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Research

Symbiotic fungi alter plant chemistry that discourages leaf-cutting ants

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Summary

- Fungal symbionts that live asymptomatically inside plant tissues (endophytes) can influence plant–insect interactions. Recent work has shown that damage by leaf-cutting ants, a major Neotropical defoliator, is reduced to almost half in plants with high densities of endophytes. We investigated changes in the phenotype of leaves that could influence ants' behavior to result in the reduction of foliar damage.
- We produced cucumber seedlings with high and low densities of one common endophyte species, *Colletotrichum tropicale*. We used the leaves in bioassays and to compare chemical and physical leaf characteristics important for ants' food selection.
- Ants cut about one-third more area of cucumber leaves with lower densities of endophytes and removed *c.* 20% more paper disks impregnated with the extracts of those leaves compared with leaves and disks from plants hosting the fungus. *Colletotrichum tropicale* colonization did not cause detectable changes in the composition of volatile compounds, cuticular waxes, nutrients or leaf toughness.
- Our study shows that endophytes changed leaf chemistry and suggests that compounds with relative low volatility released after leaf wounding are a major factor influencing foraging decisions by ants when choosing between plants with low or high endophyte loads.

Introduction

Microbial communities that live in association with animals and plants play a major role in driving the ecological and evolutionary patterns observed in nature. For example, nutrient acquisition and defense for most organisms of terrestrial and marine communities are strongly influenced by microorganisms (van der Heijden *et al.*, 1998; Lee *et al.*, 2001; Herre *et al.*, 2007; McFall-Ngai, 2008; Brownlie & Johnson, 2009). Plants, in particular, host a vast diversity of fungal symbionts with interactions that range from parasitic (e.g. pathogens) to mutualistic (e.g. mycorrhizas) and which shape ecological patterns (e.g. Gilbert, 2002; Hart *et al.*, 2003; Mangan *et al.*, 2010).

Fungal endophytes are cryptic symbionts that live inside plant tissue without causing overt signs of disease (Petrini, 1991; Schulz & Boyle, 2006). Although their presence in some cases does not seem to affect the plant's ecological interactions (Saikkonen *et al.*, 2010), endophytes can occasionally provide short-term benefits to their host. For example, they can influence the outcome of interactions between plants and their natural enemies by limiting the spread of pathogenic fungi and damage by herbivores (Clay, 1991; Arnold *et al.*, 2003; Mejía *et al.*, 2008; Van Bael *et al.*, 2009a,b; Saikkonen *et al.*, 2010). This wide variation in effects is not unique to endophytes but also occurs in fungus–plant–insect interactions involving mycorrhizas and pathogens (Hatcher, 1995; Stout *et al.*, 2006; Gehring & Bennett, 2009). Elucidating the mechanisms underlying the defense (or lack thereof) in plant–endophyte interactions is critical for understanding the variation in endophyte effects, assessing the impact that endophytes can have on the dynamics of communities, and evaluating their potential as biological control tools (Herre *et al.*, 2007).

Reduction in herbivory by plants hosting endophytes can result from directly decreasing survival rates of herbivores to indirectly affecting their developmental time, fecundity or foraging behaviors (Webber, 1981; Clark *et al.*, 1989; McGee, 2002; Jallow *et al.*, 2004; Van Bael *et al.*, 2009b; Bittleston *et al.*, 2011). Production of endophyte-specific toxins is one of the best-known examples of a defense mechanism against herbivores (Clay, 1991; Calhoun *et al.*, 1992). Nevertheless, herbivores could also be affected by the presence of endophytes, if fungi can infect insects or affect their symbiotic microbiome (Marcelino *et al.*, 2008), or by plant responses that are induced by fungal colonization such as production of secondary compounds and/or physical defenses (Heath, 2000; Van Loon, 2000; Stout *et al.*, 2006). The wide range of fungal life history traits and plant responses to microbial infections suggests that the effect of endophytes on plant–herbivore interactions also may originate from a similar wide range of mechanisms.

Leaf-cutting ants (genera *Atta* and *Acromyrmex*, Myrmicinae) are one of the most important defoliators in the Neotropics

(Cherrett *et al.*, 1989; Herz *et al.*, 2007; Costa *et al.*, 2008) and responsible for an estimated one billion US dollars per year in damage to agriculture (Hölldobler & Wilson, 1990). They maintain an obligate symbiosis with their fungal cultivar (*Leucocoprinus gongylophorus*, Agaricaceae, Basidiomycota) (Weber, 1972) that digests ant-collected plant material and serves as the main source of nutrition for the ants and their larvae. Recent studies have shown that leaf-cutting ants (*Atta colombica*) prefer harvesting leaves from plants with relatively lower densities of endophyte infections, and that they clean leaves to reduce the amount of endophytes before using them as substrate for their symbiotic fungi (Van Bael *et al.*, 2009a; Bittleston *et al.*, 2011). This finding has been consistent among laboratory colonies tested with two species of plants artificially inoculated with one endophyte species or colonized by a natural range of fungal symbionts. Previous work on selection of substrate by leaf-cutting ants found that they were highly selective with respect to the plant species and even the individual plant and the leaves within a plant that they used (Cherrett, 1968; Rockwood, 1976). Several traits influence their foraging decisions but leaf toughness and plant secondary compounds, particularly terpenoids and cuticular waxes seem to consistently explain a large part of their selectivity (reviewed in Van Bael *et al.*, 2011). How and whether fungal endophytes affect these plant traits involved in ant selectivity is unknown. Moreover, it is possible that ants are not selecting against changes in leaf traits but preventing the introduction of foreign microorganisms to their fungal garden (Fernández-Marín *et al.*, 2006).

