylum level, antibiotic applications caused an increase in taxonomic richness by 41% and an increase in diversity by 18%. At the genus level, antibiotics caused an increase in richness by 55% and an increase in diversity by 100%.

Community composition. Antibiotic applications caused substantial changes in bacterial endophyte community composition for *Tetragasris* (Table S50, $F_{1,175} = 11.90$, $P = 0.001$). For example, Actinobacteria was by far the most abundance bacterial class among control individuals, making up almost 59% of the total sequences (Fig. 26). In contrast, the relative abundance of Actinobacteria among leaves applied with antibiotics was 43.5%, 26% lower than in control leaves (Fig. 26). In addition, the relative abundances of the five next common classes were higher among leaves applied with antibiotics compared to the control. Specifically, the relative abundances of Alphaproteobacteria, Bacilli, Gammaproteobacteria, Betaproteobacteria, and Thermoleophilia were 40%, 40%, 33%, 50%, and 86% higher than leaves applied with sterile water. At the genus level, antibiotic applications reduced the relative abundance of six genera by up to 100% (Table S51). Specifically, antibiotic applications decreased the abundance of Candidatus Rhabdochlamydia and an unknown genus in the Siobacteraceae family by an order

of magnitude (T = 3.87, *P* = 0.049; T = 4.80, *P* = 0.029). Moreover, antibiotic applications decreased the abundance of Veillonella, Cellvibrio, Cupriavidus, and an unknown genus in the FAC88 family by 100% (T = 5.19, *P* = 0.023; T = 5.19, *P* = 0.023; T = 5.19, *P* = 0.023; T = 5.19, *P* = 0.023). Conversely, antibiotic applications increased the abundance of an unknown genus in the Pasteurellaceae family and an unknown genus in the Planococcaceae family by an order of magnitude (T = 3.87, *P* = 0.049; T = 4.80, *P* = 0.029).

Nutrient additions. Although antibiotic applications in general caused significant increases in relative abundance of endophytes, this varied substantially with nutrient additions. Specifically, antibiotics increased the relative abundance of Pseudonocardia by 152% when we added K; however this only occurred when N was not added. Conversely, antibiotics increased the relative abundance of Pseudonocardia by ~55% when N was added regardless of whether K was added or not (N x K interaction, F = 6.93, *P* = 0.047). In addition, antibiotics increased the relative abundance of Plesiomonas by two orders of magnitude when we added K but decreased the relative abundance of Plesiomonas by an order of magnitude when K was not added. The impact of K on Plesiomonas, however, only occurred when we did not add N (N x K interaction, F = 10.57, *P* = 0.045).

Overall, the abundance and diversity of bacterial endophytes were largely dependent on soil resource supply and host species. On the other hand, a small group of endophytes consistently occurred among all trees. Moreover, antibiotic applications decreased OTU count, however in general antibiotics increased endophyte richness, abundance and diversity. Moreover, the degree to which antibiotic applications impacted community composition differed among soil nutrient additions.

5.4 DISCUSSION

We experimentally demonstrate for the first time that soil nutrient enrichment caused substantial differences in foliar bacterial endophyte community composition. In addition, we demonstrate that endophyte diversity and composition significantly differed among five co-occurring woody species in a tropical forest. Specifically, we found strong support for three of our four hypotheses: specifically, endophyte communities varied substantially among host species (H1, Fig. 3) and with the supply of N, P, and K (H2). In addition, the degree to which nutrient enrichment (specifically P) impacted community composition varied among plant species (H3). Finally, while antibiotic applications decreased OTU count among *Tetragastris* leaves, applications increased richness and diversity compared to control leaves (H4, Figs. 25 and 26). To our knowledge, this is the first empirical study to experimentally evaluate bacterial community composition *in situ* among multiple co-occurring species. Overall, endophyte communities were highly abundant and diverse. In a recent sequencing study of bacterial communities on the phyllosphere of over 150 mid-canopy tree species in a forest in Panama, Kembel *et al.* (2014) identified just over 7,000 OTUs among 57 tree species with a mean of 418 OTUs per tree. Comparatively, we identified almost 95,000 OTUs among seedlings of five tree species, with a mean of 29,440 per seedling. Indeed, the sheer abundance and diversity of these microbes alone suggest that they may act as major determinants of seedling performance in contrasting resource environments among coexisting tree species. We ultimately suggest that endophyte communities are an independent plant functional trait with the potential to alter the rank-order performance of coexisting plant species.

5.4.1 Core microbiome or host-specific? A tale of two perspectives

Though we demonstrated that endophyte community composition substantially varied among plant species, host species only explained ~5% of the variation in bacterial community composition. On one hand, the relative abundances of almost a dozen bacterial families, including the most commonly abundant family (Mycobacteriaceae), differed among woody species. Moreover, there were many cases where particular bacterial taxa associated with particular species but not others (see Results). These findings support Kembel *et al.* (2014), who demonstrated that plant phylogeny and traits explained a large portion of phyllosphere community composition among 57 trees in Panama. On the other hand, there also is evidence that a core microbiome exists among co-occurring species. Indeed, 10 bacterial OTUs belonging to 3 phyla and 9 families were present on 95% or more of all trees sampled. In comparison, however, Kembel *et al.* (2014) found over 100 OTUs on the phyllosphere 95% or more of all trees sampled. Thus, it appears that a much larger core of bacteria occur on the leaf surface compared to inside leaves of tropical trees. We suggest that future studies empirically evaluate the impacts of host-specific bacterial clades on particular tree species.

5.4.2 Actinobacteria: the most important bacterial endophytes?

Contrary to previous findings among plant-associated microbial communities, we found that Actinobacteria represented ~40-60% of the total endophyte sequences among all species. Although Actinobacteria is some of the more common endophytes in *Arabidopsis* roots, tree branches, and conifer needles, almost all of these studies found that Actinobacteria made up less

than 10% of total endophyte sequences (Gottel *et al.* 2011, Lundberg *et al.* 2012, Schlaeppi *et al.* 2014, Shen & Fulthorpe 2015, Carrell & Frank 2014, 2015). In two recent culture-independent studies on phyllosphere (leaf surface) bacterial communities, Kim *et al.* (2012) and Kembel *et al.* (2014) demonstrated that Actinobacteria made up between 5-10% of total sequences among tropical trees in Panama and Southeast Asia. Our findings suggest that a much larger portion of these bacteria exist inside leaves, which may have large implications for plant-microbial interactions. Indeed, studies of the human microbiome and insect gut demonstrate that dominant microbial taxa often play important roles in host health (e.g., Human Microbiome Project Consortium 2012, Cho & Blaser 2012, Clemente *et al.* 2012, Engel & Moran 2013). Moreover, Actinobacteria in particular are the most abundant sources of bioactive secondary metabolites, which include a variety of antimicrobial and antifungal compounds (Qin *et al.* 2011, Bibb 2013). Notably, bacteria in the genus *Streptomyces* produce over two-thirds of clinical antibiotics, including those used in this study (Emerson de Lima Procopio *et al.* 2012, Bibb 2013). Interestingly, almost 60% of the individuals we sampled contained Streptomycete endophytes. Thus, we suggest that members of endophytic communities commonly produce secondary metabolites to exclude other bacteria and possibly fungal pathogens inside leaves (*sensu* Arnold *et al.* 2003).

Our finding that antibiotic applications decreased the relative abundance of Actinobacteria by 25% opens the door for future questions and studies on the ecology of these organisms. Our results which suggest that Actinobacteria are perhaps the most susceptible taxa to antibiotics are surprising because the majority of antimicrobial compounds originate from Actinobacterial strains (reviewed by McManus *et al.* 2002, Vidaver 2002). Indeed, strains that produce antimicrobial compounds go to great lengths to avoid self-intoxication (Demain 1974,

Vining 1979, Cundliffe 1984, 1989, Magnet & Blanchard 2005). For example, actinomycetes in particular have machinery to prevent the drug from reaching its target, discharge the drug from bacterial cells, or even modify or destroy the drug completely (reviewed by Wright 2005). Moreover, the finding that the relative abundances of almost every bacterial class besides Actinobacteria increased after antibiotic applications was also surprising. Whether these bacteria were less susceptible to antibiotics or simply colonized first after antibiotic applications is unknown. Nevertheless, such a pattern appears to be akin to Paine (1966), whereby decreasing the relative abundance in Actinobacteria opens up niche space for other more rare bacterial classes. It is clear that future studies should address the particular mechanisms by which antimicrobial products impact particular bacterial taxa.

