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Research

Pervasive interactions between foliar microbes and soil nutrients mediate leaf production and herbivore damage in a tropical forest

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Summary

• Producing and retaining leaves underlie the performance and survivorship of seedlings in deeply shaded tropical forests. These habitats are characterized by conditions ideal for foliar bacteria, which can be potent plant pathogens. Leaf production, retention and susceptibility to enemies may ultimately depend upon interactions among soil nutrients and foliar microbes, yet this has never been tested.

• We experimentally evaluated the degree that foliar bacteria and soil resource supply mediate leaf dynamics for five common tree species (five different families) in a Panamanian forest. We reduced foliar bacteria with antibiotics for 29 months and measured leaf production, retention and damage for seedlings nested within a replicated 15-yr factorial nutrient enrichment experiment (nitrogen, N; phosphorus, P; potassium, K).

• Our results demonstrate that when we applied antibiotics, soil nutrients – particularly N – *always* regulated seedling leaf production (and to a lesser extent herbivore damage) for all five tree species. In addition, it was common for two macronutrients together to negate or completely reverse the impact of applying either one alone.

• Our findings of frequent plant–microbe–nutrient interactions are novel and suggest that these interactions may reinforce plant species–environment associations, thereby creating a fairly cryptic and fine-scale dimension of niche differentiation for coexisting tree species.

Introduction

The production, retention and defense of leaves underlie the performance of seedlings and saplings that live for years in deeply shaded forest understories where soil resources are scarce and enemies cause significant mortality. In tropical forests, the amount of damage on seedlings from both pathogens and herbivores is much higher than in temperate forests (Coley & Aide, 1991; reviewed by Coley & Barone, 1996; Gilbert, 2002; but cf. Moles, 2013). Consequently, leaf production is costly for shade-tolerant species that inhabit tropical forest understories because their seedlings invest heavily in structural and chemical defenses (Coley, 1983; Coley & Aide, 1991; Reich *et al.*, 1992; Wright & Cannon, 2001; Wright *et al.*, 2004; Gilbert, 2005). Moreover, tropical leaves in resource-poor habitats persist up to nine times longer and take up to three orders of magnitude longer (in days) to payback returns on carbon investments vs leaves in high-resource habitats (Chabot & Hicks, 1982; Chazdon & Fetcher, 1984; Coley, 1988; Williams *et al.*, 1989; Kikuzawa, 1991; Sobrado, 1991; Coley & Barone, 1996; Westoby *et al.*, 2000; Wright *et al.*, 2004). Thus, because leaf production is costly and retention is essential, understanding the mechanisms that underlie leaf dynamics is critical to understanding seedling performance.

In addition to contending with nutrient-poor soils and deep shade, seedlings in tropical forests also occur in a habitat rich in foliar microbes (Chazdon & Fetcher, 1984; Vitousek & Sanford, 1986; Wright & Van Schaik, 1994; Wright *et al.*, 2004; Poorter & Bongers, 2006; Poorter *et al.*, 2009; Griffin *et al.*, 2016; reviewed by Griffin & Carson, 2015). Because UV radiation is low, and temperature and humidity are high, foliar microbes, particularly bacteria, are likely to be diverse and abundant, and thus potentially key regulators of leaf dynamics (Griffin & Carson, 2015; Griffin *et al.*, 2016). Indeed, leaves are one of the world's largest microbial habitats occupying an area twice the size of the Earth's land area (Vorholt, 2012). On average, foliar bacteria occur in densities of 1–10 million cells cm^{-2} and, moreover, an average of over 500 taxa occurred on single trees in a tropical forest in Panama (Lindow & Brandl, 2003; Delmotte *et al.*, 2009; Vorholt, 2012; Kembel *et al.*, 2014). Leaves of seedlings in the understory are almost certainly teaming with bacteria because of the numerous ways bacteria are able to colonize leaf surfaces

and subsequently access leaf interiors (reviewed by Griffin & Carson, 2015). In a recent study, Griffin *et al.* (2016) demonstrated that foliar bacteria in a tropical forest caused up to a 49% reduction in growth rates for seedlings of three common tree species. These results suggest that foliar bacteria are commonly pathogenic and may typically regulate leaf dynamics, although data are nearly nonexistent (but cf. Griffin *et al.*, 2016).

Leaf dynamics may be strongly dependent on soil nutrient availability, because the low availability of macronutrients commonly reduces plant performance even in shaded habitats (Wright *et al.*, 2011; Pasquini & Santiago, 2012; Santiago *et al.*, 2012; Pasquini *et al.*, 2015). For example, Santiago *et al.* (2012) demonstrated that 2 years of soil nutrient additions increased seedling height growth by up to 24% (see also Pasquini *et al.*, 2015). Furthermore, Griffin *et al.* (2016) found that the degree to which foliar bacteria were harmful to seedlings varied among tree species and was often ameliorated by the greater availability of soil nutrients, particularly potassium. This suggests that the impact of foliar bacteria may typically depend on the availability of macronutrients (Griffin *et al.*, 2016). Oddly, the degree to which soil nutrients interact with plant-associated microbes to regulate plant performance *in situ*, however, remains little studied. This is surprising, because plant-associated microbes are critical mediators of plant functional traits and trophic interactions (Friesen *et al.*, 2011; van der Putten *et al.*, 2013; Turner *et al.*, 2013; Averill *et al.*, 2014; van der Heijden *et al.*, 2015).

Foliar bacteria and soil nutrients may commonly interact to regulate leaf dynamics. These interactions could alter host plant nutrient status and plant defenses, thereby altering the rate of leaf loss or gain, and changing the amount and type of damage on small seedlings and saplings (Coley, 1983; Coley *et al.*, 1985; Bazzaz *et al.*, 1987; reviewed by Coley & Barone, 1996). In addition, pathogenic bacteria may, like fungal pathogens, cause premature leaf abscission when plants shed leaves in response to infection (Ostry, 1987; Eyal *et al.*, 1993; Patterson, 2001; Davidson *et al.*, 2011). Thus, interactions between foliar bacteria and soil nutrient availability may commonly regulate patterns of enemy damage. For example, studies among agricultural crop species have shown that nutrient availability may mediate the negative impacts of foliar pathogens (Dordas, 2008; Johnson *et al.*, 2010). Potassium in particular tends to mitigate the severity of pathogen damage, likely because potassium fortifies plant cell walls to confer protection from pathogen entry (Dordas, 2008). Ultimately, variation in nutrient availability may interact with bacterial communities to favor some plant species over others as resources vary patchily across the landscape (Griffin *et al.*, 2016). If so, then interactions among foliar bacteria and soil nutrient availability could reinforce plant species–environment associations, thereby creating a fairly cryptic and fine-scale dimension of niche differentiation (Griffin *et al.*, 2016).

In the present study we assess whether foliar bacteria interact with soil nutrients to govern leaf dynamics for seedlings of co-occurring tree species in the shaded understory. We tested the following hypotheses within a tropical forest in Panama: the degree to which soil nutrients mediate leaf dynamics (production, retention, and enemy damage) varies substantially among co-occurring tree species; the degree to which foliar bacteria mediate leaf dynamics varies substantially among tree species; and interactions among soil nutrients and foliar bacteria will be frequent, thereby mediating leaf dynamics among co-occurring host plant species. To address these hypotheses, we experimentally reduced foliar bacteria for 29 months for seedlings of five common tree species. These seedlings were nested within a fully factorial, well-replicated nutrient enrichment experiment (nitrogen (N), phosphorus (P), and potassium (K) and all combinations) that commenced in 1998 (Yavitt & Wright, 2008; Yavitt *et al.*, 2009, 2011; Wright *et al.*, 2011; Pasquini & Santiago, 2012; Santiago *et al.*, 2012; Pasquini *et al.*, 2015; Griffin *et al.*, 2016).

Materials and Methods

Study site and fertilization experiment

We conducted this study in a mature, seasonally moist, semi-deciduous tropical secondary forest (*c.* 200 years old) on the Gigante Peninsula in Panama, which is part of the Barro Colorado Nature Monument. The soils are oxisols, alfisols, inceptisols and acric nitisols (Turner *et al.*, 2016). The site receives 2600 mm of rainfall annually, though < 10% of this falls during the four month dry season (January–April).

