Foliar bacteria and soil fertility mediate seedling performance: a new and cryptic dimension of niche differentiation

Eric A. Griffin,[1,4] M. Brian Traw,[1] Peter J. Morin,[2] Jonathan N. Pruitt,[1] S. Joseph Wright,[3] and Walter P. Carson[1]

[1]*Department of Biological Sciences, University of Pittsburgh, A234 Langley Hall, 4249 Fifth Avenue, Pittsburgh, Pennsylvania 15260 USA*
[2]*Department of Ecology, Evolution, and Natural Resources, Rutgers University, 14 College Farm Road, New Brunswick, New Jersey 08901 USA*
[3]*Smithsonian Tropical Research Institute, Apartado 0843-03092, Balboa, Panama*

Abstract. The phyllosphere (comprising the leaf surface and interior) 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 harmful to beneficial) and this would be strongly mediated by soil resource availability. To test this, we monitored tree seedling growth responses to commercial antibiotics 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 effect of antibiotics on performance 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. Specifically, applying antibiotics had no effect when potassium was added but increased growth rate by almost 40% in the absence of potassium. Alternatively, phosphorus enrichment caused the effect of bacteria to switch from being primarily beneficial to harmful 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 of bacteria can have very strong positive and negative effects on seedling performance. Moreover, these effects were clearly mediated by soil resource availability. Though speculative, we suggest that foliar bacteria may interact with soil fertility to comprise an important, yet cryptic dimension of niche differentiation, which can have important implications for species coexistence.

Key words: *niche differentiation; phyllosphere; plant community; plant–microbe interactions; soil resource availability; species coexistence; tree diversity.*

Introduction

The phyllosphere (leaf surface and interior) 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 and 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 and 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 and Carson 2015). Very few studies have characterized foliar bacteria 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 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.

Foliar bacteria may act as an entirely independent plant functional trait and cause critical differences in

plant performance among host species. On one hand, a large majority of bacteria on the phyllosphere make up a "core microbiome," meaning that a large subset of bacterial taxa are commonly shared among individuals in the same habitat or region (Rastogi et al. 2012, Shade and Handelsman 2012, Kembel et al. 2014, reviewed by Griffin and Carson 2015). Indeed, Kembel et al. (2014) demonstrated that while less than 2% 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 occurred repeatedly among numerous tree species. Large overlaps of bacterial phyllosphere communities may have similar or vastly different impacts among host species. On the other hand, phyllosphere communities may, like insect herbivores, be host specific (e.g., Dyer et al. 2007). Kembel et al. (2014) also found that host taxonomic ranks (all levels) and host traits (e.g., leaf chemistry and morphology, plant growth rates) explained 51% of the variation in bacterial communities among tree species in Panama. Specifically, host taxonomic ranks explained 26% of variation in bacterial community structure, where the species level alone explaining a majority of this variation (51%). In addition, host traits explained 13% of the variation. Last, interactions between taxonomy and traits explained 10% of the variation. The differential impacts of these communities to plant hosts, however, were not assessed. Thus, the jury is still out on whether or not the effects of bacterial communities will be highly host specific.

The effects of bacterial communities among plant hosts may vary with soil resource availability, even in the shaded understory. 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). Specifically, 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 Amtmann et al. 2008, Dordas 2008, Johnson et al. 2010). In addition, the effects of phosphorus addition on disease severity are inconsistent and equivocal (reviewed by Dordas 2008, Johnson et al. 2010). It has recently been demonstrated that woody seedlings in tropical forests are co-limited by soil nutrients (Wright et al. 2011, Pasquini and 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, though empirical data are nonexistent. Ultimately, 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 diversity maintenance at large scales. 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 and Poorter 2008). Still, it remains unclear how these abiotic factors can facilitate the coexistence of hundreds of tree species in hyper-diverse tropical forests (e.g., Hubbell et al. 1999, Hubbell 2001, Chave 2004). Recently, however, Mangan et al. (2010) and Schnitzer et al. (2011) demonstrated that *soil* microbial communities represent a previously unappreciated biotic niche because they can cause negative feedbacks among coexisting plant species. Specifically, interactions among soil microbes and plant hosts produce local niches where in some cases common species suffer fewer negative consequences from pathogens compared to more rare species. Moreover, though speculative, abiotic factors (e.g., soil resources) may mediate biotic interactions between microbial communities and plant hosts. Thus, variations in soil nutrient availability could significantly facilitate coexistence by narrowing biotic plant–microbe niches down to finer realized niches, thereby opening niche space for other community members (Chase and Leibold 2003, Silvertown 2004).