Our results on the impacts of antibiotic applications on community composition enable us to make assertions about the impacts of foliar bacteria on *Tetragastris*. Indeed, Griffin *et al.* (in review, Chapter 3) demonstrated that antibiotic applications cause a net increase in plant growth for *Tetragastris* by 49%. In our study, we found that compared to control-treated seedlings, antibiotic applications resulted in 26% lower Actinobacterial abundances (by far the most abundant bacterial taxa). Thus, our data suggest that bacteria in this class may primarily be pathogenic to plant hosts. Indeed, almost a dozen *Streptomyces* species are prominent plant pathogens that can infect up to 60% of crops and cause scabbing lesions for carrots, radishes, beets, peanuts, and potatoes globally (reviewed by Loria *et al.* 1997, Loria *et al.* 2006). On the other hand, bacteria from other classes might primarily be mutualists and thus higher abundances may result in higher plant performance. For example, we demonstrated that classes containing some of the most prominent mutualists among agricultural crops in temperate and tropical habitats (e.g., *Azospirillum* spp., *Bacillus* spp., *Burkholderia* spp., and *Pseudomonas* spp.)

increased with antibiotic applications (reviewed by Rosenblueth & Martinez-Romero 2006). Needless to say, we recommend that high-throughput sequencing should be used in future studies to assess the degree to which experimental manipulations impact microbial community composition.

5.4.3 Implications for plant performance and trophic interactions

Our findings along other recent studies suggest that it is not possible to fully understand the mechanisms determining plant performance without considering leaf-associated bacteria (Kembel *et al.* 2014, Griffin *et al*., in review, Griffin *et al*., in prep). Indeed, the mechanisms that underlie plant performance outcomes along resource gradients may to a large degree be mediated by bacterial endophytes. For example, Griffin *et al.* (in review) demonstrated that experimental reduction of bacterial abundance and diversity in general increased plant performance and these effects differed among host species and soil nutrient additions. Thus, foliar bacteria, like herbivores, may differentially impact plant hosts as species and resources spatially vary throughout the forest. Moreover, variations in endophyte communities among species and along resource gradients may mediate trophic interactions between plants and enemies. For example, Santiago *et al.* (2012) demonstrated that P, K, and P and K in combination caused an increase in herbivore damage for seedlings of the same five species at the site in Panama. Here, we demonstrated that P, K, and P and K in combination caused substantial differences in endophyte community composition. Thus, the impacts of nutrient addition on plant-herbivore interactions may be dependent on endophyte community composition, supporting similar findings for fungal endophytes in grasses (Funk *et al.* 1983, Barker *et al.* 1984, Schardl *et al.* 2004, Tanaka *et al.* 2012, Faeth & Saari 2012). We suggest that future

studies focus on the degree to which insects or pathogens differentially impact plants based on the presence or absence of foliar endophytes using experimental inoculations. Ultimately, endophyte communities are likely an obscure yet critical component of plant life history strategies.

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5.7 TABLES AND FIGURES

Figure 21. Rarefaction curves for tree species

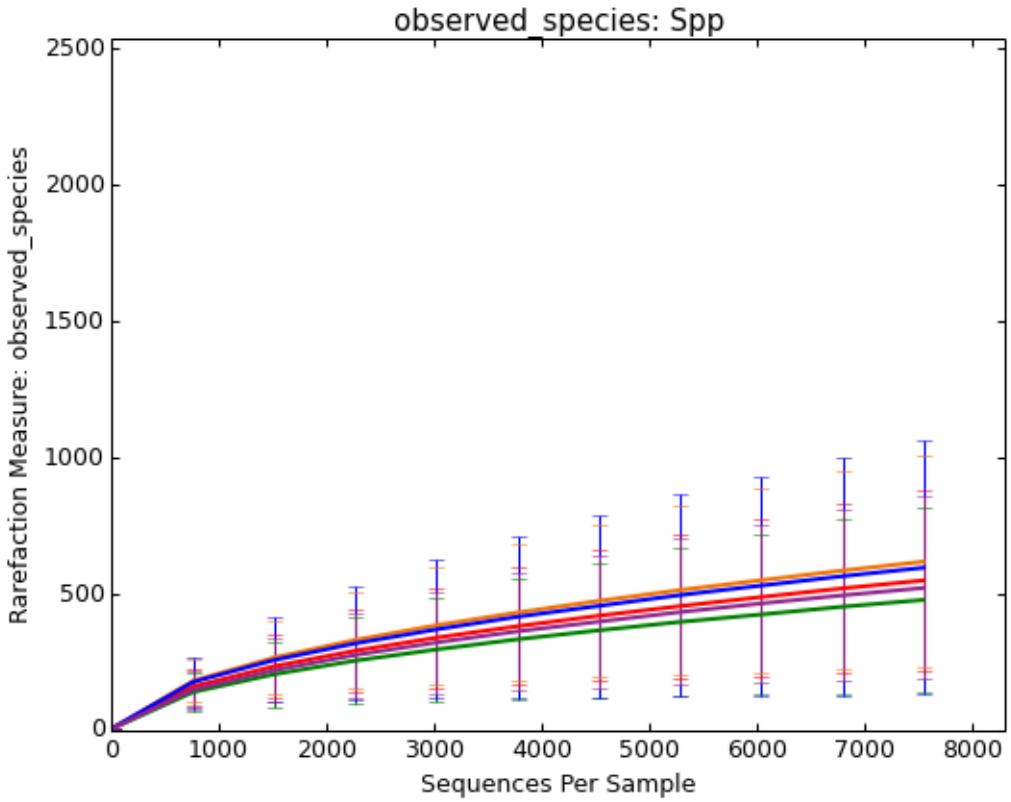

Figure 21. Rarefaction curves for five co-occurring woody species. The red curve represents *Alseis blackiana*. The orange curve represents *Desmopsis panamensis*. The blue curve represents *Heisteria concinna*. The purple curve represents *Sorocea affinis*. The green curve represents *Tetragastris panamensis*. There is no apparent asymptote in the rarefaction curves, suggesting that the sequencing depth does not encompass the full extent of OTU richness in each of the communities. Bars represent mean values (± 1 SE).