Beginning in 1998, we applied nitrogen (N), phosphorus (P) and potassium (K) by hand four times a year between June and November in a $2 \times 2 \times 2$ factorial design (Supporting Information Fig. S1). We replicated each treatment four times along a mild elevational and soil gradient, using 32 plots, measuring 40×40 m separated by at least 40 m (Yavitt *et al.*, 2009; Turner *et al.*, 2016). We applied 125 kg N ha^{-1} yr^{-1} as urea, 50 kg P ha^{-1} yr^{-1} as triple super-phosphate, and 50 kg K ha^{-1} yr^{-1} as KCl.

Study species

We selected five common and relatively shade tolerant woody species from five different families; these species vary in life history traits and spanned a wide range of maximum adult heights. *Alseis blackiana* Hems. (Rubiaceae) is a mid-canopy tree, *Desmopsis panamensis* Saff. (Annonaceae) and *Heisteria concinna* Standl. (Olacaceae) are understory treelets, *Sorocea affinis* Hemsl. (Moraceae) is a small tree, and *Tetragastris panamensis* Kunze. (Burseraceae) is a canopy tree (Dalling *et al.*, 2001; Wright *et al.*, 2003, 2010; Gilbert *et al.*, 2006; nomenclature follows Garwood, 2009). Hereafter, we refer to each species by genus or by four-letter abbreviations in figures (ALBL, *Alseis*; DEPA, *Desmopsis*; HECO, *Heisteria*; SOAF, *Sorocea*; TEPA, *Tetragastris*).

Antibiotic applications

Within the inner 30×30 m of each fertilization plot, we randomly assigned three *c.* 20–30 cm tall seedlings of each species for antibiotic treatment and another three for control treatment (sterile water; $n = 941$ seedlings). Beginning in January 2010, we carefully sprayed antibiotics or sterile water to all seedlings to

saturation every 10–15 d for 29 months. We placed a plastic sheet around the base of each seedling to prevent exposing soil microbes to either treatment; soil samples verified that neither the antibiotic, nor water, altered soil bacterial abundance or richness (see Griffin *et al.*, 2016).

We alternated the antibiotic treatments between streptomycin (up to 100 ppm of Agri-mycin 17; Hummert International #02-0150; Earth City, MO, USA) or a joint treatment of oxytetracycline and gentamicin (up to 1752 ppm of Agry-Gent Plus 800; Química Agronómica de México, Chihuahua, México). These are the three most commonly used broad-spectrum antibiotics in temperate and tropical agricultural crops and they can reduce bacterial abundance on the leaf surface as well as inside leaves by up to 85% (McManus *et al.*, 2002; Vidaver, 2002; Traw *et al.*, 2007; Griffin *et al.*, 2016). Streptomycin (Agri-mycin) and gentamycin (Agry-gent) inhibit protein synthesis for Gram-negative bacteria, and oxytetracycline (Agry-gent) inhibits both Gram-positive and Gram-negative bacteria (Chopra & Roberts, 2001; McManus *et al.*, 2002; Ding & He, 2010; Nelson & Levy, 2011). All products have limited nontarget effects, including those on fungi (Ingham & Coleman, 1984; Colinas *et al.*, 1994; Chopra & Roberts, 2001; Thiele-Bruhn & Beck, 2005).

Leaf production 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. Thus, we define the rate of leaf change for each seedling after 29 months as 'leaf production' (see Statistical analyses). In addition, we randomly selected and marked four leaves from each seedling and estimated enemy damage (see Enemy damage) at the outset as well as enemy damage and how many of the original leaves remained after 14 months of applications (retention). We recorded leaf retention and enemy damage after only 14 months because almost all leaves (*c.* 98%) had fallen before the end of the experiment (29 months). We excluded *Alseis* from the herbivore, pathogen and retention analyses at month 14 because all leaves turnover each dry season (Dalling *et al.*, 2001). Finally, all data were collected blindly so the observer was unaware of antibiotic treatment and soil nutrient additions.

Enemy damage

We estimated percentage of leaf area removed by leaf-chewing herbivores and percentage damage by pathogens (chlorosis or lesions) for four randomly selected leaves from each seedling following the protocols of Schnitzer *et al.* (2002) and Mangan *et al.* (2010). As stated above, we estimated damage at the beginning of the experiment and then 14 months after either antibiotic or control applications. We based percentage loss estimates 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). Although some insect damage may cause lesions and chlorosis (e.g. Miller & Davidson, 2005), the primary causes of this type of damage are fungi, bacteria and viruses (e.g. Garcia-Guzman & Dirzo, 2001; Myster, 2002; Mangan *et al.*, 2010; E. Griffin, pers. obs.). Hereafter, we will refer to percentage area loss by leaf-chewing insects as 'herbivore damage' and chlorosis and lesions as 'pathogen damage'.

It is important to note that we also measured the mean canopy openness (0–100%) above each seedling with a concave densitometer at beast height at each time point (0 months, 14 months, 29 months). Canopy openness had no effect on any of the models.

Statistical analyses

We performed MANOVAs to evaluate whether nutrient additions caused changes in plant performance metrics. We chose to use MANOVAs because all five species were nested within the N, P and K treatments, and MANOVAs allowed us to avoid pseudofactorialism by adjusting for correlated response variables among species (Morrison, 1976; Morin, 1983; Winer *et al.*, 1991; Hurlbert, 2013). We assessed the significance of nutrient additions using Wilks' Criterion, one of four standard test statistics commonly used to evaluate the MANOVA (Morrison, 1976), defined by (determinant (E))(determinant ($E+H$))$^{-1}$, where H is the matrix of sums-of-squares and cross products calculated among treatment means (e.g. +N and −N), and E is the element-wise squared difference between each observation and the mean vectors for that group (Morin, 1983).

We used the method of linear discriminant functions, sometimes called canonical analysis of discriminance (CAD), to identify which species contributed to significant differences among our treatments for each response (Fisher, 1936; Legendre & Legendre, 1998). CAD follows directly from the calculations used to determine test statistics in the multivariate analysis of variance (MANOVA). Species that are significantly correlated with the values of the discriminant function scores are those that contribute to significant differences among the treatments examined in the MANOVA. Although single-species ANOVAs often yield similar results to those detected by CAD, the ANOVAs can miss correlated responses among the five variables (species responses; see Methods S1), and their significance levels are not adjusted for multiple tests on potentially correlated variables (Morin, 1983). For a full explanation of the discriminant function analysis, see Methods S1.

MANOVA models for control individuals We performed a first set of MANOVAs to evaluate seedling leaf production rate (L), percentage leaf area attacked by herbivores and pathogens (H and P), and leaf retention (R). We did this to test differences among nutrient addition treatments (N, P, K and all combinations) for control seedlings only. We calculated leaf production rates (leaves/leaf month^{-1}) for each seedling as:

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

(L_0 and L_1, initial and final leaf number; $t_1 - t_0$, time period (29 months; Santiago *et al.*, 2012)).

Table 1 MANOVA results for the effects of nitrogen (N), phosphorus (P) and potassium (K) on plant performance metrics for control seedlings treated with sterile water of five species (*Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis* and *Tetragastris panamensis*)

Effect	Leaf production	Leaf retention	Herbivore damage	Pathogen damage
N	1.64	8.71***	1.79	0.87
P	1.47	1.80	4.31**	1.63
K	6.90***	2.28	2.67	1.22
N × P	2.36	5.71**	3.69*	0.62
N × K	3.38*	2.58	1.54	1.03
P × K	2.62	2.80	1.10	0.48
N × P × K	1.76	2.26	1.80	0.17

We did not include *Alseis* in the leaf retention analysis because their leaves turnover annually. Entries are F-values determined from Wilks' Criterion. Degrees of freedom are 5,20 for the leaf production, herbivore damage and pathogen damage analyses, and 4,20 for leaf retention. Detection of a significant treatment effect indicates that the mean values of the leaf metric differ for the corresponding nutrient addition.
Significance: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

We estimated herbivore and pathogen damage among all seedlings before treatments began (Time = 0 months). Estimating damage at the beginning of the experiment allowed us to evaluate how over 15 years of soil nutrient additions impacted leaf damage among all seedlings. We calculated leaf retention R by determining the proportion of four randomly selected leaves from each seedling that remained after 14 months.