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 with broad-spectrum antibiotics for 29 months and measured growth responses for seedlings of five woody species. 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 for 15 yr. We focused on growth rates because size differences among coexisting seedlings and saplings in the shaded understory determine which individuals live and die, as well as who will reach the canopy (e.g., Brown and Whitmore 1992, Boot 1996, Zagt and Werner 1998).

Methods

Study site and species

We conducted this study on the Gigante Peninsula in a mature (~200 yr old) secondary tropical forest in Panama

(9°06′31″ N, 79°50′37″ W; Appendix S1). Annual precipitation averages 2600 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).

Design of the fertilization experiment

We employed a $2 \times 2 \times 2$ factorial design with nitrogen (N), phosphorus (P), and potassium (K) and replicated the eight treatments four times (Appendix S1). The 32 experimental plots each measured 40×40 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 6-week intervals between June and November within the wet season (May-December). Each year, we applied 125 kg $N \cdot ha^{-1} \cdot yr^{-1}$ as urea, 50 kg $P \cdot ha^{-1} \cdot yr^{-1}$ as triple super-phosphate, and 50 kg $K \cdot ha^{-1} \cdot yr^{-1}$ as KCl.

Seedling measurements and antibiotic applications

In January 2010, we selected six relatively healthy individuals (minimal signs of necrosis or insect damage) of each species (~20–30 cm tall) within the inner 30×30 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 up to 100 ppm of Agri-mycin 17 (a commercial formulation of streptomycin, #02-0150; Hummert International, Earth City, Missouri, USA) and up to 1,752 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 and are highly effective under field conditions (McManus et al. 2002, Traw et al. 2007). Streptomycin (Agri-mycin) and gentamycin (Agry-gent) typically inhibit protein synthesis for Gram-negative bacteria, primarily for Proteobacteria (McManus et al. 2002, Ding and He 2010). Oxytetracycline (Agry-gent) inhibits both Gram-positive and Gram-negative bacteria and in some cases inhibits Gram-positive bacteria to a greater degree (Chopra and Roberts 2001, Ding and He 2010, Nelson and Levy 2011). Moreover, these antibiotics have limited adverse side effects, including those on fungi, which, if present, are typically short term (Ingham and Coleman 1984, Colinas et al. 1994, Chopra and Roberts 2001, Thiele-Bruhn and Beck 2005).

We measured seedling height 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 percentage of 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 in *Statistical analyses* and we present results without canopy openness.

Antibiotic effectiveness and bacterial isolate identification

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). While we are well aware that these culturing methods sample only a small portion of the total microbial diversity (e.g., Amman et al. 1995), we used culture-dependent protocols to confirm that antibiotics were effective over time and to inform us the degree to which antibiotics altered community composition (see *Antibiotic effectiveness*).