In the present study, we exploited the ability of ants to discriminate between plants with high (E_{high}) and low (E_{low}) endophyte densities to narrow the search for key chemical and physical changes responsible for ant host preferences. Using an approach of combining bioassays and chemical analyses, we tested whether the presence of endophytes altered plant traits in ways detectable to ants. Specifically, we studied the interaction between cucumber (*Cucumis sativus*), one common endophyte (*Colletotrichum tropicale*) and the leaf-cutting ant species *Atta colombica* (hereafter 'ants'). Plants manipulated to facilitate *C. tropicale* colonization were compared with untreated low endophyte control plants to measure whether endophyte presence affected (1) the plant's composition of high molecular weight compounds involved in ant choice, (2) the range of volatiles produced by leaves when ants were cutting them, (3) cuticular waxes on leaf surfaces, (4) leaf nutrient content and (5) leaf physical traits. We found evidence that the presence of endophytes affected leaf compounds with relatively high molecular weight, but not highly volatile compounds, cuticular waxes, leaf nutrient content or physical leaf traits. We discuss how endophyte-mediated changes in plant chemistry could result from plant synthesis, endophyte synthesis or their interaction.

Materials and Methods

Leaf-cutting ant colonies

We established laboratory colonies of *A. colombica* Guérin-Méneville under ambient temperature and humidity in the Gamboa laboratory of the Smithsonian Tropical Research Institute (STRI), Panama. Colonies started from either mated queens collected during the annual nuptial flight, or from small nests (incipient to 1 yr old) excavated from Gamboa fields. On a daily basis, we provided ants with leaves from local trees, mostly *Lagerstroemia speciosa* L. (Lythraceae) and *Mangifera indica* L. (Anacardiaceae), and supplemented the leaves with oatmeal flakes and corn flour.

Experimental plants

We planted *Cucumis sativus* L. seeds var. Poinsett 76 obtained commercially (Fercon SA, Cali, Colombia) in potting soil (Miracle-Gro, The Scotts Company) and transferred them after germination to growth chambers kept at 28°C, 85% humidity and with a 12-h light : dark cycle. We kept the plants endophyte free or with low rates of endophyte colonization by keeping leaf surfaces dry and providing water directly via the soil. We then inoculated seedlings of 1.5–2 months old with the endophyte *C. tropicale* Rojas, Rehner & Samuels (E_{high}), or kept others as controls (E_{low}) (see details in 'Endophyte cultures and inoculation'). We used the leaves in bioassays with ants or for chemical analyses *c.* 2 wk later. Analyses of plant chemical characteristics and bioassays involved comparisons of healthy leaves from pairs of seedlings or seedling trays (E_{low} and E_{high}) that were planted the same day and grew under similar conditions before and after endophyte inoculations. When relevant, we also matched leaves from both plant treatments by size and age. For the purpose of this work, cucumber seedlings and plants refer to vines with up to 10 leaves.

Endophyte cultures and inoculation

We inoculated experimental plants with a strain of *C. tropicale* originally isolated from leaves of *Cordia alliodora* Ruiz & Pav. (Boraginaceae) growing around Gamboa (strain Q633, Gen Bank accession code GU994350; Van Bael *et al.*, 2009a; Rojas *et al.*, 2010). *Colletotrichum* has been commonly isolated from healthy leaves of several plant species in Panama (Van Bael *et al.*, 2005), and is a common endophyte of cucumber growing outdoors in Gamboa (Van Bael *et al.*, 2012a). We cultured *C. tropicale* in plates with extracts of potato dextrose agar or 2% malt extract agar (MEA).

To prepare experimental plants we followed the methodology described in Van Bael *et al.* (2009a). We sprayed conidia directly on leaves to produce plants with a high density of *C. tropicale* (E_{high}). The inoculate spray consisted of conidia (10^6–10^7 conidia ml^{-1}) dissolved in sterile water and an emulsifying agent (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 l water). To facilitate fungal colonization after spraying conidia we kept plants in a high-humidity environment inside a 0.5 × 1 m frame covered with clear plastic for 15 h. Control plants selected to have low density of endophytes (E_{low}) received an identical treatment as E_{high} plants except that the sprayed solution lacked conidia. After 15 h plants were returned to growth chambers until they were used in experiments.

We assessed the density of endophytes in E_{high} and E_{low} leaves 7 d after the spray treatment using one healthy leaf per plant or three leaves per tray of *c.* 60 plants. We cut a portion of the foliar lamina of detached leaves in 2 × 2 mm squares with a razor blade and then sterilized the surface of leaf pieces with consecutive immersions in ethanol 70% (1 min) and 10% bleach (1 min) and plated 16 pieces per leaf on 2% MEA plates. We estimated the density of endophyte colonization in a leaf as the percentage of leaf pieces in a plate where fungal growth was observed after 7 d. Overall, inoculated E_{high} cucumber had a mean *C. tropicale* colonization of 75.7 ± 5% while only *c.* 6.6 ± 1% of the control E_{low} leaf pieces were colonized by any fungi (Wilcoxon signed-rank test, $W = 4$, $P < 0.001$). Throughout the paper means are given ± 1 SE.

Bioassays with cucumber leaves

To test for ant foraging preferences between E_{low} and E_{high} plants, we detached leaves from plants, rinsed them in water and gently dried them with paper towel immediately before tests. We then placed one leaf from each type of plant 2 cm apart on a circular arena (16 cm diameter) connected to an ant colony with a plastic strip (see the Supporting Information, Fig. S1). The elapsed time between the discovery and cutting of each leaf by ants was used as a measure of acceptance. In addition, we took a photograph of the arena at the start of the test and every 10 min until both leaves were totally removed, or after 2 h from the start of the trial. Photographs showed the number of ants recruited to each leaf and the foliar area removed by ants at different time-intervals. We used the software IMAGEJ (http://resbweb.nih.gov/ij/; NIH, USA) to measure leaf area from photographs. Our analysis only included tests where ants found and cut leaves before they wilted. Leaves for each treatment came from three different seedling trays that had a mean *C. tropicale* density of 5.6 ± 1.4% and 100% for E_{low} and E_{high}, respectively. We conducted 20 tests, each one using a different ant colony to provide 20 independent measures of preference.