The average for each species in a plot (e.g. $\bar{L}$ for leaf production) for species i was

$$\bar{L}(i) = \bar{L}(i, \text{control})$$

Because all five species were nested within treatment plots (and nonindependent), our response vectors for each metric (e.g. $\bar{L}$) in plot j was:

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

where numbers 1 through 5 represent each plant species. *Alseis* was not included in the retention analysis because their leaves had flushed within two months of the beginning of the experiment.

MANOVA models for differences among antibiotic and control individuals We performed a second set of MANOVAs to evaluate the differences in leaf production rates (δL) after 29 months of applications and herbivore damage (δH), pathogen damage (δP) and leaf retentions (δR) after 14 months of applications. We calculated the difference in plot mean values (e.g. $\delta\bar{L}$, $\delta\bar{H}$, $\delta\bar{P}$ and $\delta\bar{R}$) for replicated individuals of each species with or without antibiotic applications in each plot. Thus, for leaf production,

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

For herbivore or pathogen damage rates, we only included surviving leaves after 14 months for species (i) and evaluated as:

$$H(i) = (H_{14}, i - H_0, i)/(1 - H_0, i) \text{ or } P(i)$$
$$= (P_{14}, i - P_0, i)/(1 - P_0, i)$$

Therefore, our response vector for each performance metric (e.g. $\delta\bar{L}_j$) in 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. A MANOVA of this response vector tests whether $\delta\bar{L}_j$ differed across nutrient treatments. *Alseis* was not included in the herbivore, pathogen, or retention MANOVA analyses because its leaves flushed two months after the experiment began.

We logit transformed all proportional data (Warton & Hui, 2011). We used SAS 9.4 (SAS Institute, Cary, NC, USA) to run MANOVAs.

Results

Antibiotic efficacy

We previously demonstrated that Agry-gent and Agri-mycin significantly decreased mean abundance of epiphytic and

Table 2 Pearson correlation coefficient values (r) for correlations between the discriminant function scores of significant nutrient effects compared to the original variables (species) in MANOVAs for control seedlings treated with sterile water

Metric	Nutrient	*Alseis*	*Desmopsis*	*Heisteria*	*Sorocea*	*Tetragastris*
Production	K	0.84***	−0.61***	−0.052***	0.28	0.08
Production	N × K	0.73***	−0.62***	−0.50***	0.23	0.33
Retention	N	NA	0.39*	0.34	0.77***	0.55***
Retention	N × P	NA	0.48***	0.48***	0.84***	0.15
Herbivory	P	−0.02	−0.12	−0.32	−0.56***	0.56***
Herbivory	N × P	0.17	−0.18	0.26	0.81***	−0.44*

Significant r correlations indicate a nutrient × species interaction for the corresponding leaf metric and corresponding nutrient addition (N, nitrogen; P, phosphorus; K, potassium).
*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

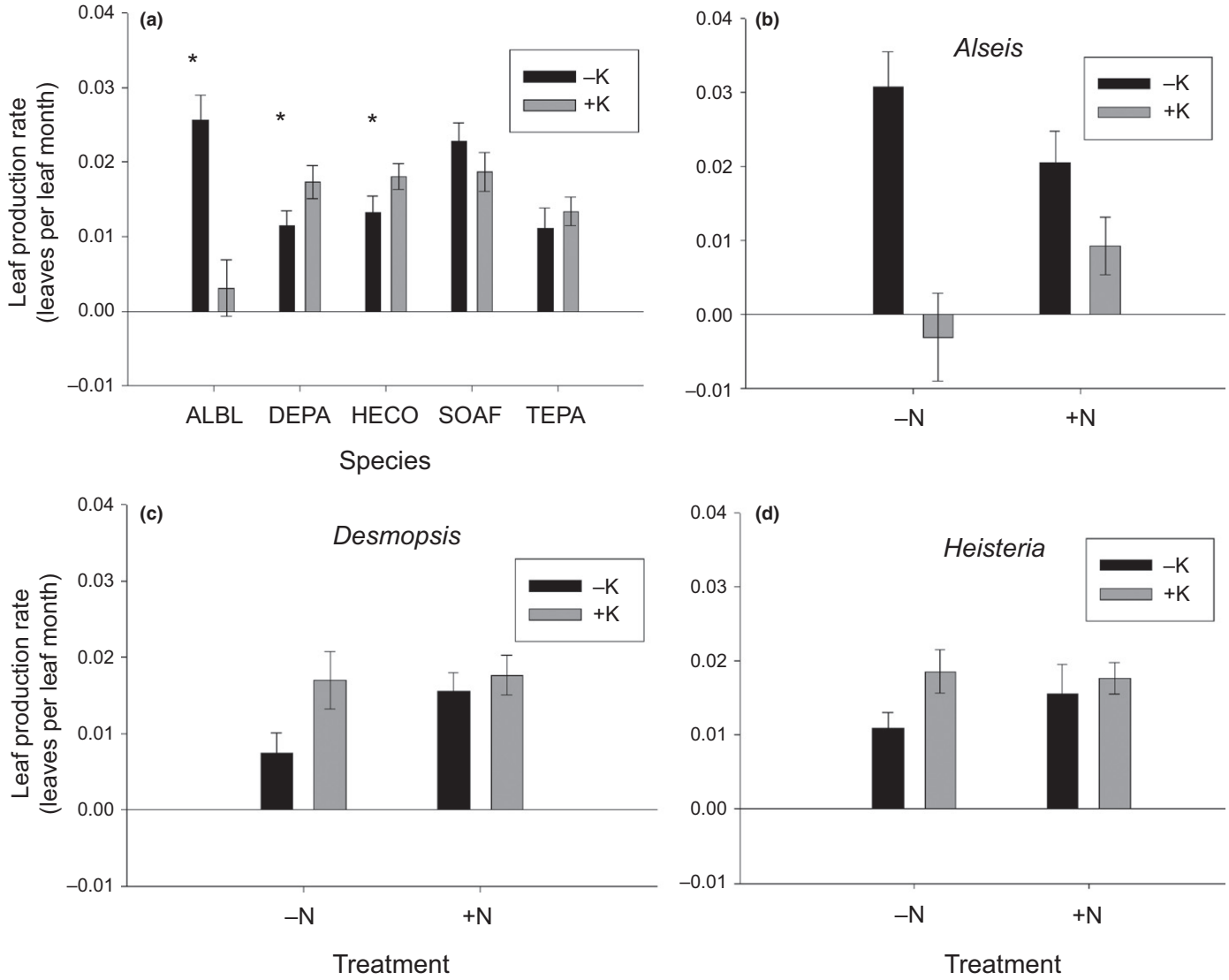

Fig. 1 Significant effects of potassium (K) and nitrogen (N) × K on leaf production rates among control seedlings of *Alseis blackiana* (ALBL), *Desmopsis panamensis* (DEPA), *Heisteria concinna* (HECO), *Sorocea affinis* (SOAF) and *Tetragastris panamensis* (TEPA) after 29 months of nutrient enrichment. (a) A significant K × species interaction effects on leaf production for *Alseis*, *Desmopsis* and *Heisteria* (K effect: $F_{5,20} = 6.90$, $P = 0.001$; *Alseis*: $P < 0.0001$; *Desmopsis*: $P = 0.0002$; *Heisteria*: $P = 0.002$). (b–d) N × K interaction effects on leaf production ($F_{5,20} = 3.38$, $P = 0.023$; (b) *Alseis*: $P < 0.0001$; (c) *Desmopsis*: $P = 0.0002$; (d) *Heisteria*: $P = 0.004$). Bars represent mean values ± SE.

endophytic bacteria by over 50% on our focal species and also decreased microbial morphotype richness by over 20% (Griffin *et al.*, 2016). Each of these antibiotic treatments were equally effective and remained so over time; also, their impact was very similar among nutrient treatments and plant species (Griffin *et al.*, 2016). Thus, our antibiotics worked in the sense that they reduced foliar bacterial loads, although our results are likely conservative because we only reduced bacterial abundance and richness by *c.* 50%.