We tested the effectiveness of each antibiotic separately vs. 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 *Antibiotic effectiveness* and Appendix S6). Briefly, we removed leaf tissue via a sterile hole punch (6.35 mm diameter) from a randomly selected leaf before and two days after antibiotic or water application. We placed each leaf disk in 200 µL sterile 10 mmol/L MgSO$_4$ buffer in 1.5-mL centrifuge tubes and immediately took them to the lab to be cultured at room temperature (23°C) for 72 h. To assess epiphytic (leaf surface) bacteria, we placed each tube on a vortex mixer for 10–15 s in order to slough off bacteria into the MgSO$_4$ buffer and then plated 30 µL 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 and Carson 2015), we sterilized leaf surfaces following Arnold and Lutzoni (2007) and Kaewkla and Franco (2013). Briefly, we immersed leaf disks in 95% ethanol (10 s), 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 mmol/L $MgSO_4$ before plating 30 μL of each sample onto plates. To quantify morphotype richness, we identified 52 distinct colony morphologies that we classified using the characteristics described by Zinniel et al. (2002) and Traw et al. (2007). In addition, we identified 13 of the morphotypes using Sanger Sequencing of the 16S rDNA gene with universal bacterial primers and MicroSeq BLAST Software (SeqWright Genomic Services, Houston, Texas, USA; Sanger et al. 1977).

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 g of topsoil (A horizon) from a randomly selected area underneath each seedling and cultured bacterial communities in the lab (for details, see Wiggins and 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 1 h at room temperature. We then cultured bacteria following the techniques already described.

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

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

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

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

Because all five 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 similar MANOVAs to evaluate whether the effects of antibiotics differed among plant species. We performed 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 ran PERMANOVAs to evaluate whether culturable epiphytic and endophytic community composition varied among plant species. Next, we ran ANOVAs to determine whether the relative abundances of the six most common epiphyte and endophyte morphotypes differed among plant species. In addition, we ran PERMANOVAs to evaluate whether antibiotics caused changes in epiphytic and endophytic communities. Finally, we ran ANOVAs to determine whether antibiotics caused changes in the relative abundances of the six most common epiphyte and endophyte morphotypes. We used False Discovery Rate (FDR) corrections as described by Pike (2011) to adjust significant P values to correct for multiple tests.

We used SAS 9.4 (SAS Institute, Cary, North Carolina, USA) for plant growth analyses and SigmaPlot 11 (Systat Software, Erkrath, Germany) and R 3.2.4 (R Foundation for Statistical Computing, Vienna, Austria) for graphing. Specifically, we used gdata and vegan packages in R for community analyses and graphing.

Results

Antibiotic effectiveness

Colony abundance.—Agry-gent and Agri-mycin each significantly decreased mean abundance of epiphytic bacteria (compared to pre-treatment) by 55% and nearly 50%, respectively (Appendix S2: Fig. S1A, B; $T_{1,\,315} = -10.80$, $P < 0.0001$; $T_{1,\,315} = -10.17$, $P < 0.0001$). The sterile

water treatments had no effect on epiphyte abundance compared to pre-treatment (Appendix S2: Fig. S1A, B; $T_{1, 316} = 1.79$, $P = 0.07$; $T_{1, 315} = 0.48$, $P = 0.63$). Further, both Agry-gent and Agri-mycin decreased mean abundance of endophytic bacteria in surface-sterilized leaves (compared to pre-treatment) by over 50% and almost 50%, respectively (Appendix S2: Fig. S1C, 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 (Appendix S2: Fig. S1C, D: $T_{1, 314} = -1.35$, $P = 0.18$; $T_{1, 315} = -0.91$, $P = 0.37$). There were almost no differences (with one exception) in the degree to which either Agry-gent or Agri-mycin reduced bacterial abundance among nutrient treatments and plant species (see Appendix S7). Our findings for both antibiotics suggest that the effectiveness of the antibiotics did not vary among resource supply treatments, among species, or through time (Appendices S6–S7).