Figure 22. Relative abundances of endophyte classes

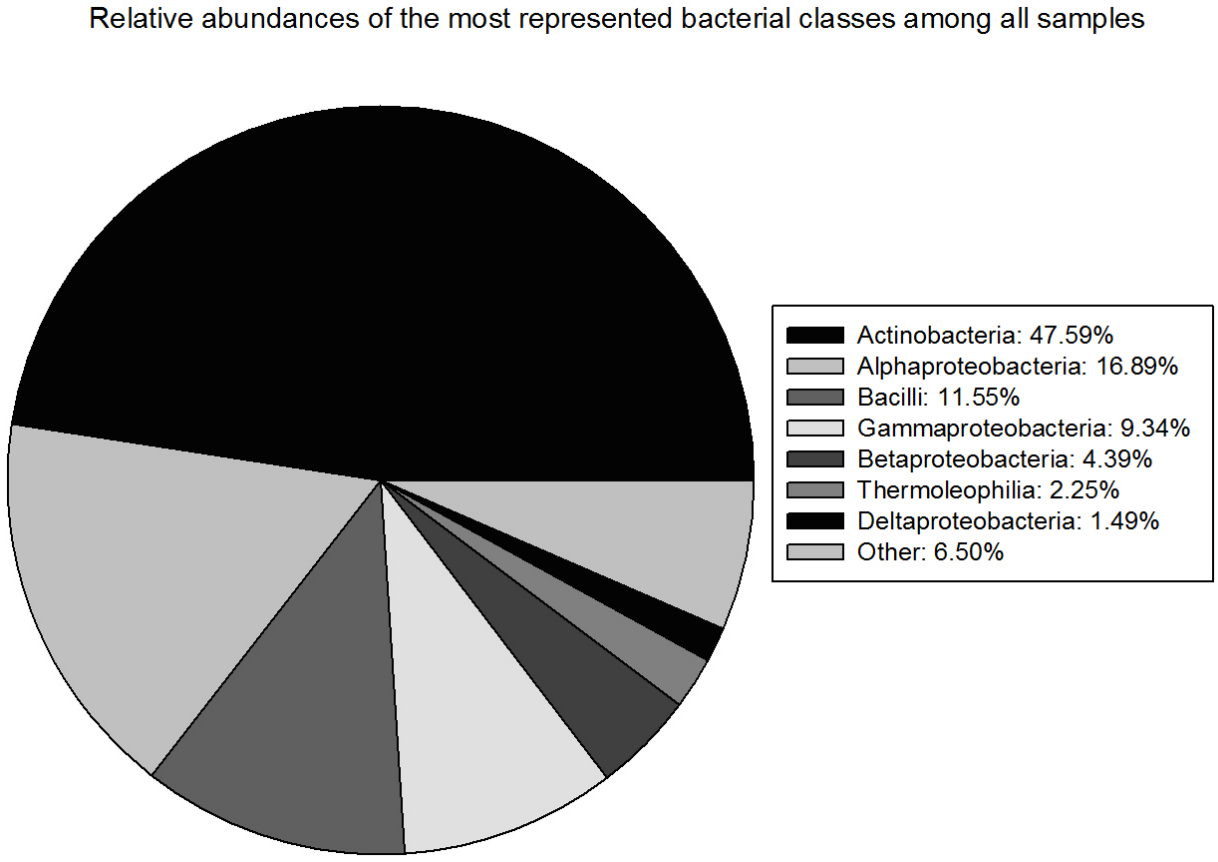

Figure 22. Relative abundances of the seven most common bacterial endophyte classes among leaves of *Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis* (N = 476). We calculated relative abundances as the percentage of sequences belonging to a particular lineage of all 16S rRNA gene sequences recovered from individual samples.

Figure 23. Bacterial endophyte community diversity among tree species

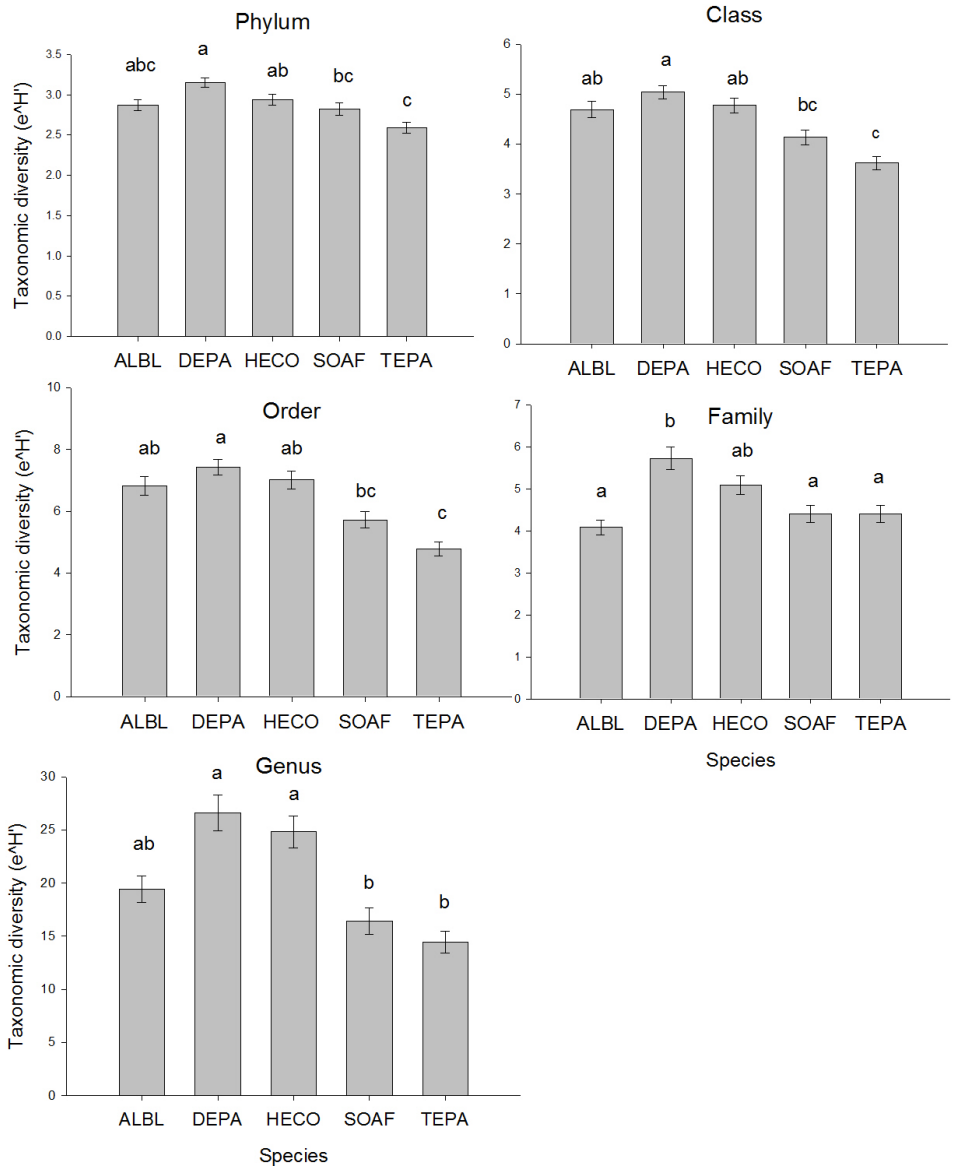

Figure 23. Endophyte community diversity among leaves of five woody species (*Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*). Letters correspond to differences determined by post-hoc Tukey studentized range tests among species. Bars represent mean values (± 1 SE).

Figure 24. Bacterial endophyte community richness among tree species

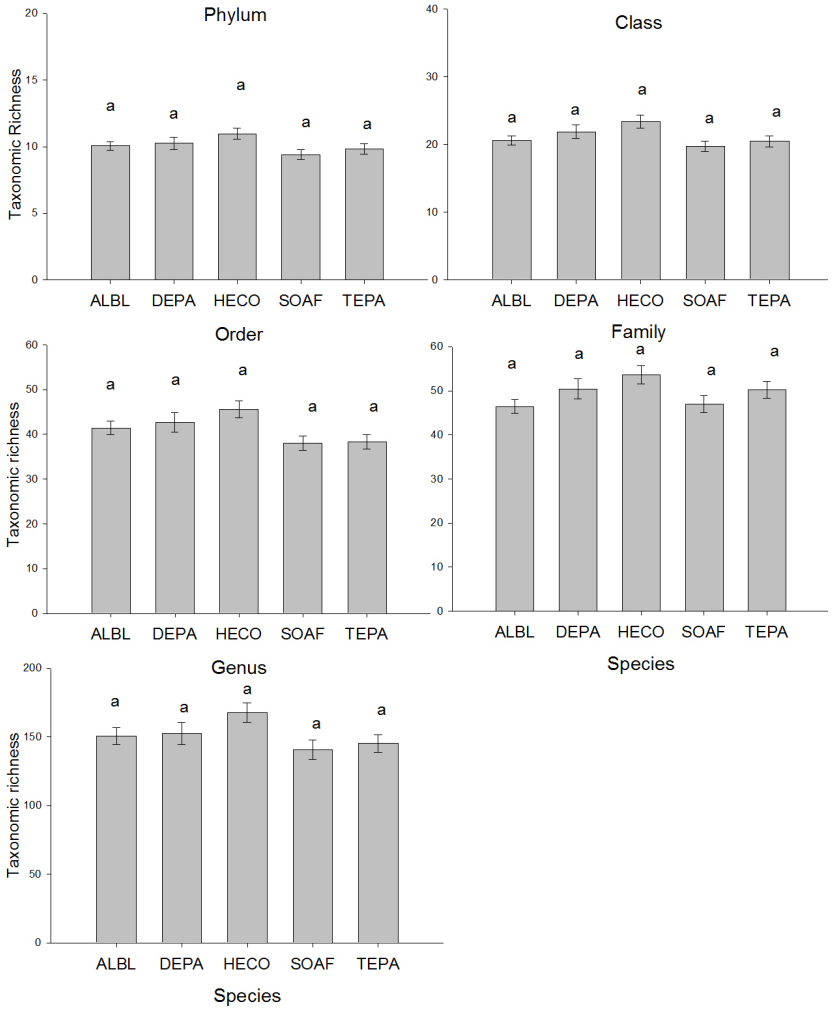

Figure 24. Taxa richness of endophyte communities among leaves of five woody species (*Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*). Letters correspond to differences determined by post-hoc Tukey studentized range tests among species. Bars represent mean values (± 1 SE).