Bioassays with leaf solvent extractions

To make solvent extractions, we rinsed detached healthy leaves in distilled water and then air-dried and ground them to powder. Approximately 3 g of the leaf powder was then extracted for 3 h in a 2 : 1 chloroform: ethanol mixture. After removing the solvent in a rotary evaporator the dark green residue left had a mean weight of 36 ± 10 mg and 26 ± 12 mg per dry leaf mass for E_{high} and E_{low} extracts, respectively. We made six leaf extracts. Three of the six used leaves from three different trays of cucumber seedlings inoculated with independent *C. tropicale* cultures and the remaining three used leaves from three control trays. The estimated mean density of endophytes was 97.5 ± 1.4% for E_{high} and 2.1 ± 0.8% for E_{low} treatments.

We used extracts to assess whether colonization with *C. tropicale* causes chemical changes that influence ants' foraging decisions. Extracts contained compounds with relatively high molecular weights (*c.* > 290). In a similar arena used for tests with detached leaves, we gave ants the choice between paper filter disks impregnated with extracts from E_{low} or E_{high} cucumber leaves. We submerged disks in 5 mg of the extract dissolved in 1 ml of dichloromethane and, once the solvent dried, distributed 20 from each treatment randomly on the arena. To discriminate between E_{low} and E_{high} disks, we marked disks of one treatment with a pencil dot. Preliminary tests showed that marks did not affect ant choices. We ran tests for 2 h or until ants removed all disks from the arena. We registered the time of first discovery and pick up of each type of disk by the colony, as well as the number of disks from each treatment left in the arena when half of the initial number of disks had been removed (20 disks). In the analysis, we only included tests where ants removed half of the disks within 2 h of the start of the trial. We tested 10 laboratory colonies with each of the three pairs of extracts, for a total of 30 independent measures of preference.

Analysis of leaf volatile organic compounds

We exposed plants to ant colonies and collected volatile compounds in the headspace of ant-cut leaf pieces immediately after at least two leaves had been partially cut. Headspace volatiles were sampled with solid phase-microextraction (SPME) using a 65 μm polydimethylsiloxane/divinylbenzene fiber (Supelco; Sigma-Aldrich). We conditioned fibers at 250°C for 30 min and then exposed to the pieces of leaf for 3 h inside a 4 ml glass vial at room temperature (24°C). The dry weight of pieces used in the analyses was, on average, 16.18 ± 2.03 mg. We analysed volatile mixtures with an Agilent 5973 Network mass selective detector (Wilmington, DE, USA) connected to a gas chromatograph 6890N using a HP-5 ms fused silica capillary column (30 m × 0.25 mm, 0.25 μm) (GC-MS). We injected in splitless mode at 250°C using helium as the carrier gas (constant flow of 1 ml min^{-1}). The temperature program started at 50°C, was held for 1 min and then rose to 300°C with a heating rate of 5°C min^{-1}. We identified compounds by comparison with mass spectra using a Wiley 7n.1 library (Wiley Registry of Mass Spectral Data; John Wiley & Sons, Inc.) and with n-alkane gas chromatographic retention indices.

We compared GC peaks between E_{low} and E_{high} samples. Analyses included compounds present in mixtures of at least two plants. For analyses, we converted peak areas into percentages, and as they conveyed relative information of compounds' abundance in a sample, we then transformed them for analysis of compositional data (Aitchison, 1986; Estrada *et al.*, 2010). First, transformations required consideration of the absence of compounds as artifacts of the measuring process and consequently applying the zero replacement technique by Fry *et al.* (2000). Zero percentages were replaced by $\tau_A = \delta(M+1)(N-M)/N^2$, and nonzero ones by $W_i \times \tau_S$, where W_i is the percentage of peak i (when $W_i > 0$), $\tau_S = \delta M(M+1)/N^2$, M is the number of zeros in an individual sample, N is the total number of peaks analysed and δ is the maximum rounding error ($\delta = 0.0001$). We then log-ratio-transformed percentage data following Reyment's formula (1989), $Z_{i,j} = \log_{10}((X_{i,j}/g(X_j))$,

where, for individual j, $X_{i,j}$ is the peak area of compound i, g (X_j) is the geometric mean of the area of all peaks and $Z_{i,j}$ is the transformed area for peak i.

We sampled leaf volatiles from seven E_{high} and eight E_{low} plants with estimated mean endophyte densities of $71.4 \pm 13.3\%$ and $2.6 \pm 1.6\%$, respectively (Wilcoxon test, $W = 0$, $P < 0.01$). Similarity of the chemical composition of these samples was visualized using nonmetric multidimensional scaling (nMDS) (Zuur *et al.*, 2007). This ordination method used the rank order of pairwise Euclidean distances among samples, based on their transformed peak areas. The minimal stress achieved, here given in percentages, measures how well distances between samples are represented by the ordination. We performed two analyses to detect statistical differences in volatile composition among treatments. First, a one-way analysis of similarity (ANOSIM) compared between E_{high} and E_{low} samples regardless of their percentage colonization by *C. tropicale*. As in the nMDS, ANOSIM was based on a Euclidean distance matrix. Second, we used a Mantel test to examine whether there was a correlation between the variation in the composition of volatile compounds among samples and variation in their estimated percentage endophyte density. The significance of the correlation was calculated after 1000 permutations. We performed statistical analyses with the software package R (version 2.12.2).

Analysis of cuticular waxes

We extracted cuticular waxes from nine E_{high} and 10 E_{low} plants with estimated mean endophyte densities of $67.8 \pm 9.3\%$ and $5.2 \pm 1.9\%$, respectively (Wilcoxon test, $W = 0$, $P < 0.01$). Leaves were dipped individually in 4 ml of chloroform for 30 s. After the chloroform was evaporated with flowing nitrogen gas, we redissolved extracts in 50 µl of dichloromethane and added 180 ng of tridecane as an internal standard. Cuticular composition of leaves was characterized with GC-MS using the same equipment described above for leaf volatiles. The temperature program started at 80°C, was held for 1 min and then rose to 320°C, with a heating rate of 6°C min^{-1}. This maximum temperature was then maintained for 15 min. We calculated the abundances of compounds identified relative to the standard, square-root transformed and analysed data using the methods described above for leaf volatiles.