Morphotype richness.—Agry-gent and Agri-mycin significantly decreased mean epiphyte morphotype richness (compared to pre-treatment) by 20% and 15%, respectively (Appendix S2: Fig. S2A, B; $T_{1, 315} = -8.30$, $P < 0.0001$; $T_{1, 315} = -5.35$, $P < 0.0001$). The sterile water treatment had no effect on morphotype richness compared to pre-treatment (Appendix S2: Fig. S2A, B; $T_{1, 316} = 1.04$, $P = 0.30$; $T_{1, 316} = -0.58$, $P = 0.56$). Further, Agry-gent and Agri-mycin decreased mean endophyte richness (compared to pre-treatment) by 35% and almost 40%, respectively (Appendix S2: Fig. S2C, D; $T_{1, 316} = -10.33$, $P < 0.0001$; $T_{1, 313} = -11.42$, $P < 0.0001$). And, the sterile water treatment had no effect on endophyte morphotype richness compared to pre-treatment (Appendix S2: Fig. S2C, D; $T_{1, 313} = 1.36$, $P = 0.18$; $T_{1, 315} = 0.72$, $P = 0.47$). There were no differences in the degree to which either Agry-gent or Agri-mycin reduced bacterial richness among nutrient treatments and plant species (see Appendix S7). Thus, our findings suggest that the effectiveness of the antibiotics did not vary among resource supply treatments, among species, or through time.

Community composition.—Overall, we found that a small number of morphotypes made up a large majority of all bacteria among all plant species, however the community structure of epiphytic bacteria substantially differed among plant species (Appendix S3: Fig. S1, $F_{1, 78} = 1.38$, $P = 0.028$). First, the most dominant bacterial morphotypes among all species on the surface and inside leaves were *Sphingobacteria* spp., which collectively were found on 97% of all samples and made up ~66% of all cultured bacteria. Out of the six most common morphotypes, which collectively made up almost 81% of all bacteria, only the relative abundance of *Stenotrophomonas rhizophila* substantially differed among host species ($F_{1, 74} = 3.90$, $P = 0.006$; Appendix S8). Specifically, *Stenotrophomonas* never occurred on *Tetragastris* but occurred on almost 25% of *Sorocea* leaves. The abundance of five other common morphotypes (MT), howev-

er, did not differ among tree species (MT1, $F_{4, 74} = 0.62$, $P = 0.65$; MT2, $F_{4, 74} = 0.41$, $P = 0.81$; MT3, $F_{4, 74} = 0.42$, $P = 0.80$; MT8, $F_{4, 74} = 0.16$, $P = 0.96$; MT9, $F_{4, 74} = 1.65$, $P = 0.17$). Thus, differences in rare morphotypes drove differences in bacterial communities among tree species. For example, though only comprising less than 2% of overall abundance, an *Enterobacteria* sp. occurred on only 3% of *Tetragastris* leaves but 25% of *Alseis* leaves (Appendix S8).

Though epiphyte community composition varied among plant species, endophyte community composition did not vary among plant species (endophytes, $F_{1, 77} = 1.31$, $P = 0.06$).

The effects of antibiotics on community composition.—Applying each antibiotic caused substantial changes in epiphyte and endophyte communities (Appendix S3: Fig. S2, Agri-mycin epiphytes, $F_{1, 79} = 3.69$, $P = 0.001$; Agri-mycin endophytes, Fig. S3: $F_{1, 77} = 2.33$, $P = 0.007$; Agry-gent epiphytes, Fig. S4: $F_{1, 77} = 2.33$, $P = 0.006$; Agry-gent endophytes, Fig. S5: $F_{1, 77} = 3.09$, $P = 0.005$). Specifically, Agri-mycin decreased the *relative* abundances of the most abundant morphotype (*Sphingobacteria*) by 16%. Agry-gent decreased the *relative* abundance of *Sphingobacteria* by 21%. Conversely, Agri-mycin caused an increase in the relative abundance of morphotypes 2, 3, 8, 9, and 15 by an average of ~5%. Moreover, Agry-gent caused an increase in the relative abundance of morphotypes 2, 3, 8, 9, and 15 by an average of ~6%. It is important to note that, while antibiotics decreased the absolute abundance of most morphotypes, applications decreased the most common morphotype compared to others.