Figure 25. The impacts of antibiotics on endophyte richness and diversity for *Tetragastris*

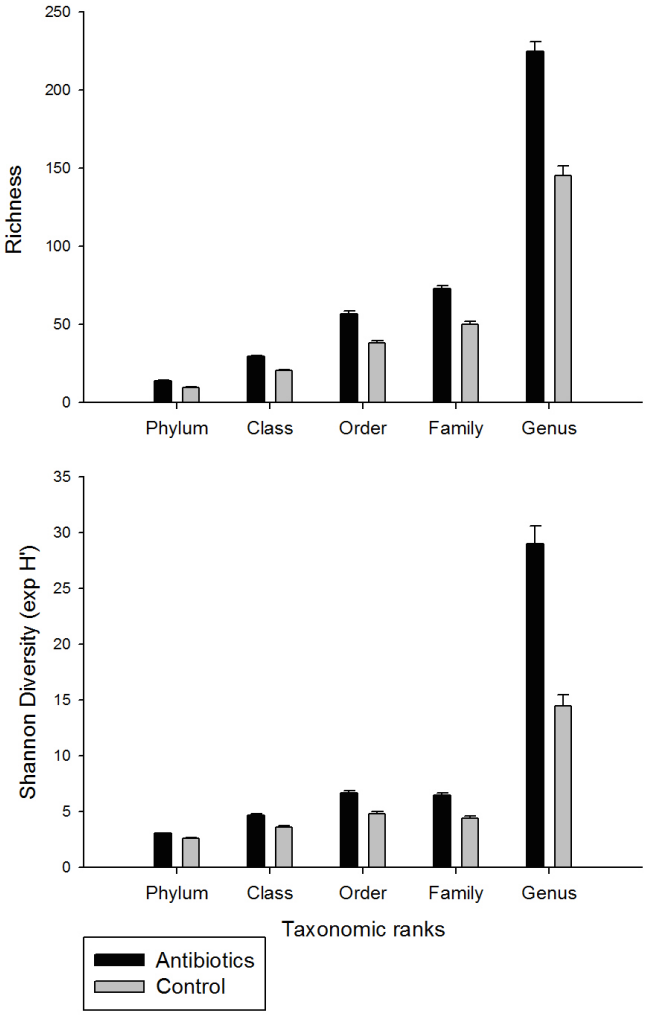

Figure 25. Endophyte taxa richness (A) and community diversity (B) among *Tetragastris* leaves of control- (N = 95) and antibiotic-treated (N = 81) seedlings after 29 months of applications. * represents significant differences among treatments ($P < 0.05$). Bars represent mean values (± 1 SE).

Figure 26. The impacts of antibiotics on endophyte community composition for *Tetragastris*

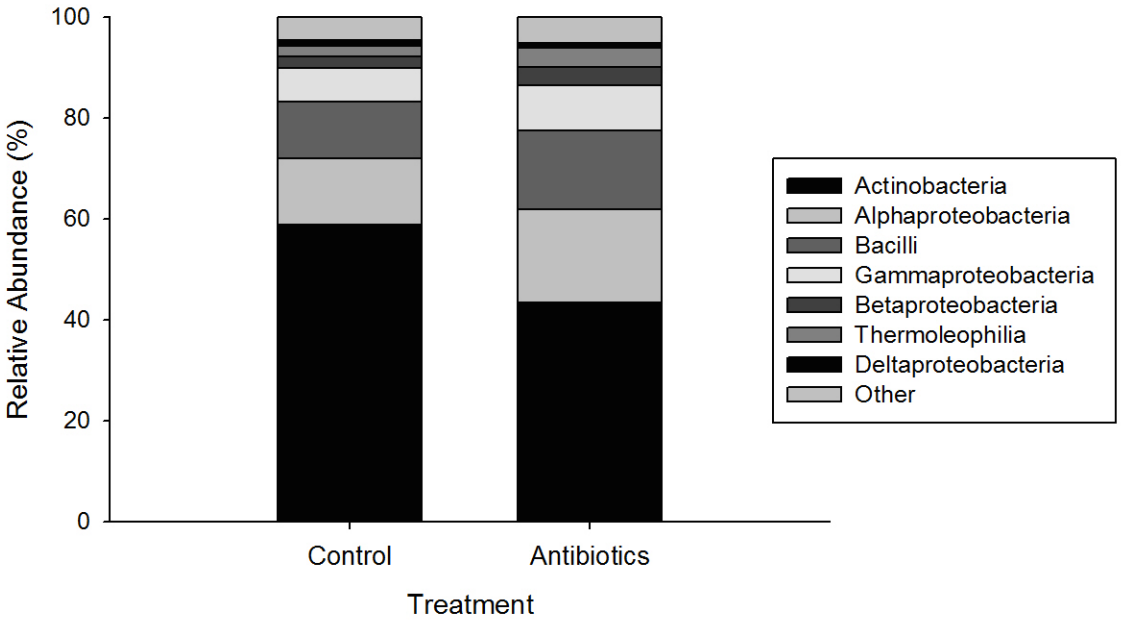

Figure 26. The impacts of antibiotic applications on endophyte community composition of *Tetragastris panamensis* compared to control-treated leaves ($F_{1,471} = 11.90$, $P < 0.001$). Classes are listed from most to less abundant in the legend.

5.8 SUPPORTING MATERIALS

Figure S27. The Gigante Fertilization Experiment

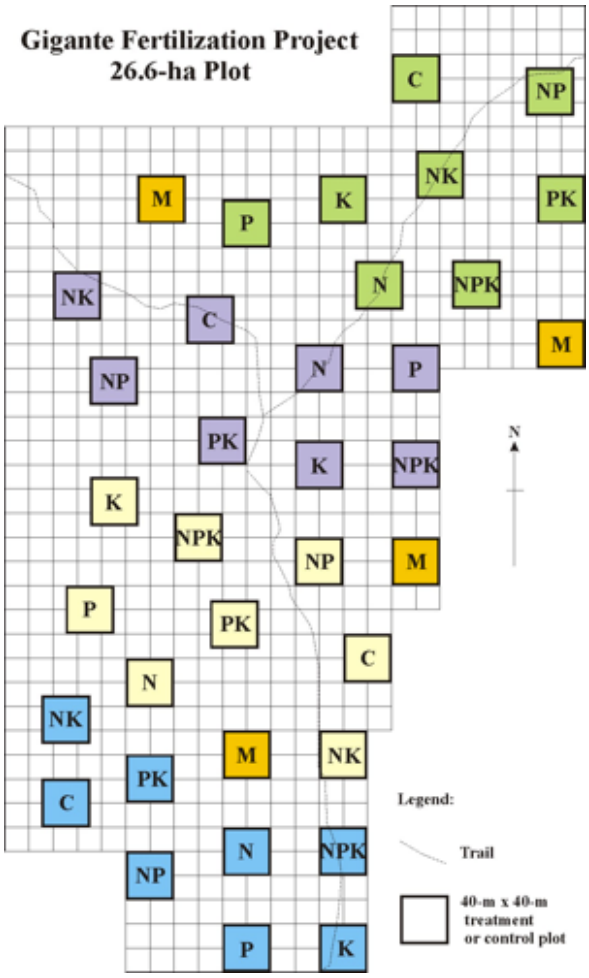

Figure S27. Map of study area showing the placement of nutrient treatments within the site. Treatments are represented by the combination of added nutrients: nitrogen (N), phosphorus (P), and potassium (K). Control plots (C) and micronutrient plots (M) are also shown. Colors represent replicates (N =4). We did not use the micronutrient treatment plots for this study.

Table S23. Core microbiome

Table S23. Operational taxonomic units (OTUs) present in 95% or more of control-treated tree samples.

Phylum	Class	Order	Family
Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae
Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae
Actinobacteria	Actinobacteria	Actinomycetales	Pseudonocardinaceae
Firmicutes	Bacilli	Bacillales	Bacillus
Firmicutes	Bacilli	Bacillales	Staphylococcus
Proteobacteria	Alphaproteobacteria	Rhizobiales	Other
Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae
Proteobacteria	Alphaproteobacteria	Sphigomonadales	Sphigomonadaceae
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae

Table S24. ANOVA results for richness differences among plant species

Table S24. ANOVA results for the effects of species on foliar bacterial endophyte community richness differences among seedlings over five species (*Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*) (N = 475).

