The role of endophyte diversity in protecting plants from defoliation by leaf-cutting ants

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Plants host a vast diversity of fungal symbionts inside their tissues that live in close proximity with each other to form rich and dynamic communities. Although endophytes can affect plant–herbivore interactions in several ways, it is still not known to what extent such effects are influenced by the properties of endophyte communities or by particular species traits. Here we compared the effects of high versus low foliar fungal endophyte diversity on the preferences of laboratory and wild colonies of leaf-cutting ants. We found that when endophyte densities were high, the ants responded similarly to leaves hosting one endophyte species, *Colletotrichum tropicale*, or those hosting a species-rich endophyte community. Results were also consistent when comparing the laboratory versus wild ant colonies. We discuss the significance of these results with respect to the ecological effects of plant–endophyte interactions in natural and agricultural ecosystems.

Keywords: *Atta colombica*, *Colletotrichum tropicale*, fungal community, herbivory, symbiosis.

Introduction

FUNGAL symbionts live asymptomatically in all plant species and all plant tissues sampled to date[1]. The associations between endophytic fungi in the family Clavicipitaceae and pooid grasses have been well studied, as these endophytes often enhance plant tolerance to biotic and abiotic stresses in a classic example of defence mutualism[2]. Much less understood are the nature and ecological significance of associations between non-clavicipitaceous fungi and their hosts, which represent by far the majority of the plant–endophyte symbioses. This is not surprising as these symbioses involve a more taxonomically diverse set of species than grass endophytes and include fungi with a wider range of life-history traits[1].

The influence of plant and non-clavicipitaceous endophyte symbioses on insect herbivores is highly variable[3,4]. Negative effects include decline in survival of herbivores developmental rates, fecundity and plant acceptance[4–8]. Most experiments conducted to evaluate these effects have manipulated endophyte density with either a single endophyte species or a mixture of multiple species inside the leaf. The influence on herbivory of endophyte species diversity has been rarely examined, even when direct and indirect interactions between symbionts are expected to have substantial impacts on the outcome of the ecology of their hosts[9–12]. Fungi can compete for resources inside leaves using antibiosis or facilitate colonization of other fungi by modifying the chemical structure of plant defensive compounds[11,13,14]. The results of these direct and indirect microbial interactions are evident by negative and positive correlations in the abundance of common species in endophyte communities[15–17], or the well-documented effect of endophytes in the success of infections by fungal pathogens[18–20].

Colonization of leaves by endophytes influences several foraging behaviours of leaf-cutting ants (*Atta*), an important Neotropical group of herbivores[6,21–23]. This plant–fungal symbiosis can also affect the development of young leaf-cutting ant colonies and alter the microbial community of their fungal gardens[24]. Using the behavioural responses of this insect we aimed to enhance our understanding of the influence of leaf endophyte diversity on the interaction of plants with herbivores in natural situations. We first compared the preferences for low versus high endophyte abundance by wild versus laboratory-grown colonies of *Atta colombica*. To compare wild and laboratory colonies, we built on previous results and conducted assays with leaves that had low versus high density of one endophyte strain, *Colletotrichum tropicale*[25]. Our previous studies have shown key evidence for chemical and nutritional changes in leaves resulting from symbiosis with *C. tropicale*, but this work has been limited to laboratory colonies that were fed artificial diets[25,26]. In our second set of experiments, we assessed the importance of endophyte diversity on leaf-cutting ant preferences. Here, we conducted choice experiments with laboratory ant colonies to assess their preferences for leaves with naturally acquired communities of endophytes versus leaves not exposed to endophyte spores. Contrasting foraging behaviours of this generalist herbivore between low and high symbiont diversity may help

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us evaluate the emergent effect of the endophyte community on plant defence.