With one exception, the degree to which both antibiotics decreased or increased the relative abundance of common morphotypes did not differ among plant species. Specifically, the degree to which Agry-gent decreased or increased the relative abundance for *Bacillus toyonensis* differed among plant species ($F_{4, 18} = 6.11$, $P = 0.003$). For example, Agry-gent decreased the relative abundance of *Bacillus* by 5% for *Heisteria* but increased the relative abundance by 19% for *Sorocea*.

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 (Appendix S4: Fig. S1A–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 (Appendix S4: 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
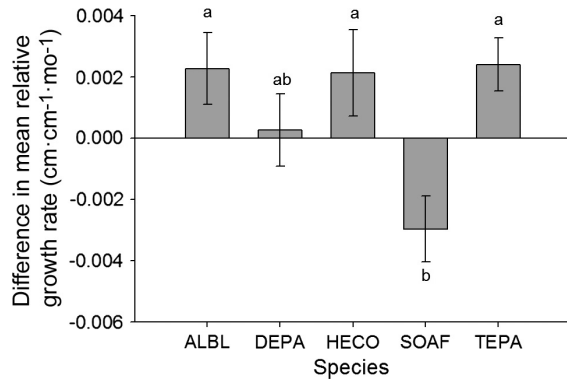
Fig. 1. The difference in mean relative growth rates of control and antibiotic treated seedlings ($\delta \bar{G}$) after 29 months of applications (letters represent significant species differences determined by Tukey tests; minimum significant difference: $P < 0.0045$). When bars are above the line, 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 ± SE. Species are *Alseis blackiana* (ALBL), *Desmopsis panamensis* (DEPA), *Heisteria concinna* (HECO), *Sorocea affinis* (SOAF), and *Tetragastris panamensis* (TEPA).

that our antibiotic or water treatments applied to leaves caused any changes in soil bacterial abundance or richness.

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.

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

Overall, applying antibiotics for 29 months consistently increased seedling growth to a lesser extent when we added K (Table 1, $F_{5, 20} = 3.19$, $P = 0.0282$). Specifically, across all species, applying antibiotics had no effect when K was added but increased growth rate by almost 40% in the absence of K, though this effect differed among species (Fig. 2). K addition caused significant changes in the effect of antibiotics on growth for both *Desmopsis* and *Heisteria* (Fig. 2; $F_{1, 31} = 6.80$, $P = 0.0155$; $F_{1, 31} = 5.86$, $P = 0.0234$). For *Desmopsis*, applying antibiotics decreased growth by 32% when K was added but conversely increased growth by 43% when K was not added. For *Heisteria*, applying antibiotics had no effect when K was added but conversely increased growth by 85% when K

was not added. K enrichment also caused 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, applying antibiotics increased plant growth by 26% when we added P but had no effect when P was not added, though this effect differed among species (Table 1, $F_{5, 20} = 3.01$, $P = 0.0349$; Appendix S5). Specifically, for *Alseis*, applying antibiotics had no effect on growth when P was added but increased growth by 138% when P was not added ($F_{1, 31} = 9.73$, $P = 0.0047$). For *Sorocea*, however, applying antibiotics had no effect on growth when P was added but decreased growth by 56% when P was not added ($F_{1, 31} = 11.00$, $P = 0.0029$).

In general, antibiotic applications had no effect on seedling growth with N addition (Table 1, $F_{5, 20} = 0.62$, $P = 0.6843$).

There was, however, a significant P × N interaction (Table 1, $F_{5, 20} = 3.66$, $P = 0.0164$), thus the effect of P on performance often depended on the presence or absence of N addition (Fig. 3). Specifically, when we applied antibiotics to *Alseis*, P addition increased growth by 34% compared to decreasing plant growth by over 67% when P was not added; however, these stark contrasts for the presence or absence of P only occurred when we added N (Fig. 3A). Conversely, when we applied antibiotics to *Heisteria*, P addition decreased growth by 80% compared to increasing plant growth by 37% when P was not added; however, this only occurred when we did *not* add N (Fig. 3B).