Nutrient analyses

We measured the content of six macronutrients (C, N, P, K, Ca, Mg), six micronutrients (B, Cu, Fe, Mn, Na, Zn) and Al for five E_{high} and five E_{low} cucumber trays. Estimated mean endophyte colonization was $57.2 \pm 13\%$ in E_{high} and $18\% \pm 6.3\%$ in E_{low} leaves (Wilcoxon test, $W = 24$, $P < 0.05$). We rinsed leaves in distilled water, allowed them to dry and ground them to powder. The Soil Laboratory at STRI determined total C and N by dry combustion using a Flash EA1112 analyser. Other mineral elements were extracted by nitric acid digestion then determined by inductively coupled plasma-optical emission spectrometry using an Optima 7300 (Perkin Elmer, Waltham, MA, USA). We compared the amounts of each element between E_{low} and E_{high} treatments with Wilcoxon signed-rank tests and related the amounts to the estimated colonization by *C. tropicale* in sampled plants using least squares regressions.

Leaf physical traits

We compared water content and specific leaf area (SLA) between E_{high} and E_{low} leaves. Percentage water was calculated from the difference between fresh and dry mass in 18 leaves from each treatment. The SLA was calculated from the ratio between area and dry mass of 14 E_{low} and 12 E_{high} leaves (Cornelissen *et al.*, 2003). Leaves had estimated mean endophyte densities of $91 \pm 6.7\%$ and $5.2 \pm 1.7\%$ for E_{high} and E_{low}, respectively (Wilcoxon test, $W = 0$, $P < 0.01$).

Results

Bioassays with cucumber leaves

Forager ants equally accepted and cut E_{high} and E_{low} cucumber leaves (Table 1). Nevertheless, in 17 out of 20 tests ants cut and removed the whole E_{low} leaf from the arena before they cut and removed the E_{high} one (G-test, df = 2, $P < 0.001$). In two of the three remaining tests where neither leaf was finished after 2 h, ants had also removed more area of the E_{low} leaf than from the E_{high} leaf one by the end of the test. Cucumber leaves for both treatments were similar in size (Table 1), yet ants had cut about one-third greater area from E_{low} leaves ($38.73 \pm 8.22\%$) by the time the E_{low} leaves were totally cut or when 2 h of the test had

Table 1 Results of choice tests by leaf-cutting ants between control (E_{low}) and *Colletotrichum tropicale*-colonized cucumber leaves (E_{high}) (mean ± 1 SE)

	Endophyte colonization			
	Low	High	Difference ($E_{low} - E_{high}$)	P
Initial leaf area (cm^2)	15.02 ± 1.18	15.75 ± 1.53	-0.73 ± 0.77	0.36
Acceptance time (min)	10.1 ± 1.7	13.25 ± 2	-3.1 ± 2.44	0.22
Recruitment (number of ants)	26.35 ± 4.92	15.14 ± 3.39	9.6 ± 2.93	< 0.01
Maximum rate of leaf removal (cm^2 min^{-1})	0.69 ± 0.06	0.51 ± 0.06	0.18 ± 0.07	0.01

Paired t-tests (df = 19) were used to compare the parameters measured in these bioassays.

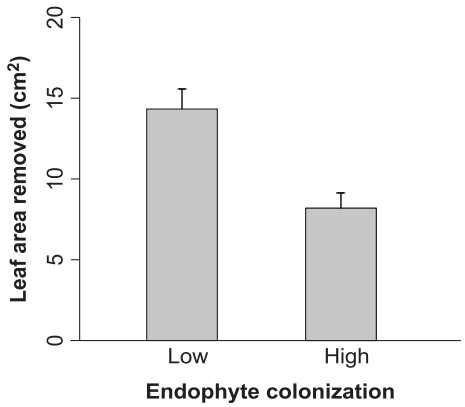

Fig. 1 *Cucumis sativus* leaf area cut and removed by *Atta colombica* laboratory colonies (mean ± 1 SE). Total area removed in choice tests after the first leaf was completely carried away from the arena or when 2 h had passed. Cucumber leaves offered to ants had low and high percentages of colonization by the endophyte *Colletotrichum tropicale*.

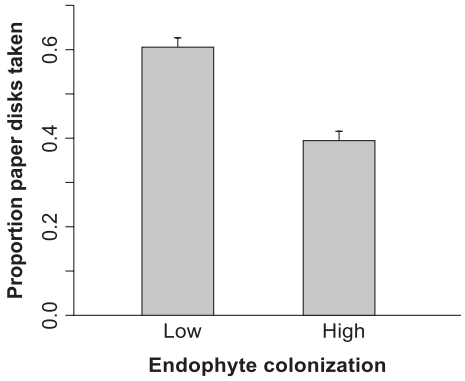

Fig. 2 Proportion of paper disks removed by *Atta colombica* laboratory colonies (mean ± 1 SE). Disks were impregnated with extracts of *Cucumis sativus* leaves with low and high percentages of colonization by the endophyte *Colletotrichum tropicale*.

elapsed (Fig. 1). The number of ants observed on E_{low} leaves was typically higher than on E_{high} leaves when both leaves were present in the arena (Table 1). Ants also totally cut and removed E_{high} leaves in 14 tests after E_{low} leaves had already been removed. However, the rate with which such leaves were cut only occasionally (seven tests) reached the maximum speed of area removal observed for leaves with fewer endophytes (Table 1).