Methods

Experimental plants

We tested the foraging preferences of leaf-cutting ants for cucumber leaves (*Cucumis sativus* var. Poinsett 76) with high and low loads of fungal endophytes. We chose cucumber for this study due to its desirable properties (viz. low genetic variability, grows fast and thrives in growth chamber conditions); cucumber has been used extensively in studies of plant responses to pathogenic infections and herbivory and there is good knowledge of its secondary metabolites. Furthermore, this research builds from previous experimental work examining the effect of leaf endophytes on *A. colombica* and its mutualistic fungi[24,25]. We used ~60 day-old seedlings that had been planted from seeds in potting soil (Miracle-Gro, The Scotts Company) and allowed to grow for 30 days before endophyte inoculations. We kept seedlings with low rates of endophyte colonization by placing them inside growth chambers and maintaining the leaf surfaces dry. Chambers had a 12 : 12 h light : dark cycle at constant 28°C and 85% humidity. Plants chosen for the high endophyte treatment (E_{high}) were exposed to fungal spores using one of two types of inoculations. For our high diversity treatment, we exposed plants to the spore rain of a natural community of endophytes by taking seedlings to the rain forest edge for 10 to 12 consecutive nights ('natural inoculation' (NI))[22]. We moved such plants at dusk (ca. 18 : 00 h), sprayed their leaves with filtered water, and left them inside a mesh cage until the next morning (ca. 9 : 00 h) when we moved them back to the chambers. Control plants selected to have low density of endophytes (E_{low}) were left inside growth chambers at all times. All plants were exposed to similar light and humidity during the daytime. The transport of seedlings for inoculation did not result in any apparent change in them. For our low diversity treatment, we sprayed conidia of *C. tropicale* (10^6–10^7 conidia ml^{-1}, strain Q633, GenBank accession code GU994350) directly on E_{high} plants. The conidia were dissolved in sterile water and an emulsifying agent (v/v 0.5% Tween 20, Sigma-Aldrich). Conidia were obtained by liquid fermentation in 1.5% molasses yeast medium (15 g molasses, 2.5 g yeast extract, 1 litre water)[6]. Control E_{low} plants were sprayed with sterile water and the emulsifying agent alone. After spraying conidia or sterile water, we left the plants in a high humidity environment for one night inside a frame covered with clear plastic and then moved them back to the chambers until they were used in experiments.

We estimated the density of endophytes seven days after inoculation. We used three healthy leaves detached from different seedlings growing in each E_{high} and E_{low} tray. We chose leaves that were fully expanded at the time of inoculation. We then cut a portion of each foliar lamina into 2 × 2 mm squares with a razor blade, sterilized their surface by consecutive immersion in 70% ethanol (1 min) and 10% bleach (1 min), and plated 16 squares on a 2% malt extract agar (MEA) plate. We estimated the density of endophyte colonization (i.e. isolation frequency) as the percentage of leaf squares in a plate where fungal growth was observed after 7 days of incubation at room temperature (24°C). Using these plates we also assessed the diversity of the endophyte community that colonized leaves in natural inoculations (E_{high}). We first established pure cultures of fungi growing from leaf squares by transferring mycelia to individual MEA plates and incubating them at room temperature for approximately 14 days. These fungal strains were then sorted by their whole colony morphology into morphotypes. We performed bioassays using leaves from three independent natural inoculation events that took place during the wet season in the Panama Canal area (September 2011, October 2011 and June 2012). For each of the first two inoculations (NI-1 and NI-2) we estimated diversity using a subset of 24 fungal strains that grew from alternate leaf squares on plates. We included all 48 isolates to estimate the diversity of endophytes in our last inoculation event (NI-3). To measure diversity we calculated the Shannon–Weaver diversity index and the estimator of species richness Chao 1 with EstimateS[27].

Bioassays

We tested leaf-cutting ant foraging preferences for E_{high} and E_{low} leaves using a choice test developed previously[25]. This test was done separately with laboratory ant colonies and then wild colonies of *A. colombica*. The laboratory-grown colonies had a choice between cucumber leaves with naturally acquired high density, high diversity endophyte communities versus control leaves with low density and low diversity endophyte communities. The wild colonies could choose between cucumber leaves inoculated with a high density of *C. tropicale* versus control leaves with low endophyte density and diversity. In both experiments, we measured preference as the relative number of ants attracted to each leaf (recruitment, number of ants cm^{-2}; see below) and the total foliar area harvested by the colony at the end of experiments (cm^2). We reported the percentage of reduction or increase of each measure in E_{high} relative to E_{low} control leaves. This relative measure controlled for the effect of colony size and allowed us to compare foraging preferences between experiments.