Factor	Df	F value	P value
Phylum	4, 159	0.68	0.604
Class	4, 159	0.66	0.622
Order	4, 159	0.97	0.424
Family	4, 159	0.53	0.710
Genus	4, 159	0.67	0.615

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S25. ANOVA results for diversity differences among plant species

Table S25. ANOVA results for the effects of species on foliar bacterial endophyte community diversity differences among seedlings over five species (*Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*) (N = 475).

Factor	Df	F value	P value
Phylum	4, 159	**7.18**	**0.0001**
Class	4, 159	**11.89**	**0.0001**
Order	4, 159	**11.97**	**0.0001**
Family	4, 159	**5.29**	**0.0005**
Genus	4, 159	**9.16**	**0.0001**

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S26. PERMANOVA results for species and N, P, and K effects on endophytes

Table S26. PERMANOVA results for the effects of species, nitrogen (N), phosphorus (P), and potassium (K) on foliar bacterial endophyte community composition among seedlings five species (*Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*; (N = 475).

Factor	Df	F value	P value	R^2
SPP	4, 471	**5.81**	**0.001**	**0.045**
N	1, 471	1.33	0.225	0.003
P	1, 471	**10.59**	**0.001**	**0.021**
K	1, 471	**3.24**	**0.047**	**0.006**
N × P	1, 471	0.65	0.472	0.001
N × K	1, 471	**4.63**	**0.013**	**0.009**
P × K	1, 471	**10.44**	**0.001**	**0.020**
N × P × K	1, 471	**5.06**	**0.015**	**0.010**
SPP × N	4, 471	0.57	0.759	0.005
SPP × P	4, 471	0.81	0.570	0.006
SPP × K	4, 471	0.34	0.961	0.003
SPP × N × P	4, 471	0.99	0.423	0.008
SPP × N × K	4, 471	1.05	0.363	0.008
SPP × P × K	4, 471	0.82	0.541	0.006
SPP × N × P × K	4, 471	0.55	0.802	0.004

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S27. Differences in relative abundances of bacterial families among plant species

Table S27. This table represents the significant differences in the relative abundances of bacterial families among *Alseis blackiana* (ALBL), *Desmopsis panamensis* (DEPA), *Heisteria concinna* (HECO), *Sorocea affinis* (SOAF), and *Tetragastris panamensis* (TEPA) and P and K additions (N = 475).

Phylum	Family	ALBL	DEPA	HECO	SOAF	TEPA	T	P
Actinobacteria	Mycobacteriaceae	.328	.280	.276	.393	.462	11.6	.021
Actinobacteria	Micromonosporaceae	.039	.011	.010	.029	.027	14.8	.005
Actinobacteria	Cellulomonadaceae	1.79 E-04	9.93E-05	1.70 E-04	1.39E-05	3.38 E-04	12.8	.012
Actinobacteria	Promicromonosporaceae	4.32 E-04	2.61E-05	2.04E-05	2.51E-06	7.68E-06	11.7	.019
Bacteroidetes	Cytophagaceae	3.02 E-03	2.02 E-03	9.62 E-04	2.81 E-03	3.01 E-03	14.8	.005
Bacteroidetes	Rhodobacteraceae	8.01E-06	0	0	9.10E-06	1.83 E-04	9.7	.046
Bacteroidetes	Rikenellaceae	0	0	5.83E-07	0	3.80E-05	12.4	.015
Chlamydiae	Waddliaceae	0	0	0	0	2.99E-05	16.3	.003
Proteobacteria	Rhodobacteraceae	0.003221204	4.38 E-04	2.54 E-04	2.09 E-04	8.89 E-04	9.8	.044
Proteobacteria	Hyphomicrobiaceae	8.05 E-03	4.21 E-03	3.71 E-03	4.59 E-03	7.56 E-03	9.6	.049
Thermi	Deinococcaceae	2.32 E-03	3.64 E-04	3.06 E-03	1.47 E-03	9.80 E-04	16.2	.003

Table S28. Differences in relative abundances of bacterial genera among plant species

Table 5S28. This table represents the significant differences in the relative abundances of bacterial genera among *Alseis blackiana* (ALBL), *Desmopsis panamensis* (DEPA), *Heisteria concinna* (HECO), *Sorocea affinis* (SOAF), and *Tetragastris panamensis* (TEPA) and P and K additions (N = 475).

Phylum	Family	ALBL	TEPA	HECO	SOAF	TEPA	T-stat	P value
Actinobacteria	Mycobacterium	331	452	350	304	339	1.60	0.021
Actinobacteria	Pseudonocardia	019	013	014	009	013	0.44	0.034
Actinobacteria	Other (F: Micromonosporaceae	027	008	005	022	019	4.66	0.005
Actinobacteria	Other (F: Micromonosporaceae	010	003	005	06	005	0.07	0.039
Actinobacteria	Leucobacter	001	001	1.67 E-04	7.00 E-04	7.65 E-04	0.26	0.036
Actinobacteria	Actinoplanes	1.43 E-04	6.62 E-04	4.14 E-04	1.02 E-04	1.95 E-04	1.58	0.021
Actinobacteria	Other (F: Nocardiodaceae)	5.54 E-04	5.23 E-04	3.16 E-04	3.72 E-04	8.33 E-04	0.93	0.027
Actinobacteria	Other (F: Propionibacteriaceae)	9.82 E-04	4.48 E-04	6.02 E-04	3.78 E-04	4.91 E-04	1.47	0.022
Actinobacteria	Cellulomonas	1.05E -04	9.93 E-05	1.60 E-04	1.39 E-05	3.84 E-04	3.81	0.008
Actinobacteria	Janibacter	2.18 E-04	1.91 E-05	9.41 E-05	7.24 E-06	1.06 E-04	1.47	0.022
Actinobacteria	Other (F: Actinosynnemataceae	5.40 E-05	3.31 E-04	1.20 E-06	2.70 E-05	7.60 E-06	2.12	0.016
Actinobacteria	Arthrobacter	2.27 E-05	1.39 E-05	8.82 E-06	1.09 E-04	4.27 E-05	3.58	0.009
Actinobacteria	Rathayibacter	3.92 E-05	6.02 E-05	5.68 E-05	7.04 E-06	4.84 E-05	4.25	0.007
Actinobacteria	Xylanimicrobium	6.27 E-05	1.26 E-05	7.15 E-06	2.51 E-06	2.87 E-06	1.42	0.022
Bacteroidetes	Spirosoma	.001	.001	.001	.002	.002	0.02	0.040
Bacteroidetes	Chitinophaga	1.28 E-04	4.43 E-05	4.91 E-05	3.45 E-05	5.50 E-05	0.19	0.037
Chlamydiae	Waddlia	0	0	0	0	2.99E -05	6.29	0.003
Fermicutes	Clostridium	5.53 E-04	5.58 E-05	3.58 E-04	3.90 E-04	8.35 E-05	1.47	0.022
Fermicutes	Other (F: Ruminoccaceae)	1.50 E-06	.006	4.24 E-05	1.14 E-05	3.03 E-06	4.46	0.006
Fermicutes	Other (F: Gemellaceae)	5.76 E-05	1.89 E-05	8.60 E-05	5.70 E-05	8.41 E-05	1.68	0.020

Fermicutes	Tetragenoccocus	6.51 E-07	7.39 E-07	0	0	5.51 E-06	0.25	0.001
Proteobacteria	Neisseria	5.00 E-04	3.64 E-04	1.46 E-04	9.04 E-05	4.32 E-04	1.59	0.021
Proteobacteria	Other (F: Rhodobacteraceae)	3.25 E-04	3.99 E-05	4.03 E-05	7.62 E-05	5.66 E-04	2.85	0.012
Proteobacteria	Other (F: Moraxellaceae)	2.54 E-05	4.47 E-05	1.82 E-05	1.19 E-05	1.06 E-05	.62	0.047
Proteobacteria	Other (F: Rhodobacteraceae)	5.04 E-05	1.53 E-05	1.89 E-05	1.61 E-06	5.52 E-05	0.72	0.030
Proteobacteria	Kaistia	2.54 E-06	5.95 E-06	0	5.54 E-06	4.76 E-05	2.44	0.014
Proteobacteria	Roseococcus	8.11 E-05	2.73 E-06	4.79 E-06	0	0	1.71	0.020
Tenericutes	Other (F: Anaeroplasmataceae)	0	0	0	0	6.39 E-05	2.17	0.016
Thermi	Deinococcus	2.32 E-04	3.64 E-04	3.06 E-04	1.47 E-04	9.80 E-04	6.15	0.003

Table S29. MANOVA results for the effects of N, P, and K on genus richness

Table S29. MANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) on bacterial **genus** taxonomic richness among seedlings over five species (*Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*) and four replicates of all factorial combinations of N, P and K (N = 475).