Bioassays with leaf solvent extractions

Ants showed preferences for paper disks impregnated with extracts that originated from E_{low} leaves rather than E_{high} leaves (Fig. 2). Although ants carried both types of disk to their nest, in 24 out of 30 tests ants removed more disks with E_{low} than E_{high} extracts when half of the disks had been taken from the arena (G-test, df = 2, $P < 0.001$). In two of the six remaining tests the number of disks chosen from both treatments was the same. The difference in proportion of disks taken ($E_{low} - E_{high}$) at that moment varied from −0.2 (ants carried four more E_{high} disks) to 0.7 (ants carried 14 more E_{low} disks), with a mean difference significantly greater than zero (0.2 ± 0.04, t-test, df = 29, $P < 0.01$, Fig. 2). Ant colonies consistently preferred disks with E_{low} extracts over E_{high} extracts, but preferences varied significantly among the pairs of extracts tested (mean proportion difference = 0.07 ± 0.07, 0.2 ± 0.04, and 0.36 ± 0.08 for extract pairs 1–3, respectively, Kruskal–Wallis test, df = 2, $P = 0.03$). The difference was only marginally significant between extracts 1 and 3 (Wilcoxon test with Bonferroni correction, $P = 0.017$) and is not correlated with differences in percentage of colonization by *C. tropicale* between pairs of plants used to make extractions. Differences in percentage of colonization between E_{high} and E_{low} treatments ($\%E_{high} - \%E_{low}$) were 99.9, 93.8 and 97.1% for extracts 1, 2 and 3, respectively.

Analysis of leaf volatile organic compounds

Volatile mixtures of ant-cut leaf pieces contained 45 compounds that eluted in 41 chromatographic peaks. The occurrences of volatile compounds detected in control and treated plants are summarized in Table S1 and Fig. S2. A typical volatile mixture included green-leaf volatiles, aldehydes and ketones reported for undamaged and mechanically wounded cucumber leaves and fruits. The two major chromatographic peaks contained 2-hexenal, (*Z*)-3-hexen-1-ol and 1-hexanol, which eluted together and 3-hexen-1-ol acetate. The dominance of 2-hexenal and (*Z*)-3-hexen-1-ol in the mixtures differed across samples but this variation was independent of plant treatment. Similarly, a few samples from E_{high} and E_{low} plants contained β-ocimene and 4,8-dimethyl-1,3,7-nonatriene – compounds typically induced by biotic stress in cucumber. Odors that originated from ants could also be part of the mixtures in headspace samples. For example, we found 2-heptanone, a major component of the alarm pheromone produced by *Atta*, in two E_{high} samples (Table S1).

Comparisons among samples showed that high levels of *C. tropicale* density did not cause detectable changes in the composition of volatile mixtures when they were measured soon after tissue damage. Volatile emissions measured as the total peak area per dry weight of leaf were similar between E_{low} and E_{high} samples ($2.8E+07 \pm 6E+06$ and $2E+07 \pm 6E+06$ for E_{low} and E_{high}, respectively, Wilcoxon tests, $P > 0.05$). Similarly, variations in chemical composition among samples were not related to treatment (E_{low} vs E_{high}, ANOSIM, $R = -0.08$, $P = 0.81$) or to estimated percentage of colonization by *C. tropicale* among plants (Mantel test, $r = -0.02$, $P = 0.49$). This lack of clear distinction in chemical mixtures between treatments is illustrated by the nMDS plot (Fig. 3).

Analysis of cuticular waxes

Gas chromatographic analysis from chloroform extracts of cucumber leaf surfaces showed 25 compounds eluted in 24 peaks. Dominant compounds in the wax mixture were saturated straight-chain alkanes with 29, 31 and 33 carbon atoms. Minor compounds included other n-alkanes with chains between 25 and 32 carbons, aldehydes of even-numbered n-alkanes from 26

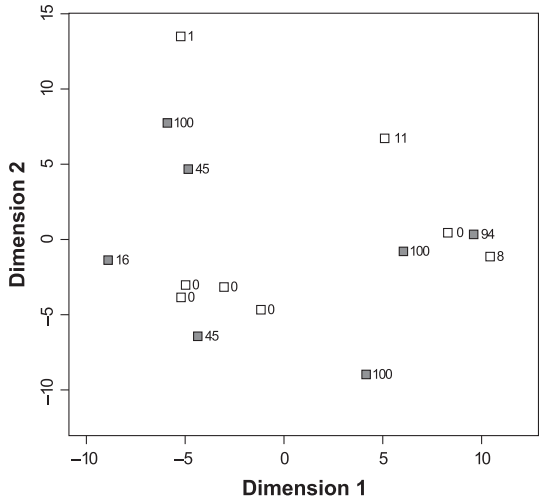

Fig. 3 Similarity in the composition of volatile mixtures emitted by ant-cut leaf pieces from control (open squares) and endophyte-treated (tinted squares) *Cucumis sativus* plants (nMDS, stress = 8.24). Each symbol represents a sample with numbers indicating the estimated density of *Colletotrichum tropicale* in the plant exposed to ants.

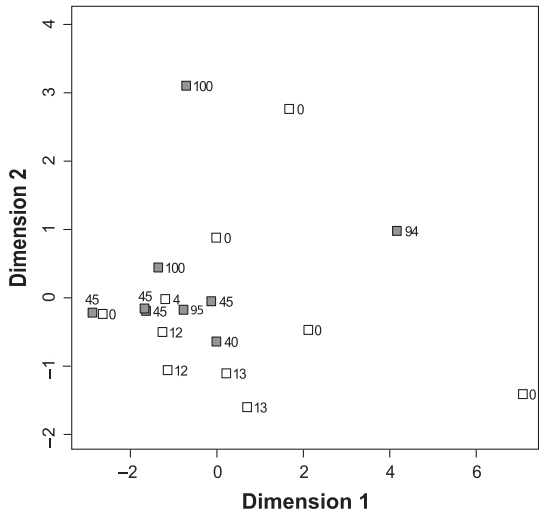

Fig. 4 Similarity in the composition of chloroform extracts of leaf surfaces from control (open squares) and endophyte-treated (tinted squares) *Cucumis sativus* plants (nMDS, stress = 8.51). Each symbol represents a sample with numbers indicating the estimated density of *Colletotrichum tropicale* in the plant.

to 32 carbons, sterols, tocopherols, triterpenes and triterpene alcohols (Table S2, Fig. S3).