Between July and August 2012, we carried out trials with 13 wild colonies of *A. colombica* distributed around Gamboa (9°070 N, 79°420 W) in the Republic of Panama. These colonies varied in size, ranging from nests with a

few entrances and worker ants of a relatively uniform body size, harvesting mostly understorey plants, to mature colonies with multiple nest entrances, workers with highly variable body sizes and ants cutting leaves from trees. For each trial we placed one cucumber leaf of each treatment in a 25 × 15 cm plastic tray that served as a foraging arena. Leaves of comparable size were detached from the plants, rinsed in filtered water and dried. To prevent their premature dehydration we wrapped the petioles in moist tissue paper and foil. We then placed the arena alongside a well-established foraging trail facing its entrance toward the direction of ants moving away from their nest. To direct ants to the arena, we added to its entrance soil and litter pieces from the adjacent trail of ants which presumably contained pheromones deposited by foraging workers to direct others toward food sources. We measured the time taken by ants to first find and then start cutting each leaf, considering the time interval between these two events as a measure of acceptance. In addition, we took a photograph of the arena every 5 min until one leaf was totally harvested or after 120 min from the start of the trial. We considered that a leaf had been totally cut when only the base of the petiole was left in the arena. From the photographs we counted the number of ants recruited to each leaf and calculated the foliar area remaining at each time interval. The software ImageJ (http://resbweb.nih.gov/ij/; NIH, USA) was used to measure leaf area from the photographs. We further estimated endophyte densities that were typical for the leaf material harvested by the ant colonies from neighbouring vegetation at the time of the experiments. We collected a few leaf pieces carried by ants and estimated endophyte density by plating 32 leaf squares for each ant colony in the assay, following the methodology described above.

To test the effect of natural endophyte inoculations on the foraging behaviour of *A. colombica*, we used 31 one to two-year-old laboratory colonies established from either mated queens collected during the annual nuptial flight, or one-year-old colonies excavated from Gamboa fields. We maintained colonies in the Gamboa laboratory of the Smithsonian Tropical Research Institute under ambient temperature and humidity, and with a diet consisting of oatmeal flakes and new leaves from locally growing trees, *Lagerstroemia speciosa* and *Mangifera indica*. Choice tests were carried out between September 2011 and July 2012 using 18, 5 and 8 ant colonies for NI-1, NI-2 and NI-3 respectively. To start a trial we connected each colony to the experimental arena with a plastic bridge and then measured their preference for E_{low} versus E_{high} leaves with the same methods described above for wild colonies.

Statistical analyses

Our measures of foraging preferences resulted in negative percentages, showing a decrease in recruitment or foliar area harvested from E_{high} relative to E_{low} leaves, or positive percentages showing the opposite. For each set of trials, we used Student's *t*-test to assess whether mean percentages for each of these variables were different from zero. In addition, we used general linear models to compare the magnitude of preference between published results[25] from trials that used *C. tropicale*-infected leaves and laboratory-grown ant colonies with those reported here for wild and laboratory-grown colonies. Specifically, we tested the effect of type of inoculation (*C. tropicale* versus NI), type of ant colony (laboratory versus wild), and inoculation event on the mean relative ant recruitment and mean relative area harvested. As *post hoc* tests we calculated pairwise *t*-tests with Bonferroni correction. Deviations from a 1 : 1 ratio in acceptance and total leaf harvested between E_{low} and E_{high} were tested with log likelihood ratio statistic (*G*-test). We performed analyses using the software package R (R Development Core Team, version 2.15.1). Unless otherwise specified, we report mean ± 1 SE of preference measures throughout this article.