Factor	Df	F value	P value
N	5, 20	0.09	0.9926
P	5, 20	0.54	0.7425
K	5, 20	1.22	0.3371
N × P	5, 20	0.65	0.6661
N × K	5, 20	1.27	0.3144
P × K	5, 20	0.45	0.8067
N × P × K	5, 20	0.12	0.9872

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S30. MANOVA results for the effects of N, P, and K on genus diversity

Table S30. MANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) on bacterial **genus** taxonomic diversity among seedlings over five species (*Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*) and four replicates of all factorial combinations of N, P and K (N = 475).

Factor	Df	F value	P value
N	5, 20	0.46	0.7986
P	5, 20	0.46	0.8037
K	5, 20	0.62	0.6884
N × P	5, 20	0.59	0.7099
N × K	5, 20	1.27	0.3144
P × K	5, 20	0.53	0.7514
N × P × K	5, 20	0.39	0.8469

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S31. MANOVA results for the effects of N, P, and K on family richness

Table S31. MANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) on bacterial **family** taxonomic richness among seedlings over five species (*Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*) and four replicates of all factorial combinations of N, P and K (N = 475).

Factor	Df	F value	P value
N	5, 20	0.22	0.9490
P	5, 20	0.59	0.7092
K	5, 20	1.07	0.4084
N × P	5, 20	0.61	0.6932
N × K	5, 20	1.30	0.3048
P × K	5, 20	1.29	0.3078
N × P × K	5, 20	0.28	0.9206

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S32. MANOVA results for the effects of N, P, and K on family diversity

Table S32. MANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) on bacterial **family** taxonomic diversity among seedlings over five species (*Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*) and four replicates of all factorial combinations of N, P and K (N = 475).

Factor	Df	F value	P value
N	5, 20	0.74	0.6030
P	5, 20	0.29	0.9140
K	5, 20	0.47	0.7976
N × P	5, 20	0.92	0.4906
N × K	5, 20	1.37	0.2777
P × K	5, 20	0.66	0.6595
N × P × K	5, 20	1.25	0.3243

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S33. MANOVA results for the effects of N, P, and K on order richness

Table S33. MANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) on bacterial **order** taxonomic richness among seedlings over five species (*Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*) and four replicates of all factorial combinations of N, P and K (N = 475).

Factor	Df	F value	P value
N	5, 20	0.17	0.9714
P	5, 20	0.36	0.8690
K	5, 20	0.54	0.7414
N × P	5, 20	0.34	0.8802
N × K	5, 20	1.15	0.3690
P × K	5, 20	0.61	0.6939
N × P × K	5, 20	0.31	0.8990

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S34. MANOVA results for the effects of N, P, and K on order diversity

Table S34. MANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) on bacterial **order** taxonomic diversity among seedlings over five species (*Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*) and four replicates of all factorial combinations of N, P and K (N = 475).

Factor	Df	F value	P value
N	5, 20	0.46	0.8002
P	5, 20	0.37	0.8639
K	5, 20	0.45	0.8094
N × P	5, 20	0.47	0.7929
N × K	5, 20	0.48	0.7835
P × K	5, 20	0.77	0.5826
N × P × K	5, 20	0.62	0.6896

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S35. MANOVA results for the effects of N, P, and K on class richness

Table S35. MANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) on bacterial **class** taxonomic richness among seedlings over five species (*Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*) and four replicates of all factorial combinations of N, P and K (N = 475).

Factor	Df	F value	P value
N	5, 20	0.19	0.9634
P	5, 20	0.53	0.7501
K	5, 20	0.45	0.8106
N × P	5, 20	0.46	0.7988
N × K	5, 20	1.50	0.2331
P × K	5, 20	0.33	0.8919
N × P × K	5, 20	0.52	0.7559

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S36. MANOVA results for the effects of N, P, and K on class diversity

Table S36. MANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) on bacterial **class** taxonomic diversity among seedlings over five species (*Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*) and four replicates of all factorial combinations of N, P and K (N = 475).

Factor	Df	F value	P value
N	5, 20	0.38	0.8565
P	5, 20	0.37	0.8629
K	5, 20	0.33	0.8859
N × P	5, 20	0.46	0.8815
N × K	5, 20	0.34	0.7835
P × K	5, 20	0.84	0.5350
N × P × K	5, 20	0.51	0.7619

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S37. MANOVA results for the effects of N, P, and K on phylum richness

Table S37. MANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) on bacterial **phylum** taxonomic richness among seedlings over five species (*Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*) and four replicates of all factorial combinations of N, P and K (N = 475).

Factor	Df	F value	P value
N	5, 20	0.30	0.9097
P	5, 20	0.61	0.6965
K	5, 20	0.46	0.8009
N × P	5, 20	0.67	0.6495
N × K	5, 20	1.64	0.1962
P × K	5, 20	0.34	0.8799
N × P × K	5, 20	0.64	0.6717

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S38. MANOVA results for the effects of N, P, and K on phyla diversity

Table S38. MANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) on bacterial **phylum** taxonomic diversity among seedlings over five species (*Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis*, and *Tetragastris panamensis*) and four replicates of all factorial combinations of N, P and K (N = 475).

Factor	Df	F value	P value
N	5, 20	0.42	0.8316
P	5, 20	0.43	0.8216
K	5, 20	0.64	0.6744
N × P	5, 20	0.84	0.5372
N × K	5, 20	0.62	0.6871
P × K	5, 20	1.29	0.3081
N × P × K	5, 20	0.59	0.7104

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S39. Significant genera differences among N, P, K additions and host species

Table S39. This table lists the genera whose relative abundances differed with particular soil nutrient enrichment (N, P, and K) and host species (SPP) interactions.

Phylum	Genus	Treatment	F value	FDR- P value
Firmicutes	Streptococcus	P × K	31.90	1.19 E-06
Firmicutes	Streptococcus	N × P	18.64	1.99 E-04
Proteobacteria	Haemophilus	N × P × K	17.86	1.99 E-04
Proteobacteria	Haemophilus	K	17.39	1.99 E-04
Actinobacteria	Mycobacterium	K	17.32	1.99 E-04
Proteobacteria	Pseudomonas	**N**	16.38	2.61 E-04
Proteobacteria	Haemophilus	P × K	15.87	2.86 E-04
Proteobacteria	Sphingomonas	**K**	15.04	3.23 E-04
Firmicutes	Streptococcus	N × P × K	14.99	3.23 E-04
Proteobacteria	Haemophilus	N × P	14.90	3.23 E-04
Firmicutes	Staphylococcus	N × P × K	13.79	4.88 E-04
Actinobacteria	Actinomyces	SPP × P	5.59	4.88 E-04
Actinobacteria	Acinetobacter	N × P × K	13.41	5.20 E-04
Firmicutes	Paenibacillus	N	12.99	5.94 E-04
Fusobacteria	Fusobacterium	P × K	12.79	6.15 E-04
Fusobacteria	Fusobacterium	K	12.64	6.22 E-04
Proteobacteria	Methylobacterium	P × K	11.83	8.81 E-04
Firmicutes	Streptococcus	SPP × P	4.98	8.94 E-04
Actinobacteria	Actinomyces	N	11.51	9.25 E-04
Actinobacteria	Pseudonocardia	SPP × P	4.77	0.001
Firmicutes	Gemella	N × P × K	10.86	0.001

Data presented are *P*-values for fixed effects. Bolded values are statistically significant (False Discovery Rate- corrected *P* values).

Table S40. ANOVA results for the effects of antibiotic applications and N, P, and K on phylum richness

Table S40. ANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) and antibiotic applications on bacterial **phylum** taxonomic richness among seedlings of *Tetragastris panamensis* (N=176).