Extracts of leaf surfaces of E_{high} and E_{low} plants contained similar compounds in comparable amounts. E_{high} leaves tended to show lower amounts of most compounds, except for the putative acetates of the triterpene alcohols β- and α-amyrin and isomultiflorenyl, which appeared to be present in higher amounts than in E_{low} samples. Nevertheless, differences between treatments in average amounts detected for each compound (or group of compounds when they eluted together) were not significant (Wilcoxon tests, $P > 0.05$). Furthermore, multivariate analyses of chemical variation among extracts did not separate (nMDS, Fig. 4) or find significant differences in composition between E_{low} and E_{high} plants (ANOSIM, $R = -0.03$, $P = 0.72$). Similarly, there was no correlation between chemical similarity in the cuticular mixtures and degree of leaf colonization by *C. tropicale* (Mantel Test, $r = 0.06$, $P = 0.26$).

Nutrient analyses

The content of the mineral elements analyzed were not different between E_{low} and E_{high} treatments (Wilcoxon tests, $P > 0.05$, Table 2). Nevertheless, there was a positive relationship between the amount of Al, Ca and Fe, and the estimated density of *C. tropicale*. More than 40% of the variation in these three elements can be explained by variation in the percentage of the fungal symbiont inside leaves (Table 2, Fig. S4).

Leaf physical traits

Colonization by *C. tropicale* did not result in detectable changes in water content ($90 \pm 0.7\%$ and $89 \pm 0.8\%$ for E_{low} and E_{high}, respectively) or SLA of cucumber (0.87 ± 0.04 and 0.82 ± 0.08 for E_{low} and E_{high} respectively, Wilcoxon tests $P > 0.05$ in both comparisons).

Discussion

A previous study showed that leaf-cutting ants prefer to cut leaves with low rather than high colonization by endophytic fungi (Bittleston *et al.*, 2011). Here we show that changes in the chemical characteristics of leaves resulting from their symbiosis with leaf endophytes are associated with most of the observed ant preferences. In our experiments ants demonstrated a preference for paper disks impregnated with leaf extracts from E_{low} cucumber plants that was similar in magnitude to the observed preference for E_{low} detached leaves relative with E_{high} treatments. Ants cut about one-third more area of E_{low} cucumber leaves and removed *c.* 20% more paper disks impregnated with E_{low} extracts compared with E_{high} leaves and disks, respectively.

Leaves from both plant treatments were often consumed but ants consistently cut and carried E_{low} leaves to their nests faster than E_{high} leaves. This overall difference in the speed in which leaves were cut can partly be explained by the difference in recruitment of ants to both types of leaves (Table 1, Bittleston *et al.*, 2011). The number of ants recruited to a resource is dynamically modulated by individual workers through deposition of pheromones and correlates with food quality (Littledyke & Cherrett, 1978a,b; Jaffe & Howse, 1979; Roces, 1990). The chemical profile of leaves that ants use during host selection includes compounds from the cuticle cover, volatile compounds released to the surface, and chemicals that can be smelled or tasted while cutting (Van Bael *et al.*, 2011). Discerning minor differences of cucumber leaf quality by ants was done using components of the chemical phenotype that were included in leaf extracts added to paper disks. Leaf extracts contained compounds from a wide range of polarity and relatively high molecular weight (C. Estrada, S. A. Van Bael & W. T. Wcislo, unpublished). Variations in the chemical composition of leaf volatile

Table 2 Content of macronutrients, micronutrients and aluminum in *Colletotrichum tropicale*-treated and control cucumber leaves

Element	Low endophyte	High endophyte	Slope coefficient	R^2	F
Al	0.08 ± 0.01	0.12 ± 0.03	1.33E-3 ± 4.41E-4	0.53	9.18*
B	0.06 ± 0.01	0.06 ± 8E-3	5.65E-5 ± 1.00E-4	0.03	0.26
Ca	11.50 ± 0.32	11.67 ± 0.57	2.36E-2 ± 8.16E-3	0.51	8.37*
Cu	0.03 ± 3E-3	0.03 ± 1E-3	−6.25E-5 ± 4.77E-5	0.18	1.71
Fe	0.13 ± 0.01	0.17 ± 0.04	1.29E-3 ± 5.14E-4	0.44	6.28*
K	51.85 ± 1.28	48.58 ± 2.59	-3.01E-2 ± 5.37E-2	0.04	0.31
Mg	8.76 ± 0.17	8.27 ± 0.51	9.64E-3 ± 9.28E-3	0.12	1.08
Mn	0.16 ± 0.01	0.17 ± 0.02	1.79E-4 ± 4.63E-4	0.02	0.15
Na	0.56 ± 0.05	0.54 ± 0.07	8.44E-4 ± 1.55E-3	0.04	0.30
P	7.81 ± 0.68	7.39 ± 0.87	1.45E-2 ± 1.89E-2	0.07	0.59
Zn	0.12 ± 5E-3	0.13 ± 5E-3	8.97E-5 ± 1.27E-4	0.06	0.50
Total C	35.98 ± 0.45	36.60 ± 0.18	4.53E-3 ± 6.77E-3	0.05	0.45
Total N	4.3 ± 0.97	4.02 ± 0.42	3.38E-03 ± 9.54E-03	0.02	0.13

Total C and total N are measured in percentage of dry leaf mass while values for other elements are given in milligrams per gram of dry leaf mass (mean ± 1 SE).
*F-values with $P < 0.05$ obtained from linear regressions performed between the amount of an element and the estimated colonization by the endophyte in sampled leaves.

compounds and cuticular waxes were independent of the degree of colonization by *C. tropicale*. Furthermore, observed colony preferences during tests with leaves typically happened after ants had started cutting both types of leaves. Thus, overall, our results suggest that compounds with relatively low volatility exposed during tissue wounding play major role in ants' selectivity. Alternatively, changes may have been subtle and went undetected in our chemical analyses.