DISCUSSION

We demonstrated that the net effects of broad-spectrum antibiotic applications were highly host specific and

TABLE 1. 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).

Factor	df	F	P
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

Notes: 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)). Data presented are for fixed effects. Values in boldface type are statistically significant ($P < 0.05$).
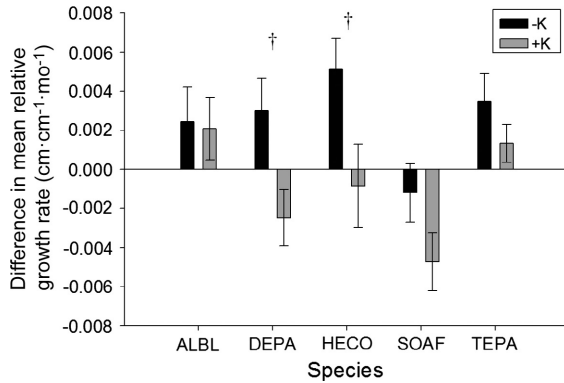
FIG. 2. Significant effects of potassium (K) enrichment on mean relative growth rates of control and antibiotic treated seedlings ($\delta \bar{G}$) after 29 months among five species. Dagger (†) represents significant Tukey test differences between potassium treatments for single species: $P < 0.0043$ 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 ± SE.

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). Antibiotic applications consistently increased plant growth to a lesser extent when we added K (Fig. 2). P and N enrichment caused an interaction so that antibiotic applications either decreased or increased plant growth depending on combinations of P, N, and plant species (Fig. 3). Thus, plant responses to antibiotic applications 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% (Appendix S6). 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 effects 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 (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 effects of antibiotics and thus bacterial community differences in situ among multiple coexisting species. Overall, foliar bacteria appear to be 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 underlie the formation of cryptic biotic niches and that variations in abiotic factors (soil fertility) may decrease niche space

among co-occurring plant species and thus promote the maintenance of plant diversity.