At the beginning of the experiment, mean leaf numbers were 7.53 for *Alseis* ($n = 181$), 10.59 for *Desmopsis* ($n = 188$), 5.81 for *Heisteria* ($n = 191$), 5.32 for Sorocea ($n = 187$) and 6.70 for *Tetragastris* ($n = 193$). Below, if we do not report the impact of the antibiotic or specific nutrient or nutrient combination, it is because it did not have a significant impact on leaf dynamics. It is important to note that *c.* 10% seedlings died during the entirety of the experiment, and mortality did not differ among tree species or nutrient or antibiotic applications.

The impacts of macronutrients on leaf dynamics separate from the effects of antibiotics

Leaf production For three species, N and K interacted to cause increases or decreases in leaf production by 55–88% (Tables 1, 2; Fig. 1). K addition caused *Alseis* to produce far fewer leaves, but adding K and N together significantly reduced the negative effects of K alone (Tables 1, 2; Fig. 1b). For two other species, K addition alone caused the production of significantly more leaves

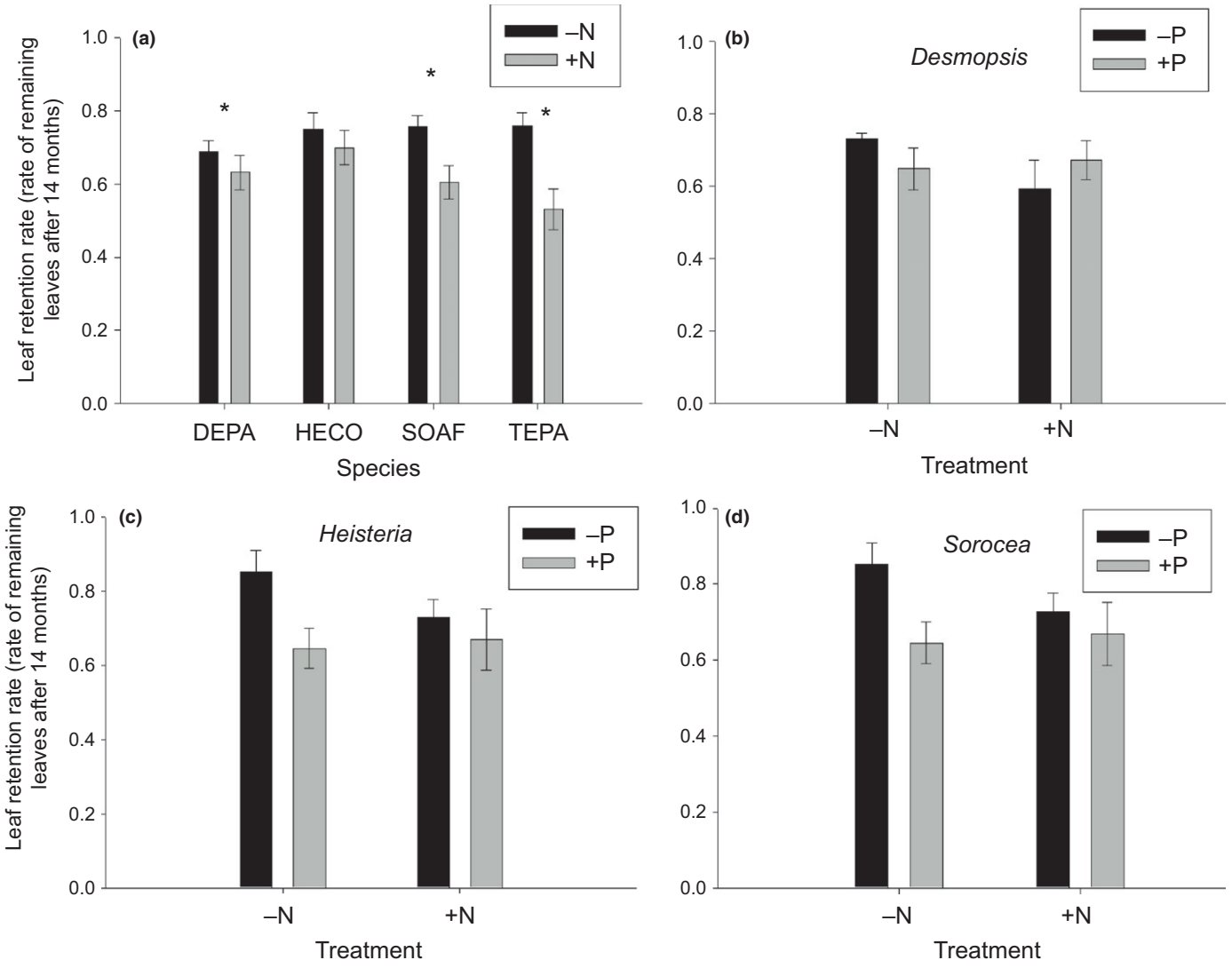

Fig. 2 Significant effects of nitrogen (N) and N × phosphorus (P) on leaf retention rates among control seedlings of *Desmopsis panamensis* (DEPA), *Heisteria concinna* (HECO), *Sorocea affinis* (SOAF) and *Tetragastris panamensis* (TEPA) after 14 months of nutrient enrichment. (a) A significant N × species interaction effect on leaf retention for *Desmopsis*, *Sorocea* and *Tetragastris* (N effect: $F_{4,20} = 8.71$, $P = 0.0003$; *Desmopsis*: $P = 0.027$; *Sorocea*: $P < 0.0001$; *Tetragastris*: $P = 0.001$). (b–d) N × P interaction effects on leaf retention ($F_{4,20} = 5.71$, $P = 0.003$; (b) *Desmopsis*: $P = 0.005$; (c) *Heisteria*: $P = 0.005$; (d) *Sorocea*: $P = 0.001$). Bars represent mean values ± SE.

(Fig. 1a), as did adding N alone, but adding both K and N together did not cause an additive increase in leaf production (significant N × K interaction, Fig. 1c,d).

Leaf retention We found a very consistent and significant effect of adding N alone, or adding N and P together on leaf retention. N addition alone decreased leaf retention for all four species (significant for three or four) but this decrease disappeared or was even reversed (*Desmopsis*) when N and P were added together (N × P interactions, Tables 1, 2; Fig. 2a–d; *Alseis* was not included). These findings demonstrate that a strong effect of adding one soil resource could be entirely offset by the simultaneous addition of another.

Herbivore and pathogen damage For two species, significant N × P interactions mediated herbivore damage (Tables 1, 2; Fig. 3). For *Sorocea*, adding P reduced damage, however, adding N and P together increased damage (Tables 1, 2; Fig. 3b). Conversely, for *Tetragastris*, adding P more than doubled herbivore damage but this effect disappeared when we added N and P together (Tables 1, 2; Fig. 3c). Nutrient additions never significantly altered pathogen damage. Overall, mean herbivore damage across all tree species was 8.5% (± 0.35 SE) and pathogen damage was 6.1% (± 0.26 SE; Fig. 4).