Results

Effect of C. tropicale on foraging preferences of wild A. colombica

Foraging ants from wild colonies entered test arenas and found cucumber leaves within 10 min after the start of experiments (0.73 ± 0.41 min). Although E_{high} and E_{low} leaves were found at approximately the same time, in 10 out of 13 tests, ants accepted and started to cut E_{low} leaves first (*G*-test $df = 2$, $P < 0.01$). In a typical experiment ants were quickly attracted to the E_{low} leaves, which they started to cut and carry toward their nest using their previously established trails. Ants also harvested E_{high} leaves. They accepted these leaves less readily than E_{low} leaves, but later increased their cutting rate, particularly when E_{low} leaves had started to show signs of water loss. Nevertheless, relative to E_{low} leaves about 51% fewer ants per leaf area where observed on E_{high} leaves (1.92 ± 0.4 and 0.73 ± 0.19 ants cm^{-2} in E_{low} and E_{high} leaves respectively; relative difference = –51 ± 13%, *t*-test $df = 12$, $P < 0.01$, Figure 1). Similarly, at the end of experiments ants had cut on average 18% less area from E_{high} leaves relative to E_{low} leaves, although this reduction was only marginally significant (41.1 ± 4.45 and 32.18 ± 4.31 cm^2 cut from E_{low} and E_{high} leaves respectively; relative difference = –18 ± 10%, *t*-test $df = 12$, $P = 0.09$; Figure 1).

We isolated fungal endophytes from about 59% of the squares (range = 13–97%, $N = 416$) cut from leaf pieces harvested by ants from neighbouring plants. This natural endophyte density was intermediate to that found on cucumber leaves used in our trials. The estimated colonization by *C. tropicale* in E_{high} leaves was 100%, while no fungi were detected from E_{low} controls.

Effect of natural endophyte communities on foraging preferences of A. colombica laboratory colonies

Laboratory colonies found and accepted control and naturally inoculated leaves equally (48% colonies accepted E_{low} leaves faster). Nevertheless, relative to E_{low} leaves recruitment of ants was about 33% lower in E_{high} leaves (1.76 ± 0.21 and 1.02 ± 0.11 ants cm^{-2} for E_{low} and E_{high} leaves respectively; relative difference $-33\% \pm 8$, t-test $df = 30$, $P < 0.01$; Figure 1), and thus E_{low} leaves were more often finished first (G-test $df = 2$, $P < 0.01$). At the end of experiments, ants had harvested about 18% more area of E_{low} than E_{high} leaves (22.56 ± 1.73 and 18.85 ± 2.28 cm^{-2} for E_{low} and E_{high} leaves respectively; relative difference $-18 \pm 7\%$, t-test $df = 30$, $P = 0.01$; Figure 1).

Although we found significant negative effects of endophyte-infected leaves on the preferences of ants when all trials were considered, we observed remarkable variation in the strength of preferences among sets of trials from different NIs (Table 1 and Figure 2). Linear models showed a significant effect of the inoculation event on both relative measures of preference ($F = 6.29$, $df = 2, 28$, $P < 0.01$ for area cut and $F = 14.35$, $df = 2, 28$, $P < 0.01$ for recruitment). The foraging behaviour of ants towards leaves in NI-2 was significantly different relative to the other two events (pairwise t-tests $P \leq 0.05$). In NI-1 and NI-3, we found significant reductions of recruitment and area harvested on E_{high} relative to E_{low} leaves (relative differences t-tests $P \leq 0.02$). In contrast, in three out of five NI-2 trials ants recruited more ants and cut more area from E_{high} leaves, which resulted in an overall trend to prefer those over E_{low} leaves. The mean increase of ant recruitment and area cut of E_{high} relative to E_{low} leaves, however, was not significantly different from zero (relative differences t-test $df = 4$, $P = 0.16$ for recruitment and $P = 0.27$ for area; Figure 2).

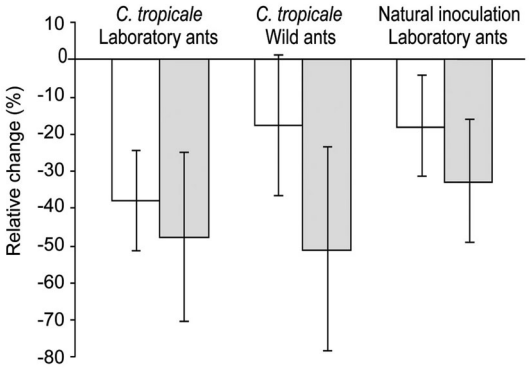

Figure 1. Mean percentage (± CI; confidence intervals) decrease (or increase) in area harvested (white bars) and number of ants recruited per leaf area (grey bars) for E_{high} relative to E_{low} leaves. We show the effects of *Colletotrichum tropicale* inoculations in the foraging preferences of *Atta colombica* wild colonies ($N = 13$ colonies) and those of natural endophyte inoculations on laboratory-grown colonies of the same leaf-cutting ant species ($N = 31$ colonies are shown). The results are compared with published data obtained from tests of *A. colombica* laboratory colonies with *C. tropicale*-infected leaves ($N = 20$ colonies)[25].