Our results showed no consistent qualitative or quantitative differences in the composition of volatile compounds emitted by the control and endophyte-colonized cucumber. The few studies that have evaluated the volatile composition resulting from plant –endophyte symbioses have shown alteration of such mixtures compared with endophyte-free controls, although not with predictable patterns. For example, while emissions of most terpenoids from tomato decreased by half in plants hosting a root fungal endophyte compared with control plants (Jallow *et al.*, 2008), the same group of chemicals increased significantly in peppermint colonized by a growth-promoting endophyte (Mucciarelli *et al.*, 2007). However, in the latter study it is unclear whether the increase in emissions is caused by the generally larger size of infected plants and leaves. Volatile emissions from endophyte- and pathogen-infected plants have also shown compounds that presumably have a fungal origin (3-octanone, 1-octen-3-ol) (Yue *et al.*, 2001; Cardoza *et al.*, 2002). Several endophytes can release volatiles in culture and have been shown to have antibiotic and insecticidal effects (Strobel *et al.*, 2001; Daisy *et al.*, 2002; Mucciarelli *et al.*, 2007). *Colletotrichum tropicale*, in particular, produced small quantities of > 15 volatile organic compounds in *in vitro* cultures (C. Estrada, S. A. Van Bael & W. T. Wcislo, unpublished), most of which are likely sesquiterpenoids, compounds typically associated with host rejection by leaf-cutting ants (Van Bael *et al.*, 2011). Nevertheless, neither those sesquiterpenoids nor the typical fungal compounds found in other studies were detected in volatile mixtures from E_{high} leaves. This is not surprising given that production of secondary compounds varies considerably with the medium where fungi grows (Ezra & Strobel, 2003) and that leaf endophyte biomass, usually restricted to intercellular spaces, is much lower than the biomass of *in vitro* cultures (Cabral *et al.*, 1993; Schulz *et al.*, 2002). This means that fungal emissions *in planta* may fall below the detection sensitivity of chemical instruments.

Volatile compounds emitted by leaves from both plant treatments were mixtures typically found in undamaged or mechanically wounded cucumber (Kemp *et al.*, 1974; Takabayashi *et al.*, 1994). This is a group of chemicals stored permanently inside leaves and released soon after tissue damage. Biotic and abiotic stresses also induce complex networks of signaling cascades in plants that result in emission of a different set of volatile compounds (Thomma *et al.*, 2001; Kessler & Baldwin, 2002; Chisholm *et al.*, 2006; Dudareva *et al.*, 2006). Our results do not show evidence of activation of such defense biochemical pathways in cucumber in response to colonization by *C. tropicale*. First, samples lacked methyl salicylate, a volatile ester derived from the hormone salicylic acid (SA) that plants emit when they are infected by pathogenic fungi (Cardoza *et al.*, 2002). Salicylic acid-dependent defenses are typically activated in plants in response to infections by biotrophic fungi (Thomma *et al.*, 2001; Chisholm *et al.*, 2006). Both SA and its airborne signal, methyl salicylate, also generate systemic acquired resistance (SAR), a long-lasting disease resistance induced by early microbial or viral pathogenic infections (Van Loon, 2000). Second, volatile terpenoids known to be regulated in cucumber by the jasmonic acid (JA) pathway, β-ocimene and 4,8-dimethyl-1,3,7-nonatriene (Takabayashi *et al.*, 1994; Mercke *et al.*, 2004), were detected in a few of the plants but their occurrence was independent of the degree of colonization by *C. tropicale*. Both JA and ethylene are hormones involved in the induced systemic resistance (IRS), disease resistance caused by nonpathogenic root-colonizing microorganisms (Van Loon, 2000; Shoresh *et al.*, 2005). These signaling molecules also activate defense mechanisms against herbivores and infections by necrotrophic fungi (Thomma *et al.*, 2001; Chisholm *et al.*, 2006).

Most compounds found in our leaf surface chloroform extracts had been reported as cuticular waxes or components of the cell membrane of leaves, fruits and seeds of cucumber (Steinmüller &

Tevini, 1985; Akihisa *et al.*, 1986, 1988; Hartmann, 1998; Chun *et al.*, 2006). Our results showed that high levels of *C. tropicale* colonization did not cause significant changes in the composition of cuticular waxes of leaves. However, while all major compounds were present in both treatments in about the same proportions, the content of individual chemicals tended to be higher in E_{low} leaves than in E_{high} leaves where only traces of some minor compounds were found (e.g. aldehydes and triterpene alcohols). Our results were unexpected, as changes in cuticular waxes after biotic and abiotic challenges have been reported from cucumber leaves. First, irradiation with enhanced UV-B levels caused an increase in total wax in cotyledons, particularly in aldehyde and alkane content (Steinmüller & Tevini, 1985). Second, resistance of cucumber to *Colletotrichum largenarium* owing to an early inoculation with the same pathogen (Kuć & Richmond, 1977) results in part from chemical changes on the leaf surface that repress fungal penetration to epidermal cells (Xuei *et al.*, 1988; Kováts *et al.*, 1991).

Overall, neither volatile compound nor cuticle chemical composition indicated that *C. tropicale* activated defense signaling cascades (JA, SA) in cucumber in the manner described for infections by pathogens or root-colonizing fungi and bacteria (SAR, IRS). However, this does not indicate that activation of plant defenses in these or alternative pathways does occur. A closer examination of hormone presence or gene expression downstream in defense signaling pathways is necessary to determine the extent of the involvement of plant defense mechanisms. Indeed, evidence exists for activation of defense genes in cacao plants colonized by *C. tropicale* (L. C. Mejía & E. A. Herre, pers. comm.). Changes in cucumber leaf chemistry are suggested by the preferences of our ant colonies for paper disks impregnated with E_{low} extracts rather than E_{high} extracts. We are currently investigating the identity of the chemicals influencing ants' foraging decisions. Qualitative or quantitative changes in leaf chemistry could be caused by enzymes or antibiotic compounds expressed by cucumber in response to *C. tropicale* or toxins from the endophyte that are constitutive or induced by cucumber defense responses. Some forms of resistance by plants toward fungal strains that cause diseases in genetic varieties of the same plant species or in other species (non-host resistance) include the accumulation of antimicrobial compounds induced by fungal colonization (Heath, 2000). Moreover, chemical warfare appears to be common among fungal root endophytes and their hosts, suggesting that many of these interactions are antagonistic, although maintained at equilibrium where none of the partners is favored ('balanced antagonism', Schulz *et al.*, 1999, 2002; Schulz & Boyle, 2005).