The impact of antibiotics and macronutrients on leaf dynamics

Leaf production The regular application of antibiotics and soil nutrients over 29 months caused increases or decreases in leaf production for all five species ranging from 38% to 140% (Tables 3, 4; Figs 5, 6). However, the effects of antibiotics were

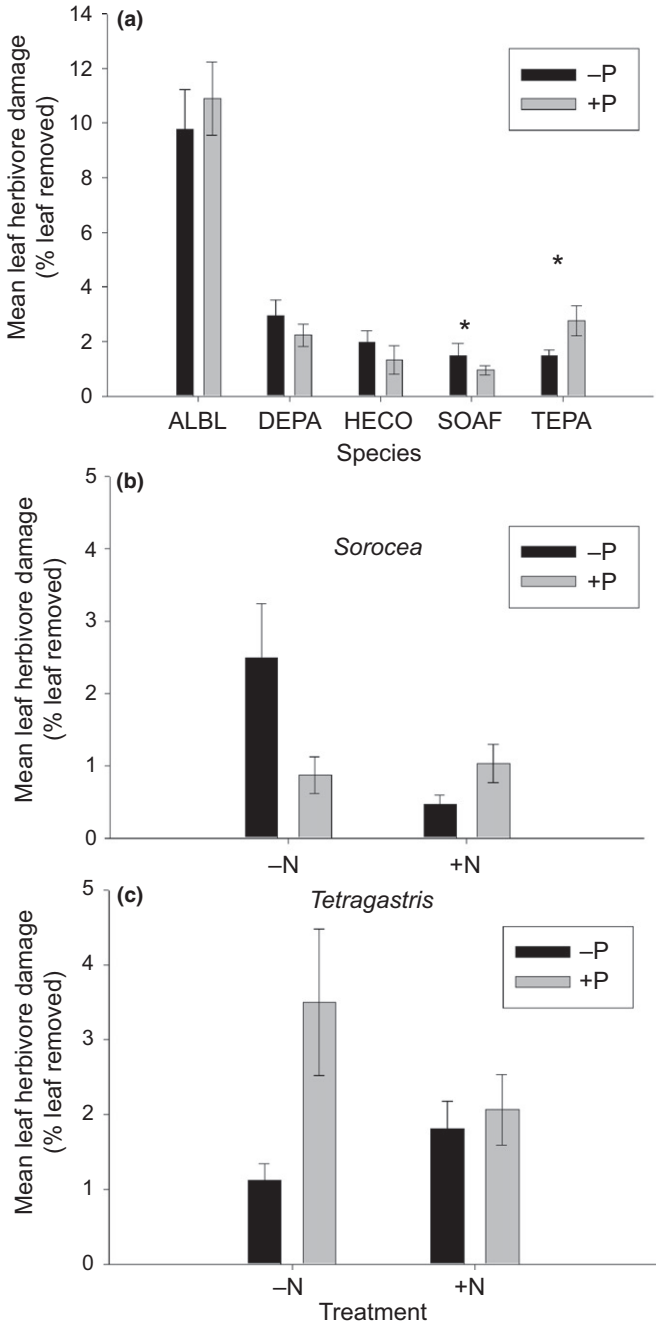

Fig. 3 Significant effects of phosphorus (P) and nitrogen (N) × P on leaf herbivore damage rates among control seedlings of *Alseis blackiana* (ALBL), *Desmopsis panamensis* (DEPA), *Heisteria concinna* (HECO), *Sorocea affinis* (SOAF) and *Tetragastris panamensis* (TEPA) at the beginning of the experiment (Time = 0 months). (a) A significant P × species interaction effect on herbivore damage for *Sorocea* and *Tetragastris* (P effect: $F_{5,20} = 4.31$, $P = 0.01$; *Sorocea*: $^*P = 0.001$; *Tetragastris*: $^*P = 0.001$). (b, c) N × P interaction effects on herbivore damage ($F_{5,20} = 3.69$, $P = 0.016$; (b) *Sorocea*: $P < 0.0001$; (c) *Tetragastris*: $P = 0.013$). Bars represent mean values ± SE.

always governed by N × K interactions, and to a lesser extent N × P interactions (Table 3). For example, for *Desmopsis*, antibiotics increased leaf production in plots fertilized with P but this was reversed in plots fertilized with both P and N (Table 4; Fig. 5b). For *Sorocea*, antibiotics increased leaf production but only in plots fertilized with both N and P (Fig. 5c).

For four of five species, the impact of antibiotics was strongly governed by N × K interactions (Table 4; Fig. 6). For three of five species, the response was consistent: adding antibiotics and K together increased leaf production; however, antibiotics had no effect when K and N were added together (Figs 6a,c,d, 3a). For a fourth species, *Heisteria*, applying antibiotics and K together decreased leaf production unless N and K were applied together

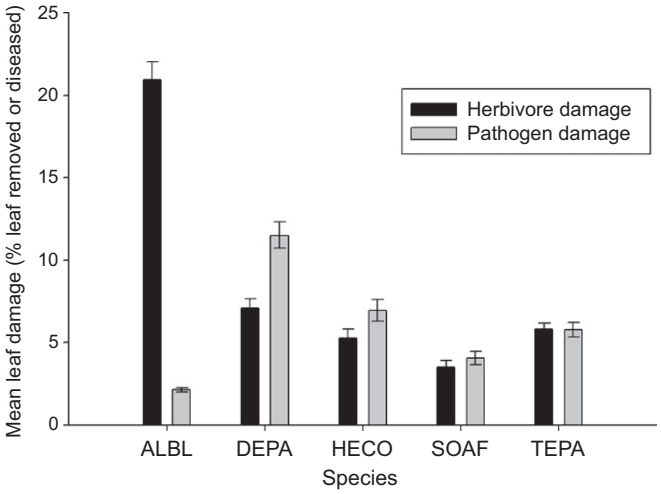

Fig. 4 Mean insect herbivore and pathogen damage (chlorosis or lesions) among leaves of *Alseis blackiana* (ALBL), *Desmopsis panamensis* (DEPA), *Heisteria concinna* (HECO), *Sorocea affinis* (SOAF) and *Tetragastris panamensis* (TEPA) at the beginning of the experiment. We estimated percentage leaf area removed by leaf-chewing herbivores and percentage damage by pathogens for four randomly selected leaves from each seedling before we began applying antibiotics ($n = 3572$ leaves). Bars represent mean values ± SE.

Table 3 MANOVA results for the effects of nitrogen (N), phosphorus (P) and potassium (K) on plant performance metric differences between antibiotic and control seedlings of five species (*Alseis blackiana*, *Desmopsis panamensis*, *Heisteria concinna*, *Sorocea affinis* and *Tetragastris panamensis*)

Effect	Leaf production	Leaf retention	Herbivore damage	Pathogen damage
N	1.97	1.76	1.51	2.48
P	1.49	0.90	0.19	1.13
K	3.52*	0.25	0.20	0.97
N × P	3.39*	0.37	0.93	2.12
N × K	4.45**	2.41	3.19*	1.20
P × K	0.56	1.13	1.94	0.92
N × P × K	1.16	1.18	2.58	1.05

Detection of a significant treatment effect indicates that the mean values of the leaf metric differ among antibiotic and control individuals for the corresponding nutrient addition. We did not include *Alseis* in the leaf retention analysis because their leaves turnover annually. Entries are F-values determined from Wilks' Criterion. Degrees of freedom are 5,20 for leaf production, and 4,20 for the leaf retention, herbivore and pathogen damage analyses.
Significance: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

Table 4 Pearson correlation coefficient (*r*) for correlations between the discriminant function scores of significant nutrient effects compared to the original variables (species) in MANOVAs of antibiotic and control differences

Metric	Nutrient	*Alseis*	*Desmopsis*	*Heisteria*	*Sorocea*	*Tetragastris*
Production	K	0.93***	−0.01	−0.32	0.31	0.36*
Production	N × P	0.15	0.86***	0.04	−0.46*	0.02
Production	N × K	0.47***	−0.26	−0.52*	0.57**	0.78***
Herbivory	N × K	NA	0.50**	0.84*	0.59***	−0.25

Significant *r* correlations indicate nutrient × species × antibiotic interactions for the corresponding leaf metric and nutrient addition (N, nitrogen; P, phosphorus; K, potassium). *Alseis* was not included in the herbivore, pathogen or retention analyses because its leaves turnover annually.
*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

(Fig. 6b). Overall, the effects of the antibiotics were often reversed when two macronutrients were added together.

Leaf retention We never detected any impact of antibiotics on leaf retention under any combination of our nutrient treatments (Table 3). Note that in the second set of analyses we evaluated the *difference* in means between the antibiotic and control individuals. This simply means that there were no differences in how antibiotic individuals responded to nutrient enrichment vs control individuals, not that there were no nutrient effects on retention (e.g. Tables 1, 2; Fig. 2a–d).