Soil resource supply mediated plant responses to foliar bacteria

The effects of antibiotic applications on host performance varied strongly with soil nutrient supply. Antibiotic applications consistently increased plant growth to a lesser extent when we added K, suggesting that K enrichment mitigates the negative effects of bacteria on plant 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 host 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 effects of phosphorus fertilization 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, but only in the presence of N. For a second species (*Heisteria*), P addition caused a decrease in plant performance when we reduced bacteria, but only in the absence of N. We do not want this complexity to get in the way of a key take-home message: variation in the effects of antibiotics depended upon interactions with soil resource supply and this in turn varied by species (Fig. 3). These findings *suggest* that plant-foliar bacteria interactions might contribute to a much greater degree of fine-scale habitat heterogeneity than previously recognized. Indeed, Silvertown (2004) suggested that plant–microbe interactions may function in this way, however, to date, there has been little 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 plant-foliar bacteria interactions. Ultimately, we suggest that as with plant–soil-microbes, plant-foliar bacteria interactions help create fine-scale biotic niches which contribute to plant species coexistence. Moreover, our results suggest that the strength and direction of these plant-foliar bacteria interactions (positive or negative) will vary among plant host species as well as among patches of forest soil that vary in fertility (e.g., Mangan et al. 2010, Schnitzer et al. 2011).
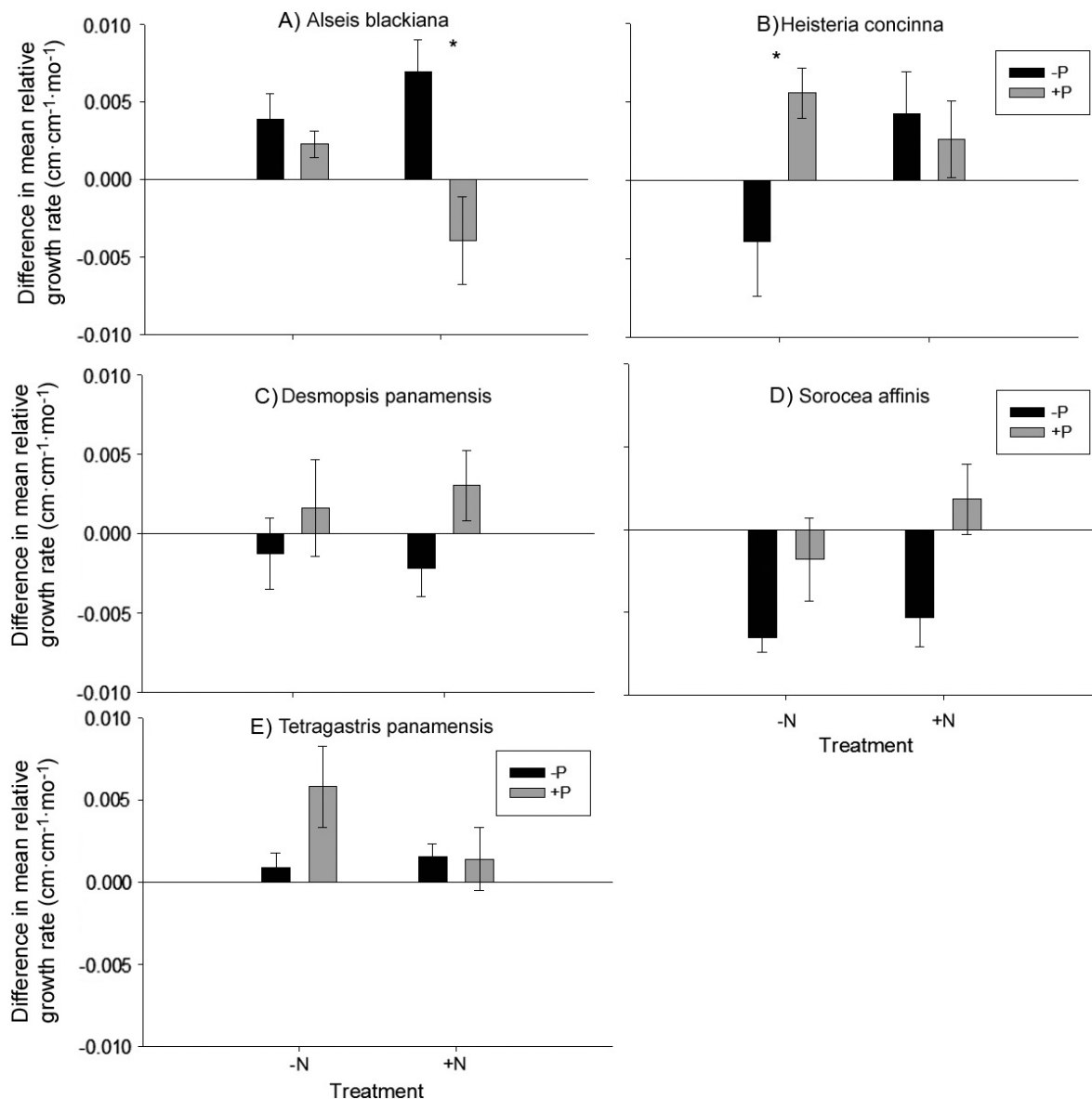
Fig. 3. Growth responses of the five species to the antibiotic treatment in the presence and absence of P addition (black vs. gray bars) and the presence vs. absence of N (*x*-axis). When bars are above the line, antibiotic applications increased plant relative growth rates and, when below the line, 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 ($\delta \bar{G}$) 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 ± SE. *Alseis* and *Sorocea* respond in the opposite direction to N addition (species effects) whereas *Alseis* and *Heisteria* illustrate the significant N × P interaction. ($F_{1,31} = 5.40$, $P = 0.0290$; $F_{1,31} = 5.86$, $P = 0.0343$, respectively).