Leaf-cutting ant colonies encountered E_{high} and E_{low} leaves that differed in both density and diversity of endophyte species. Exposure of plants to the natural spore rain resulted in leaves with an endophyte density of about $99 \pm 0.6\%$, while control plants kept inside growth chambers at all times had a density of only $21 \pm 8\%$. This difference in endophyte loads between leaf treatments was significant (t-test $df = 8.12$, $P < 0.01$). Both density and diversity of the endophytes varied slightly between inoculation events (Table 1). Overall, leaves from NI-2 had a lower difference in endophyte loads between E_{high} and E_{low} treatments (52%) compared to the other two events. Similarly, the diversity (H') and estimated richness (Chao 1) of morphotypes isolated from E_{high} leaves in NI-2 was relatively low, although not significantly different from the other two inoculation events (CI for Chao 1 estimations overlap). We tentatively identified the dominant morphotypes as *Colletotrichum* and *Xylaria* spp. in naturally assembled endophyte communities using colony morphology and reproductive structures (Figure 3). The other genera present in lower frequency included *Pestalotiopsis*, *Phomopsis* and *Endomelanconiopsis*. The occurrence of the most common endophyte morphotypes was similar among the three leaves used to assess endophyte diversity for each NI. Therefore, we are confident that within sets of trials ants were exposed to leaves with a comparable assemblage of endophytes. Contamination with endophytic fungi in control leaves included up to three morphotypes, from which *Aspergillus* sp. was the most common and was the only strain found in NI-2 control samples.

Comparison among trials

Linear models showed that mean preferences for E_{low} leaves were comparable between trials with laboratory and wild colonies ($F = 1.61$, $df = 1, 62$, $P = 0.21$ for area

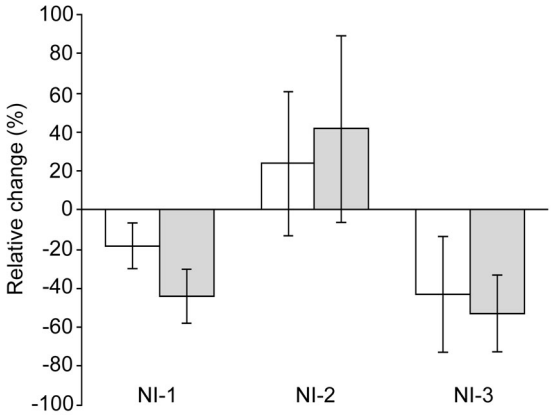

Figure 2. Foraging preferences of *A. colombica* laboratory-grown colonies using leaves inoculated with natural endophyte communities in three independent events (NI). Bars represent the mean percentage (± CI) of change in area harvested (white) and the number of ants recruited per leaf area (grey) for E_{high} relative to E_{low} leaves. Eighteen, 5 and 8 ant colonies were tested for NI-1, NI-2 and NI-3 respectively.

Table 1. Density and diversity of the fungal endophyte community and results of choice tests for the three independent natural inoculation events (NI)

Natural inoculation events	Density (%) (E_{high}–E_{low})	Diversity[a]	Chao 1[b]	Area harvested[c]		Ant recruitment[d]	
				E_{low}	E_{high}	E_{low}	E_{high}
NI-1	98–4	2.2 (13)	35 (18–106)	22.97 (1.40)	18.95 (1.91)	2.28 (0.24)	1.22 (0.14)
NI-2	100–48	1.83 (8)	9 (8–18)	18.73 (4.83)	23.48 (1.63)	0.4 (0.17)	0.96 (0.24)
NI-3	100–10	2.23 (15)	22 (16–51)	24.01 (5.41)	15.74 (7.85)	1.42 (0.36)	0.60 (0.18)

[a]Shannon–Weaver index (H') and number of morphotypes for E_{high} leaves. [b]Estimation of richness Chao 1 (95% CI) for E_{high} leaves. [c]Mean (1 SE) leaf area cut by ants (cm^2). [d]Mean (1 SE) number of ants per leaf area (ants cm^{-2}).