Factor	Df	F value	P value
N	1, 15	0.08	0.7723
P	1, 15	0.96	0.3328
K	1, 15	0.00	0.9830
N × P	1, 15	0.72	0.4007
N × K	1, 15	1.81	0.1848
P × K	1, 15	0.03	0.8568
N × P × K	1, 15	0.01	0.9280
TRT	1, 15	**25.33**	**<0.0001**
TRT × N	1, 15	0.17	0.6837
TRT × P	1, 15	0.15	0.6967
TRT × K	1, 15	0.10	0.7555
TRT × N × P	1, 15	0.56	0.4562
TRT × N × K	1, 15	0.80	0.3766
TRT × P × K	1, 15	0.15	0.6966
TRT × N × P × K	1, 15	0.31	0.5828

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S41. ANOVA results for the effects of antibiotic applications and N, P, and K on phylum diversity

Table S41. ANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) and antibiotic applications on bacterial **phylum** taxonomic diversity among seedlings of *Tetragastris panamensis* (N=176).

Factor	Df	F value	P value
N	1, 15	0.63	0.4303
P	1, 15	3.29	0.0756
K	1, 15	0.01	0.9152
N × P	1, 15	1.32	0.2563
N × K	1, 15	0.19	0.6614
P × K	1, 15	0.36	0.5535
N × P × K	1, 15	0.47	0.4955
TRT	1, 15	**21.27**	**<0.0001**
TRT × N	1, 15	0.85	0.3608
TRT × P	1, 15	1.79	0.1871
TRT × K	1, 15	1.05	0.3098
TRT × N × P	1, 15	1.69	0.2002
TRT × N × K	1, 15	0.37	0.5485
TRT × P × K	1, 15	**5.30**	**0.0257**
TRT × N × P × K	1, 15	0.15	0.7011

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S42. ANOVA results for the effects of antibiotic applications and N, P, and K on class richness

Table S42. ANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) and antibiotic applications on bacterial **class** taxonomic richness among seedlings of *Tetragastris panamensis* (N=176).

Factor	Df	F value	P value
N	1, 15	0.03	0.8631
P	1, 15	1.11	0.2971
K	1, 15	0.00	0.9808
N × P	1, 15	0.60	0.4407
N × K	1, 15	1.99	0.1650
P × K	1, 15	0.05	0.8257
N × P × K	1, 15	0.00	0.9757
TRT	1, 15	**22.99**	**<0.0001**
TRT × N	1, 15	0.20	0.6595
TRT × P	1, 15	0.29	0.5906
TRT × K	1, 15	0.17	0.6847
TRT × N × P	1, 15	0.55	0.4632
TRT × N × K	1, 15	0.74	0.3947
TRT × P × K	1, 15	0.08	0.7850
TRT × N × P × K	1, 15	0.07	0.7974

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S43. ANOVA results for the effects of antibiotic applications and N, P, and K on class diversity

Table S43. ANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) and antibiotic applications on bacterial **class** taxonomic diversity among seedlings of *Tetragastris panamensis* (N=176).

Factor	Df	F value	P value
N	1, 15	0.49	0.4862
P	1, 15	1.85	0.1804
K	1, 15	0.17	0.6818
N × P	1, 15	0.91	0.3459
N × K	1, 15	0.10	0.7502
P × K	1, 15	0.48	0.4919
N × P × K	1, 15	0.39	0.5337
TRT	1, 15	**21.75**	**<0.0001**
TRT × N	1, 15	0.53	0.4696
TRT × P	1, 15	0.67	0.4162
TRT × K	1, 15	0.86	0.3570
TRT × N × P	1, 15	0.47	0.4963
TRT × N × K	1, 15	0.90	0.3485
TRT × P × K	1, 15	**5.00**	**0.0301**
TRT × N × P × K	1, 15	0.04	0.8455

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S44. ANOVA results for the effects of antibiotic applications and N, P, and K on order richness

Table S44. ANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) and antibiotic applications on bacterial **order** taxonomic richness among seedlings of *Tetragastris panamensis* (N=176).

Factor	Df	F value	P value
N	1, 15	0.01	0.9082
P	1, 15	1.10	0.2988
K	1, 15	0.03	0.8599
N × P	1, 15	0.55	0.4613
N × K	1, 15	1.75	0.1920
P × K	1, 15	0.02	0.8916
N × P × K	1, 15	0.01	0.9160
TRT	1, 15	**23.93**	**<0.0001**
TRT × N	1, 15	0.19	0.6668
TRT × P	1, 15	0.33	0.5656
TRT × K	1, 15	0.17	0.6841
TRT × N × P	1, 15	0.62	0.4342
TRT × N × K	1, 15	0.40	0.5293
TRT × P × K	1, 15	0.10	0.7510
TRT × N × P × K	1, 15	0.04	0.8482

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S45. ANOVA results for the effects of antibiotic applications and N, P, and K on order diversity

Table S45. ANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) and antibiotic applications on bacterial **order** taxonomic diversity among seedlings of *Tetragastris panamensis* (N=176).

Factor	Df	F value	P value
N	1, 15	0.54	0.4654
P	1, 15	2.23	0.1421
K	1, 15	0.78	0.3820
N × P	1, 15	0.78	0.3820
N × K	1, 15	0.05	0.8286
P × K	1, 15	0.30	0.5849
N × P × K	1, 15	0.11	0.7391
TRT	1, 15	**20.96**	**<0.0001**
TRT × N	1, 15	0.44	0.5109
TRT × P	1, 15	0.90	0.3470
TRT × K	1, 15	0.83	0.3660
TRT × N × P	1, 15	0.50	0.4815
TRT × N × K	1, 15	0.98	0.3263
TRT × P × K	1, 15	**5.39**	**0.0245**
TRT × N × P × K	1, 15	0.00	0.9747

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S46. ANOVA results for the effects of antibiotic applications and N, P, and K on family richness

Table S46. ANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) and antibiotic applications on bacterial **family** taxonomic richness among seedlings of *Tetragastris panamensis* (N=176).

Factor	Df	F value	P value
N	1, 15	0.08	0.7813
P	1, 15	1.26	0.2678
K	1, 15	0.00	0.9868
N × P	1, 15	0.44	0.5085
N × K	1, 15	1.95	0.1690
P × K	1, 15	0.00	0.9632
N × P × K	1, 15	0.09	0.7617
TRT	1, 15	**30.41**	**<0.0001**
TRT × N	1, 15	0.14	0.7061
TRT × P	1, 15	0.59	0.4445
TRT × K	1, 15	0.06	0.8052
TRT × N × P	1, 15	0.17	0.6783
TRT × N × K	1, 15	1.15	0.2888
TRT × P × K	1, 15	0.29	0.5940
TRT × N × P × K	1, 15	0.02	0.8925

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S47. ANOVA results for the effects of antibiotic applications and N, P, and K on family diversity

Table S47. ANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) and antibiotic applications on bacterial **family** taxonomic diversity among seedlings of *Tetragastris panamensis* (N=176).

Factor	Df	F value	P value
N	1, 15	1.17	0.2854
P	1, 15	2.49	0.1212
K	1, 15	0.23	0.6314
N × P	1, 15	0.03	0.8560
N × K	1, 15	0.16	0.6886
P × K	1, 15	0.00	0.9638
N × P × K	1, 15	0.02	0.8778
TRT	1, 15	**25.87**	**<0.0001**
TRT × N	1, 15	0.76	0.3886
TRT × P	1, 15	1.10	0.3001
TRT × K	1, 15	0.40	0.5286
TRT × N × P	1, 15	1.74	0.1936
TRT × N × K	1, 15	0.01	0.9349
TRT × P × K	1, 15	3.05	0.0871
TRT × N × P × K	1, 15	0.30	0.5849

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S48. ANOVA results for the effects of antibiotic applications and N, P, and K on genus richness

Table S48. ANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) and antibiotic applications on bacterial **genus** taxonomic richness among seedlings of *Tetragastris panamensis* (N=176).

Factor	Df	F value	P value
N	1, 15	0.00	0.9973
P	1, 15	1.30	0.2602
K	1, 15	0.04	0.8424
N × P	1, 15	0.65	0.4224
N × K	1, 15	1.78	0.1879
P × K	1, 15	0.02	0.8772
N × P × K	1, 15	0.20	0.6581
TRT	1, 15	**29.21**	**<0.0001**
TRT × N	1, 15	0.10	0.7496
TRT × P	1, 15	0.50	0.4836
TRT × K	1, 15	0.06	0.8070
TRT × N × P	1, 15	0.11	0.7473
TRT × N × K	1, 15	0.64	0.4280
TRT × P × K	1, 15	0.20	0.6528
TRT × N × P × K	1, 15	0.02	0.8917

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S49. ANOVA results for the effects of antibiotic applications and N, P, and K on genus diversity

Table S49. ANOVA results for the effects of nitrogen (N), phosphorus (P), and potassium (K) and antibiotic applications on bacterial **genus** taxonomic diversity among seedlings of *Tetragastris panamensis* (N=176).