Herbivore damage For three species, the impact of antibiotics was consistent and regulated entirely by N × K interactions (Tables 3, 4; Fig. 7). Specifically, antibiotics decreased damage when neither N nor K was applied, or when both were applied together; conversely, antibiotics increased damage when just N was applied (Table 4; Fig. 7). This was most pronounced for *Heisteria* and *Sorocea* (Fig. 7b,c). These results mirror the strong N × K interactions that regulated leaf production (Fig. 6). To our surprise, we never detected any impact of the antibiotic on pathogen damage under any combination of nutrient treatments (Table 3).

Discussion

Our results demonstrate that soil nutrients and broad-spectrum antibiotics regulate leaf dynamics among five species in a deeply shaded forest understory. Specifically, potassium (K) was a key resource in regulating leaf production (K and N × K interactions), nitrogen (N) was key for leaf retention (N and N × P interactions), and phosphorus (P) was key for regulating herbivore damage (P and N × P interactions; Tables 1, 2; Figs 1–3). Notably, antibiotics caused increases in leaf production for four of five species (Fig. S2). More importantly, the impact of broad-spectrum antibiotics and soil nutrients interact to regulate leaf dynamics and, to a lesser extent, herbivore damage. This was true for all five of our focal tree species, which came from five different plant families, and vary in adult stature and other life history traits. The direction and magnitude of the impact of antibiotics were entirely dependent upon interactions with soil nutrients for all five species (Table 4). N was a key resource involved in each of these interactions for both leaf production and herbivore damage (i.e. significant N × K and N × P interactions). For example, it was striking that applying antibiotics caused a dramatic increase (up to 140%) in leaf production in plots enriched with K, but this increase was entirely reversed if N was applied together with K (e.g. *Alseis* (Figs 5a, 6a) showed highly significant N × K interactions). These types of reversals occurred for two other species (N × K interactions for leaf production; Fig. 5c,d) and also for three species for the effects of antibiotics on herbivore damage (N × K interactions; Fig. 7a–c). Our results provide compelling evidence that the impact of foliar microbial communities depends upon soil resource availability and, in particular on two macronutrients, N and K. Furthermore, our results strongly suggest that the application of antibiotics in combination with a single macronutrient (typically K) decreases the abundance of pathogenic bacteria, often increasing leaf production. However, the benefits of reducing bacteria, or the indirect effects of reducing bacteria (see Potential mechanisms underlying plant–microbe–nutrient interactions), were often negated when N and another macronutrient were applied together. Overall, our findings support recent studies that challenge the conventional wisdom that light is only a limiting resource for tropical seedlings and moreover demonstrate that soil nutrient availability and foliar bacteria are major drivers of plant performance (e.g. Pasquini & Santiago, 2012; Santiago *et al.*, 2012; Pasquini *et al.*, 2015; Griffin *et al.*, 2016).

Our results support all three hypotheses and demonstrate that antibiotics alter foliar bacteria to such a degree that it can substantially mediate leaf production and herbivore damage. Moreover, the *magnitude* of the impacts of nutrients on leaf production was host specific, and N and K availability regulated these responses. Thus, we suggest that without considering microbial communities, it is difficult to understand the mechanisms that regulate seedling performance and dynamics among co-occurring species and thus key aspects of forest regeneration (e.g. Mangan *et al.*, 2010). Moreover, these interactions appear strong enough to frequently change the rank-order performance of seedlings of different species across forest understories because soil resources and most likely bacteria as well, are patchily distributed at both large and small spatial scales (e.g. John *et al.*, 2007; Baldeck *et al.*, 2013; Condit *et al.*, 2013; Kembel *et al.*, 2014). For example, for our five species, antibiotic applications changed the rank-order performance for two of five species for leaf-production and

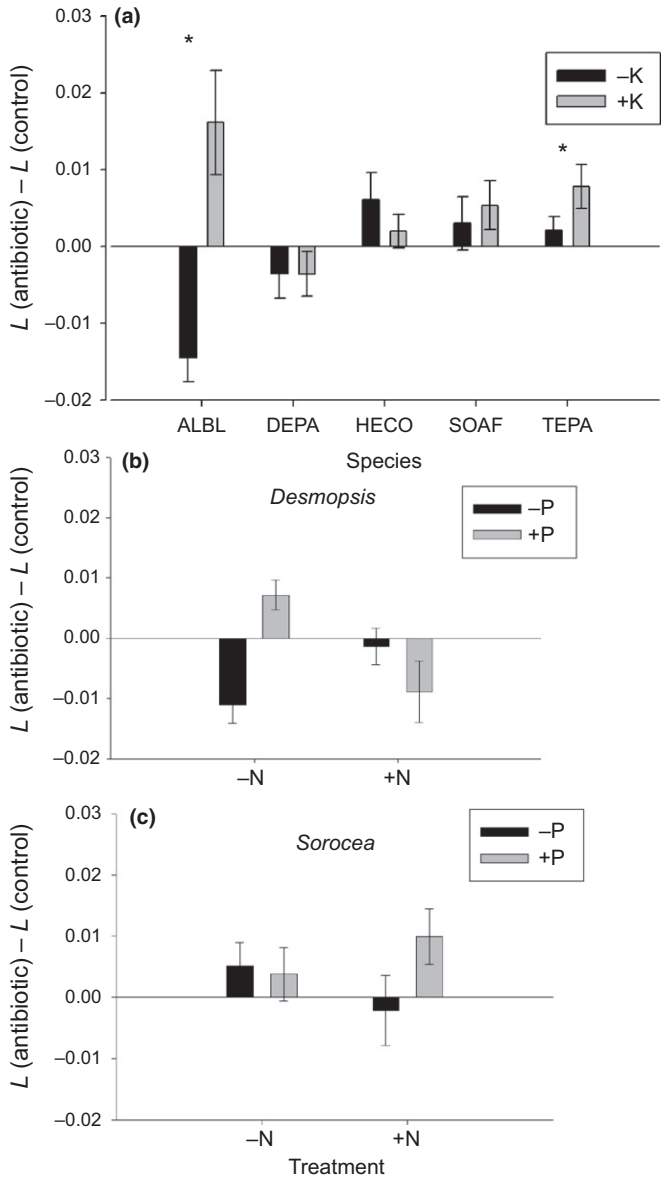

Fig. 5 Significant effects of antibiotic applications and potassium (K) and nitrogen (N) × phosphorus (P) on leaf production rates (*L*) among seedlings of *Alseis blackiana* (ALBL), *Desmopsis panamensis* (DEPA), *Heisteria concinna* (HECO), *Sorocea affinis* (SOAF) and *Tetragastris panamensis* (TEPA) after 29 months of applications and nutrient enrichment. When bars are above the line, antibiotic applications increased leaf production and when below the line, antibiotic applications decreased production. (a) For *Alseis* and *Tetragastris*, applying antibiotics increased leaf production when K was added (K effect: $F_{5,20} = 3.52$, $P = 0.0192$; *Alseis*: $^*P < 0.0001$; *Tetragastris*: $P = 0.0427$). (b, c) For *Desmopsis* and *Sorocea*, antibiotics and N × P regulated leaf production (significant antibiotics × N × P interaction, $F_{5,20} = 3.35$, $P = 0.0233$; *Desmopsis*: $P < 0.0001$; *Sorocea*: $P = 0.0141$). Bars represent mean values ± SE.

herbivore damage, and four of five species for seedling growth (Fig. S2; growth data from Griffin *et al.*, 2016). Notably, however, Griffin *et al.* (2016) only detailed the interplay between soil nutrients and foliar bacteria and their impacts on seedling growth among tree species. Here, we went one step further to measure the impacts of these interactions on leaf production and retention, two key underlying mechanisms for seedling growth. Additionally, we demonstrate that interactions between antibiotics and soil nutrients impacted plant growth and leaf production differently under different experimental conditions and sometimes in opposing directions. For example, Griffin *et al.* (2016) demonstrated that antibiotics mediated seedling growth when P, K and N × P were added; however, here we demonstrated that antibiotics mediated leaf production when K, N × P and N × K were added. In another example, Griffin *et al.* (2016) demonstrated that antibiotics increased *Desmopsis growth rates* when N and P were added; however, in this study antibiotics decreased *Desmopsis leaf production* rates when N and P were added. Thus, species-specific impacts of foliar bacteria vary among nutrient additions for different performance metrics, even if those metrics correlate with one another to a certain degree (e.g. growth and leaf production). For these reasons, we argue that foliar bacteria should be considered an entirely independent yet cryptic plant functional trait that regulates plant phenotypes and performance metrics among species. Ultimately, we hypothesize that these interactions may well represent a novel dimension of fine-scale niche differentiation, thus promoting the long-term coexistence of plant species (Griffin *et al.*, 2016).