Our results for the mineral content of leaves also suggest that a balanced antagonism could occur between *C. tropicale* and cucumber. Although most of the elements analysed are constituents of the fungal cell (Martin, 1979), and thus could increase with fungal biomass inside leaves, only the amounts of Ca, Fe and Al showed a positive correlation with the estimated degree of colonization by *C. tropicale*. Such relationships for the first two compounds could be linked to the plant responses to fungi and subsequent fungal resistance. Calcium is crucial for regulating many responses of plant cells to their environment (Dodd *et al.*, 2010). In particular, calcium crosslinks pectin molecules in cell walls and thus its content in leaf tissue increases during reinforcement of the cell wall induced by fungal infections (Moerschbacher & Mendgen, 2000). By contrast, iron is essential for fungal growth and influential in the stability of plant–fungal interactions (Johnson, 2008). Furthermore, iron-containing antioxidant enzymes in fungi degrade antimicrobial active oxygen molecules produced by plants as an early response to halt fungal colonization (Mayer *et al.*, 2001). However, fungal antioxidant enzymes that use Zn, Cu or Mn also exist, yet these elements did not vary with colonization of leaves by *C. tropicale*. Moreover, changes in content of Ca and Fe were not detected in leaves colonized by a natural community of endophytes relative to control plants (Van Bael *et al.*, 2012a). Clearly, further investigation is necessary to explain the observed increase of both elements with the dynamics of cucumber–*C. tropicale* symbiosis. The increase of Al with *C. tropicale* colonization is puzzling as, to our knowledge, neither cucumber nor *Colletotrichum* accumulate this metal. Interestingly, the amount of Al could have negatively influenced the foraging preferences of ants in one study (Folgarait *et al.*, 1996; but see Mundim *et al.*, 2009); thus an increase of this element with *C. tropicale* colonization could have accounted for part of the observed ants' preference for E_{low} treatments.

Along with leaf chemistry, toughness is one of the more important traits that ants use during host selection (Van Bael *et al.*, 2011). Changes in the physical properties of leaves owing to endophyte colonization could also contribute to the observed foraging patterns in our arenas using detached leaves. It is known that a plant's typical response to detection of microbes consists of adding lignin, cellulose and other components of their cell walls as a mechanism to decrease the chance that the infection will reach the cell's cytoplasm (Heath, 2000; Moerschbacher & Mendgen, 2000; Chisholm *et al.*, 2006). This effect, which has been documented for fungal pathogens in host and nonhost plants, also occurs after colonization of leaves of *Theobroma cacao* by *C. tropicale* (S. Maximova & E. A. Herre unpublished), and *Juncus* spp. by several species of fungal endophytes (Cabral *et al.*, 1993). Although the reinforcement of the cell walls is confined to cells surrounding the point of fungal growth (Moerschbacher & Mendgen, 2000), this reinforcement in highly endophyte-colonized leaves could result in an overall increase in toughness, making leaves less appealing to ants. Nevertheless, several studies, including ours, have found no evidence that endophytes trigger leaves to become harder when conventional surrogate methods such as total carbon, water content and SLA have been used (Witkowski & Lamont, 1991; Bittleston *et al.*, 2011). Similar results have been obtained when using more direct toughness measures such as resistance by puncturing and tearing (Bittleston *et al.*, 2011). Experiments are thus necessary to determine whether deposition of cell wall components is a general response of plants to colonization by endophytic fungi and whether such reinforcements make leaf cutting more difficult for ants.

Each tropical plant can host dozens of endophyte species at any given time (Arnold *et al.*, 2000; Suryanarayanan & Johnson,

2005; Van Bael *et al.*, 2005). Our simplified system is therefore a first step to identify the mechanisms involved in endophyte-mediated protection of tropical plants. *Colletotrichum tropicale* influences leaf chemistry and makes leaves less appealing to leaf-cutting ants but likely offers little protection to individual plants as endophyte-colonized leaves are also consumed. Nevertheless, for ants, leaves hosting fungal endophytes take longer to process than those free of the symbiont (Van Bael *et al.*, 2012b), and this additional cost decreases colony development, at least for young colonies whose risk of mortality is the greatest (Van Bael *et al.*, 2012a). It is still an open question whether plant fitness is affected by short-term benefits resulting from ants' food selection or by changes in colony populations resulting from plant-endophyte symbiosis. More importantly, this study shows that fungal communities in leaf tissues can create a mosaic of palatability driven by leaf and endophyte chemistry within and between plants beyond the existing variance resulting from plant genotype, environment and leaf age or location. Such variability can be high if leaf responses are tuned to endophyte species and if the local diversity of fungal endophytes results in a similar diversity of fungal toxins. Symbiosis with fungi can then influence the ecological interactions of plants with their natural enemies by making leaf defenses less predictable and thus more difficult to adapt to by herbivores (Herre *et al.*, 2007).

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Bioassay to test preferences for cucumber leaves or paper disks with leaf extracts by laboratory colonies of leaf-cutting ants (*Atta colombica*).

Fig. S2 Chemical composition of volatile mixtures of ant-cut leaf pieces.

Fig. S3 Chemical composition of chloroform extracts of leaf surfaces.

Fig. S4 Plots of the amount of micronutrients, macronutrients and aluminum (Al) in *Cucumis sativus* leaves against the estimated density of *Colletotrichum tropicale* in sampled plants.

Table S1 Composition of volatile compounds detected in ant-cut leaf pieces of *Cucumis sativus* sampled with solid-phase microextraction (SPME)

Table S2 Composition of chloroform extracts of *Cucumis sativus* leaf surfaces

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