Linking antibiotic applications to plant performance: potential mechanisms and future directions

Though the scope of our culture-based work is limited, we make assertions about our findings and suggest potential mechanisms. First, our finding that *Sphingobacteria* was particularly common among all plant species is consistent with recent studies using high-throughput sequencing to quantify bacterial community structure on the leaf surface and inside leaves of tropical trees. For example, Kembel et al. (2014) demonstrated that *Sphingobacteria* was the third most common operational taxonomic unit (OTU) found on the leaf surface of over 95% of trees in an adjacent Panamanian forest (including all of our focal species except *Tetragastris*). In addition, E. A. Griffin, S. W. Kembel, A. A. Carrell, S. J. Wright, and W. P. Carson (*in preparation*) identified *Sphingobacteria* inside leaves in 75% of seedlings from the same

species used in this study using high-throughput techniques. On average, antibiotic applications decreased the *relative* abundance of *Sphingobacteria* by up to 20% among all host species, suggesting that *Sphingobacteria* is much more susceptible to antibiotics than other more rare morphotypes. Because (1) the abundances of dominant morphotypes were similar among species and (2) antibiotics caused similar decreases or increases in dominant morphotypes, we suggest that common bacterial species have similar effects among plant hosts. Thus, because antibiotic applications resulted in increased plant growth for three species, we suggest that *Sphingobacteria* typically act as pathogens or other morphotypes conversely act as mutualists, or both. Conversely, we expect that *Shingobacteria* act as mutualists and other dominant morphotypes act as pathogens for *Sorocea*, where antibiotic applications decreased plant growth by 19%. Further, because Agry-gent applications resulted in a 19% increase in *Bacillus toyonensis* for *Sorocea*, we suggest that *Bacillus* is primarily pathogenic for *Sorocea*. Indeed, *Bacillus* spp. are some of the most pervasive bacterial pathogens in agriculture and make up almost 12% of bacterial endophytes in tropical tree species (e.g., Hosford 1982, Galal et al. 2006, Peng et al. 2013; Griffin et al., *in preparation*). Nevertheless, the overarching message is clear: changes in foliar bacterial community structure cause substantial decreases or increases in plant growth.

The mechanisms whereby bacteria mediate plant performance are speculative, though our results call for studies that evaluate a suite of alternatives. Our findings are consistent with a trade-off 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, 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 and 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 and 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 and Lindow 1999, Morris and Monier 2003). Conversely, in some cases and under varying levels of macronutrients (e.g., for *Sorocea*), bacterial reductions reduced plant growth rates. Under these circumstances, bacteria may function to some degree as mutualists by competitively excluding pathogenic fungi or even inducing plant resistance to fungal pathogens (reviewed by Griffin and Carson 2015, see also Arnold et al. 2003, Herre et al. 2007, Mejia et al. 2008, 2014 for beneficial leaf fungal endophytes). However, as with endophytic fungi, there may be costs associated with hosting

beneficial bacteria, including reduced photosynthetic capacity, increased water loss, differential allocation of carbon, and altered host metabolism (e.g., Mejia et al. 2014). In addition, interactions between foliar bacteria and fungi likely contribute to plant performance outcomes, and therefore future studies addressing the degree to which antibiotics affect interactions among microorganisms should be addressed. Moreover, we acknowledge the potential limitations in characterizing foliar bacteria to function because we did not fully inhibit these organisms and it is possible that there were antibiotic application effects on non-target fungi. Nevertheless, our study demonstrates the *net* effects of antibiotic applications 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.

Implications for species diversity

Our findings suggest that important yet cryptic interactions between foliar bacteria and plant hosts may contribute to plant diversity at large scales. 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, biotic interactions between plants and their foliar bacteria provide an additional and novel dynamic on a long recognized abiotic niche axis (soil nutrient availability). Ultimately, our findings build upon recent research demonstrating the critical implications of plant–microbe interactions for plant diversity (e.g., Mangan et al. 2010, Schnitzer et al. 2011, reviewed by Bever et al. 2015, Christian et al. 2015).

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SUPPORTING INFORMATION