Although speculative, we argue that combinations of two nutrients together (particularly N) make leaves more vulnerable to enemies, thus negating or reversing the effects of enrichment on performance. These results are consistent with findings by both Andersen *et al.* (2010) and Santiago *et al.* (2012), who suggested that enhanced herbivory caused by nutrient enrichment could mask growth responses to fertilization. This conclusion, however, runs counter to our result where the effects of nutrients, the application of antibiotics, or their interaction never significantly altered pathogen damage, and had significant but variable effects on herbivore damage for three species. It is possible that we did not pick up many significant herbivore or pathogen effects because the majority of leaves we selected were fully expanded and potentially already accrued most of their damage. For example, almost 70% of lifetime enemy damage to tropical leaves occur within the first few weeks of leaf expansion (Coley & Aide, 1989; Kursar & Coley, 2003; reviewed by Coley & Barone, 1996). Moreover, it is important to point out that plants infected with foliar pathogens may suffer reduced performance yet often show no visible symptoms (reviewed by Griffin & Carson, 2015). For example, *Pseudomonas tomato*, a plant pathogen, can decrease tomato leaf production by as much as 30% without showing any sign of infection (Bashan & Okon, 1981). Indeed, we found that antibiotics or their interactions with nutrients caused no differences in foliar pathogen damage yet caused increases in leaf production by up to 140%. Thus, our results lead us to suggest that high nutrient tissue concentrations, particularly for N and K together and N and P together, may commonly make host plants more vulnerable to plant enemies. Indeed, some authors have argued that increased vulnerability to enemies may select against luxury consumption under low light and nutrients, particularly for N (Ostertag, 2010; Sayer & Banin, 2016). Nevertheless, our study demonstrates there were pervasive interactions between antibiotic and macronutrient treatments,

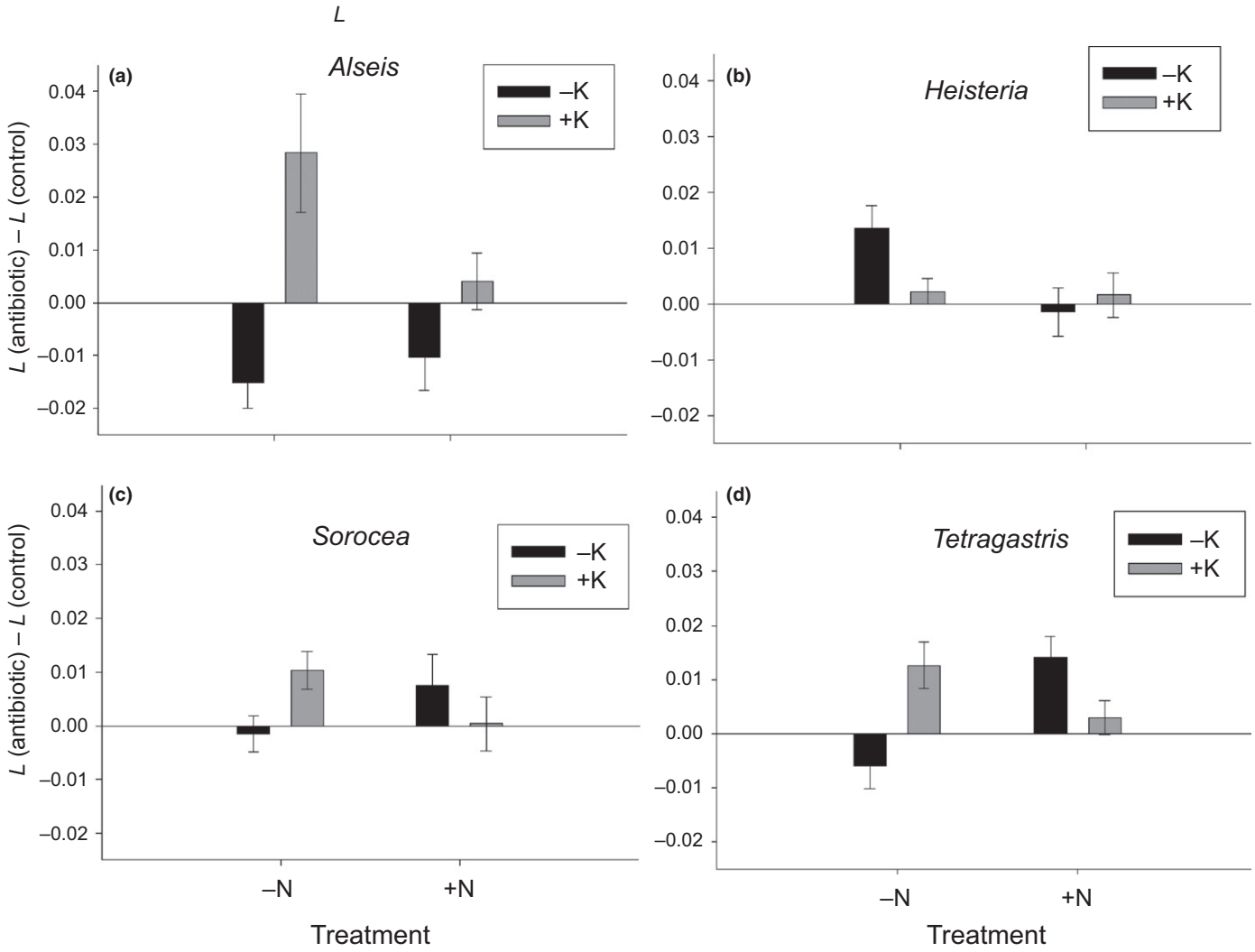

Fig. 6 Significant effects of antibiotic applications and nitrogen (N) × potassium (K) on leaf production rates (*L*) among seedlings of *Alseis blackiana*, *Heisteria concinna*, *Sorocea affinis* and *Tetragastris panamensis* after 29 months of applications and nutrient enrichment. When bars are above the line, antibiotic applications increased leaf production and when below the line, antibiotic applications decreased production. All panels illustrate significant effects of antibiotics and N × K on leaf production rates (significant antibiotics × N × K interaction; $F_{5,20} = 4.45$, $P = 0.0069$). (a, c, d) For *Alseis*, *Sorocea* and *Tetragastris*, applying antibiotics increased leaf production when K was added; however, antibiotics had no effect on leaf production when K and N were added together (*Alseis*: $P = 0.0064$; *Sorocea*: $P = 0.0006$; *Tetragastris*: $P < 0.0001$). (b) For *Heisteria*, applying antibiotics decreased leaf production when K was added, however, antibiotics had no effect on leaf production when K and N were added together ($P = 0.0022$). Bars represent mean values ± SE.

particularly N treatments, on leaf production for all five tree species. Ultimately, foliar bacteria appear to be a highly cryptic component of plant performance, and future studies should consider the impacts of pathogens even when there are no visible symptoms on plant hosts.