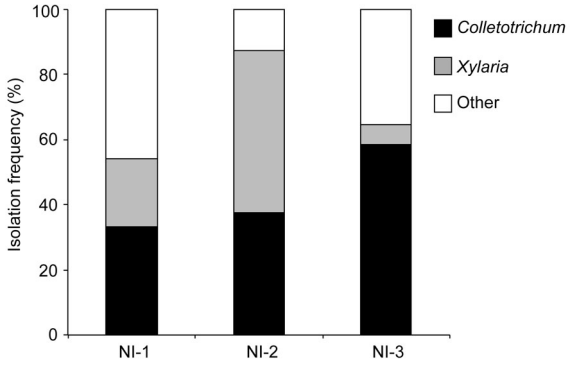

Figure 3. Frequency of isolation of endophyte strains in the genera *Colletotrichum*, *Xylaria* or other unidentified species from cucumber natural inoculations.

harvested and $F = 1.79$ d$f = 1, 62$, $P = 0.19$ for ant recruitment), and also between *C. tropicale* and NI ($F = 0.46$ d$f = 1, 62$, $P = 0.51$ for area harvested and $F = 0.67$, d$f = 1, 62$, $P = 0.42$ for ant recruitment). In contrast, when we compared preferences between trials with *C. tropicale* and those from each NI independently, our model found an effect on both measures of preference ($F = 4.33$, d$f = 3, 60$, $P < 0.01$ for area harvested and $F = 6.54$, d$f = 3, 60$, $P < 0.01$ for ant recruitment). Colonies tested with NI-2 had a significantly different mean area harvested than those tested with leaves colonized by *C. tropicale* or by multiple endophytes found in NI-3 (pairwise *t*-tests $P \leq 0.01$). The mean relative ant recruitment for NI-2 trials was also different from recruitment in all other trials (pairwise *t*-tests with $P < 0.01$).

Discussion

Our results are consistent with previous studies in showing that plant–endophyte symbioses negatively affect the foraging preferences of leaf-cutting ants[6,22,25]. Relative to control leaves, ant colonies recruited fewer ants to cut cucumber leaves filled with endophytes at a slower rate. Endophyte diversity did not have a significant effect on the preference of ants for E_{low} leaves. Nevertheless, we observed a trend for weaker mean preferences when laboratory colonies encountered E_{high} leaves inoculated with naturally acquired endophyte communities. This was due to a high variation in the outcome of these trials. In particular, our second inoculation event resulted in a lack of preferences, with ants showing a tendency to favour E_{high} leaves. Several factors may have contributed to the contrasting results in NI-2. The difference in endophyte density between E_{high} and E_{low} leaves in NI-2 dropped by about 40% compared with other NIs or inoculations with *C. tropicale* (Table 1). A contributor to this difference was contamination of our control plants with *Aspergillus* sp., which may be pathogenic to the ants or their fungal garden[28]. Thus, the ants could have been sensing this particular endophyte and avoiding it to favour the E_{high} leaves. Furthermore, compared to the other two inoculations events, endophyte richness and diversity in NI-2 was slightly lower. It is probable that the particular species composition hosted by NI-2 leaves was different from other trials and may have influenced leaf acceptance by the ants.

The endophyte communities found in the high diversity treatments were dominated by strains of *Colletotrichum* and *Xylaria*. In each inoculation event we found up to three strains that were morphologically distinguishable in MEA plates for each of these genera. They coexisted with several less frequently isolated fungi, typically found as singletons. Our measures of diversity isolating endophytes by cultivation and using colony morphology alone are problematic and can only give us a rough approximation of the species richness and abundance found in cucumber leaves exposed to natural inoculations[29,30]. This, together with a lack of unification in morphotype assignation across our inoculation events and with controls, prevents us from understanding which components of the community could have influenced the preferences of ants, particularly for NI-2 trials. Nevertheless, the scope of this research was to contrast the behaviour of leaf-cutting ants towards leaves with low (only *C. tropicale*) versus high endophyte diversity. The variation in ant responses to diverse endophyte communities enhances our understanding of endophyte effects on plants and herbivores, and suggest avenues for further experiments.