Factor	Df	F value	P value
N	1, 15	0.27	0.6054
P	1, 15	0.87	0.3550
K	1, 15	0.01	0.9330
N × P	1, 15	0.19	0.6661
N × K	1, 15	0.14	0.7102
P × K	1, 15	0.06	0.8131
N × P × K	1, 15	0.01	0.9359
TRT	1, 15	**27.97**	**<0.0001**
TRT × N	1, 15	0.11	0.7371
TRT × P	1, 15	0.68	0.4151
TRT × K	1, 15	1.18	0.2837
TRT × N × P	1, 15	0.61	0.4392
TRT × N × K	1, 15	0.42	0.5189
TRT × P × K	1, 15	**4.05**	**0.0497**
TRT × N × P × K	1, 15	0.04	0.8448

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S50. PERMANOVA results for the effects of antibiotic applications and N, P, and K on *Tetragastris* endophytes

Table S50. PERMANOVA results for the effects of nitrogen (N), phosphorus (P), potassium (K), and antibiotic applications on foliar bacterial endophyte community composition among seedlings of *Tetragastris panamensis* (N = 176).

Factor	Df	F value	P value	R^2
N	1, 175	0.39	0.742	0.002
P	1, 175	1.13	0.291	0.006
K	1, 175	0.40	0.724	0.002
N × P	1, 175	**2.68**	**0.044**	**0.013**
N × K	1, 175	0.37	0.757	0.002
P × K	1, 175	1.67	0.180	0.003
N × P × K	1, 175	**3.78**	**0.020**	**0.019**
ANTIB	1, 175	**11.90**	**0.001**	**0.059**
ANTIB × N	1, 175	2.95	0.055	0.015
ANTIB × P	1, 175	1.00	0.336	0.005
ANTIB × K	1, 175	**2.86**	**0.049**	**0.014**
ANTIB × N × P	1, 175	0.66	0.553	0.003
ANTIB × N × K	1, 175	**4.89**	**0.010**	**0.024**
ANTIB × P × K	1, 175	**5.53**	**0.011**	**0.028**
ANTIB × N × P × K	1, 175	0.74	0.472	0.004

Data presented are *P*-values for fixed effects. Bolded values are statistically significant ($P < 0.05$).

Table S51. Significant differences in genera abundances between antibiotic and control plants

Table S51. This table lists the significant differences in the relative abundances of bacterial genera among antibiotic and control-treated individuals of *Tetragastris* (N = 176).

Phylum	Genus	A mean	C mean	Test-statistic	P value
Chlamydiae	Candidatus Rhabdochlamydia	1.58E-05	3.10E-04	3.87	0.049
Elusimicrobia	Other (O: FAC88)	0	1.78E-04	5.19	0.023
Firmicutes	Veillonella	0	4.06E-05	5.19	0.023
Firmicutes	Other (F: Planococcaceae)	6.40E-04	5.69E-05	4.01	0.045
Proteobacteria	Cellvibrio	0	1.52E-05	5.19	0.023
Proteobacteria	Cupriavidus	0	8.04E-05	5.19	0.023
Proteobacteria	Other (F: Pasteurellaceae)	7.77E-05	6.35E-06	8.27	0.004
Proteobacteria	Other (F: Sinobacteraceae)	8.41E-04	1.59E-03	4.80	0.029

6.0 CONCLUSION

Though it has been known for decades that plant-associated bacteria have striking impacts on plant host performance, the degree to which plant-microbial interactions are pervasive or particular to co-occurring plant species scale up to potentially affect community processes is a novel area of great interest. In my dissertation work, I first reviewed the natural history of bacteria that reside on and inside leaves. Next, I empirically assessed how microhabitat variations impacted plant-foliar bacterial interactions among woody species in a hyper diverse plant community. Last, I quantified bacterial endophyte community structure among co-occurring plant species and along a key soil nutrient gradient. Below, I address the major findings and implications of my work.

In my review (Chapter 2), I conclude that bacteria are likely to be particularly potent and important to plant performance in the understory of tropical forest habitats. The basic ecology and even identity of these organisms, unfortunately, is almost entirely unknown. Much of what we know about the ecology of foliar bacteria comes almost exclusively from agro-ecosystems or lab species such as *Aradidopsis thaliana*. I outline a theoretical framework for my own research questions in hopes of assessing the relative impacts of foliar bacteria, many of who are likely to be potent pathogens and can function as major players in plant diversity maintenance processes. I make a call-of-arms for further work on plant-foliar bacterial interactions in shaded understory habitats.

Through my empirical work, I discovered that foliar bacteria in general caused enormous,

primarily negative, effects on plant performance among seedlings in a tropical forest. Specifically, I found that experimentally reducing bacteria *in situ* for almost three years caused increases in plant performance (Chapter 3) and leaf number (Chapter 4). In many instances, I found that soil nutrients mediated plant performance, but these effects were only seen when we experimentally manipulated bacterial loads. Because these results were pervasive for every metric measured (e.g., relative growth rate of height, leaf production, herbivore damage, pathogen damage), my work suggests that foliar bacteria are an absolutely critical component of plant performance for seedlings in this habitat. Thus, this work raises the question as to the degree that these organisms influence plant phenotypes in other systems. Perhaps more importantly, my findings suggest that studies which have failed to include foliar bacteria are missing the big (or more precisely, the small) picture.

Notably, I found that for every metric measured, the degree to which soil nutrients mediated plant-bacterial interactions differed among host species. It remains unclear which bacterial taxa were driving these performance outcomes. On one hand, a small portion of bacterial taxa which are unique to each species may cause drastic differences in plant performance. On the other hand, unique interactions among similar taxa or core microbiota (however this is defined) result in the plant phenotypes I observed. Indeed, I did in fact discover that endophyte community structure significantly differed among host species (Chapter 5), however host species did not explain a large portion of the variation in endophyte community structure (~5%). Thus, further studies should be conducted to empirically address which bacterial taxa and under what conditions cause the greatest impacts to plant performance.

Further, high-throughput sequencing provided novel insight into endophyte community structure and can act as a model for future work. In the first-ever replicated long-term empirical

manipulation of foliar bacterial communities, I demonstrate unequivocally that endophyte community structure significantly differed among host species (Chapter 5). Further, I experimentally determined that soil nutrient enrichment caused substantial differences in foliar bacterial endophyte community composition. Moreover, I found that while antibiotic applications decreased bacterial OTU count, antibiotics actually caused an increase in endophyte richness and diversity (Chapter 5). Future work will be needed to assess the effects of antibiotics on particular bacterial taxa, especially is these products are used to address scientific questions in the field. Moreover, my results suggest that we should begin to assess bacterial community effects at the community- rather than species-level. In the future, I can begin to synthesize results from high-throughput sequencing (Chapter 5) and empirical work in the field (Chapters 3 and 4) to make assertions and formulate hypotheses about which bacterial taxa cause the greatest impacts on plant performance.

To my knowledge, my work is the first to provide evidence that foliar bacteria may build up to structure and maintain plant community structure. Studies to date have demonstrated that co-occurring tree species respond differently along a soil nutrient resource gradient, whereby species perform better or worse than others as soil nutrient availability varies (e.g., Harms *et al.* 2001, John *et al.* 2007, Russo *et al.* 2008). This degree of specialization of species to particular resource availability is thought to facilitate the maintenance of species diversity as resources vary across large spatial scales (Wright 2002, Silvertown 2004). Here, I find evidence that foliar bacteria compose an entirely novel dimension of plant performance among resource supplies, whereby plant species respond differently along a nutrient axis and interactions with foliar bacteria. Thus, what was thought to be an entirely independent gradient of plant performance variation may be more finely tuned via plant-bacterial interactions. Ultimately, my findings

suggest that the mechanisms that maintain species diversity in plant communities are much more complex than previously realized.

In sum, I have used a system largely unconsidered in ecology to test theory about plant-microbe interactions and to hypothesize how these interactions may scale up to cause trophic cascades and even structure plant diversity at large scales. Ultimately, I hope that my research has demonstrated the importance of the little things in ecology. Moreover, I hope that this work demands consideration and the inclusion of bacteria (particularly bacterial communities) in ecological experiments and inspires future questions that consider foliar bacteria as an entirely autonomous model system.