Potential mechanisms underlying plant–microbe–nutrient interactions

Although our antibiotic treatments reduced bacterial abundance and richness (Griffin *et al.*, 2016), it remains unclear whether the impacts of antibiotics are due to bacterial reduction, changes in the species composition of bacterial communities, indirect effects (e.g. changes in fungal pathogens due to reductions in bacteria), or a combination of these. Indeed, whereas many species of bacteria are plant pathogens, others are mutualists, and thus like some fungal endophytes, produce compounds such as growth hormones that increase plant resistance to pathogens and herbivores (see Arnold *et al.*, 2003; Herre *et al.*, 2007; Mejia *et al.*, 2008, 2014 for leaf fungal endophytes; mutualist bacteria reviewed by Griffin & Carson, 2015). Griffin *et al.* (2016) recently demonstrated that antibiotics in general increased tropical seedling growth and here, antibiotics increased leaf production for four of five species (Fig. S2). Moreover, we found that in four cases, antibiotics, in combination with the addition of a single macronutrient, increased leaf production (Figs 5, 6). Thus, our findings suggest that bacteria typically function as plant pathogens. In one case, however, antibiotics in combination with a single macronutrient decreased performance (Figs 5, 6). Because the mechanisms are

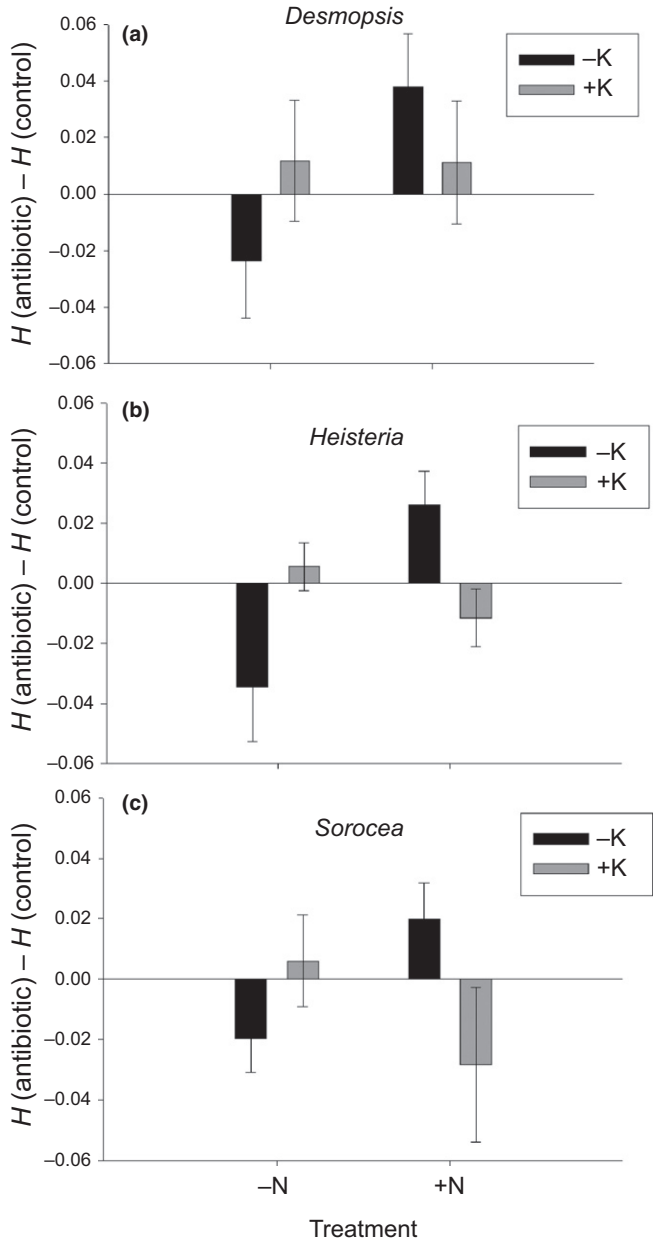

Fig. 7 Significant effects of antibiotic applications and nitrogen (N) × potassium (K) on herbivore damage rates (*H*) among seedlings of *Desmopsis panamensis*, *Heisteria concinna*, and *Sorocea affinis* after 14 months of applications and nutrient enrichment. When bars are above the line, antibiotic applications increased herbivore damage and when below the line, antibiotic applications decreased herbivore damage. (a–c) For *Desmopsis*, *Heisteria* and *Sorocea*, applying antibiotics decreased damage when neither N nor K was applied, or when both were applied together; conversely, antibiotics increased damage when just N was applied (significant antibiotics × N × K interaction; $F_{4,20} = 5.05$, $P = 0.0353$; *Desmopsis*: $P = 0.0041$; *Heisteria*: $P < 0.0001$; *Sorocea*: $P = 0.0005$). Bars represent mean values ± SE.

still unclear, future experiments are needed using genomic techniques to critically evaluate how antibiotics alter bacterial *communities* to allow for a deeper understanding of how particular bacterial taxa drive leaf dynamics. Moreover, it is possible that antibiotic applications cause direct effects (e.g. toxic) on nontarget organisms (e.g. fungi); however, empirical studies have looked for this and found little evidence (Ingham & Coleman, 1984; Colinas *et al.*, 1994; Chopra & Roberts, 2001; Thiele-Bruhn & Beck, 2005). Nevertheless, more reductionist approaches are imperative to link bacterial communities to plant physiology and plant performance.

We suggest that future studies address the mechanisms and pathways by which foliar bacteria and other enemies impact plant hosts *in situ*. On the one hand, enemy attacks often trigger plants to produce proteins, phenolics and alkaloids for protection from both pathogens and insects; thus, bacterial infection may induce plant defenses against insect enemies (Tierens *et al.*, 2001; Thomma *et al.*, 2002; Wittstock & Gershenzon, 2002; Haq *et al.*, 2004; Kniskern *et al.*, 2007). On the other hand, pathogen infection may upregulate the salicylic acid pathway. If so, this directly inhibits the jasmonic acid pathway, which is a key pathway that enhances plant defense against herbivores (e.g. Traw *et al.*, 2003; reviewed by Stout *et al.*, 2006; Bari & Jones, 2009; Pieterse *et al.*, 2009; Thaler *et al.*, 2012). Thus, upregulation of microbial defenses in response to attack may leave the host plants more vulnerable to herbivore attack (Glazebrook, 2005; Koornneef & Pieterse, 2008). Studies addressing the mechanisms underlying enemy attack, however, have almost exclusively focused on *Arabidopsis thaliana* or agricultural crop species. Thus, future studies should address the interactions among microbes, herbivores and plant hosts, in species-rich ecosystems where enemy pressure is substantial.

Implications for plant diversity maintenance and niche differentiation

Studies have demonstrated that increased soil nutrient availability decreases realized niche space of co-occurring species, yet it remains uncertain whether soil nutrients alone can maintain hyper-diversity of plant species in tropical forests (e.g. Hubbell *et al.*, 1999; Hubbell, 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, spatial variation in soil nutrient availability favors different species in different places. Here, we demonstrate that foliar bacteria are critical mediators of interactions between plants and soil resource availability, supporting other studies that demonstrate the importance of plant–microbial interactions in diversity maintenance (e.g. Mangan *et al.*, 2010; Schnitzer *et al.*, 2011; Pendergast *et al.*, 2013). In fact, some have proposed that plant-associated microbes can act as stabilizing factors to increase differences in species' performance outcomes along resource gradients or among interactions with other trophic levels (e.g. Chesson, 2000; reviewed by Bever *et al.*, 2010; Mordecai, 2011). In this framework, such stabilizing processes cause intraspecific effects to be more negative than interspecific differences (Chesson, 2000). Thus, when any single species increases in abundance, its per capita growth rate slows relative to growth rates of other species, which aids in species coexistence (Chesson, 2000). Indeed, we found that foliar bacteria caused co-occurring plant species to

perform in some cases better or in other cases worse among different soil resource treatments. This suggests that bacteria interact with a familiar and long-standing key niche axis (soil nutrient availability and its degree of patchiness). Although speculative, we argue that plant–bacterial interactions more finely divide niche differences among coexisting plant species and thus function to promote plant diversity.

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Author contributions

E.A.G., S.J.W. and W.P.C. planned and designed the experiment; E.A.G. performed the experiment and conducted fieldwork; P.J.M. analyzed the data; and E.A.G., S.J.W. and W.P.C. wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Map of study area showing the placement of nutrient treatments within the study site.

Fig. S2 The impacts of antibiotic applications on rank-order performance among plant performance metrics (leaf production, growth rate and herbivore damage).

Methods S1 Full description of linear discriminant function analysis.

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