The ecological effects of endophytes on their hosts are both plant- and endophyte-specific, and often context dependent[20,31]. This is not surprising given the multiple ways in which endophytes interact with plants and other fungi. First, endophyte species affect, and are affected by their host defence responses in different ways[16,32]. For example, tolerance and detoxification of host antifungal compounds which is common among plant-colonizing fungi, differ considerably even among congeneric

endophyte species[13,33]. Second, herbivores may encounter different chemical environments in leaves due to interactions among endophytes, or between endophytes and other plant symbionts. Fungal endophytes are known to produce a wide diversity of organic compounds *in vitro*[34]. It is expected that these compounds are part of the strategy of the fungus to interact with plants and other fungi, although little is known about their production *in vivo*. Furthermore, many microbial competitive traits are likely to be switched on only when fungi are challenged by neighbouring microbes, as has been shown by the detection of secondary metabolites that only appear in mixed *in vitro* cultures[35,36]. Thus, combinations of metabolites from different endophyte species may additively or synergistically enhance plant toxicity and thereby influence herbivore behaviour.

Surprisingly, only a few studies have directly or indirectly evaluated the effects of endophyte diversity on the interaction between plants and antagonistic organisms[37,38]. In one study, inoculations with single or dual endophyte species resulted in significant changes in the endophyte assemblages hosted later by new leaves[10]. This variation in species composition did not affect the growth of a generalist herbivore but reduced feeding by a specialist, particularly in plants that had received dual inoculations. Similarly, increasing the number of mycoparasitic endophyte strains included in an inoculum mixture applied to a plant decreased the symptoms produced by its fungal pathogens[39]. However, here also the effects varied considerably with the pathogenic strain and the particular combination of endophytes applied. Finally, a recent study in our system showed that a high abundance of endophytes increases the time that leaf-cutting ants need to cut, clean and plant leaf material into their fungal garden[21]. This study found that the endophyte diversity did not have an effect on the leaf-processing rates of ants, although their preferences were not assessed.

A. colombica harvest leaves with lower endophyte densities than the average values found in neighbouring leaves in the wild[40]. Although endophyte abundance covaries with other characteristics of leaves[41,42], our results showing preferences for E_{low} cucumber leaves in wild colonies suggest that this harvesting pattern resulted in part from the active selection by ants of material with fewer symbionts. In our assays, the amount cut from E_{high} and E_{low} leaves was only marginally different, despite the presence of a higher number of ants recruited in E_{low} leaves. This was likely the result of a premature wilting in favoured leaves, typically E_{low} leaves. Cutting pieces accelerated water loss and made the leaves harder to cut. When we removed physical factors from leaves and tested ants with paper disks infused with leaf chemical extracts, we found preferences for E_{low} extracts in wild colonies similar to those found in laboratory ants. Wild colonies took from foraging arenas $26 \pm 6\%$ ($N = 11$ colonies) more paper disks infused with E_{low} than with E_{high} leaf extracts (data not shown). This difference in laboratory-grown colonies was $20 \pm 4\%$ (ref. 25).

Much research has been devoted to understanding the ecology of plant–endophyte interactions, their variability, and their significance in natural and agricultural systems. Although our knowledge is still limited, previous studies and results from the present study suggest that the effect of multiple endophyte strains on plant defence can be as variable as that of a single endophyte strain. This variation likely results from the particular characteristics of the species involved in these complex, multi-species system of interactions. Further, variation in endophyte effects on herbivores may stem from biotic and abiotic stress conditions, nutrient availability, temperature and the timing of fungal colonization[9,20,43]. We propose that future research should continue to experimentally simplify the endophyte community to a degree where we can identify basic patterns and mechanisms of interactions. Additionally, we suggest that studies of plant–endophyte defence be included in agricultural systems, where many biotic and abiotic variables are simplified and multiple genetic tools are available. Once these patterns are established, the next step would be to add complexity and diversity to assess whether these patterns are maintained in natural situations. This approach has been used successfully in studies of plant–mycorrhizal symbioses, leading to a better understanding of how mycorrhizal diversity influences their interactions with plants[12,44]. Such experiments will illuminate the extent to which endophyte species play unique or redundant roles in the ecology of their hosts.

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