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Endophytic fungi reduce leaf-cutting ant damage to seedlings

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Our study examines how the mutualism between *Atta colombica* leaf-cutting ants and their cultivated fungus is influenced by the presence of diverse foliar endophytic fungi (endophytes) at high densities in tropical leaf tissues. We conducted laboratory choice trials in which ant colonies chose between *Cordia alliodora* seedlings with high (E_{high}) or low (E_{low}) densities of endophytes. The E_{high} seedlings contained 5.5 times higher endophyte content and a greater diversity of fungal morphospecies than the E_{low} treatment, and endophyte content was not correlated with leaf toughness or thickness. Leaf-cutting ants cut over 2.5 times the leaf area from E_{low} relative to E_{high} seedlings and had a tendency to recruit more ants to E_{low} plants. Our findings suggest that leaf-cutting ants may incur costs from cutting and processing leaves with high endophyte loads, which could impact Neotropical forests by causing variable damage rates within plant communities.

Keywords: Attini; herbivory; symbiosis; endophytes; mutualism

1. INTRODUCTION

Mutualism is an important evolutionary strategy, as organisms take advantage of other species' unique abilities in order to increase survival and reproduction. Leaf-cutting ants are members of a well-known mutualistic association involving a coevolved fungal symbiont (*Leucocoprinus gongylophorus*): the ants cut and prepare leaf pieces as a fungal growth substrate, and the cultivar provides the ants with nutrition and a matrix for brood-rearing, among other benefits [1]. This ant-centred mutualism interacts antagonistically with another suite of mutualistic organisms, which are vastly more widespread yet little studied, involving foliar endophytic fungi (hereafter; endophytes) and plants [2]. Endophytes are present without symptoms of disease in the photosynthetic tissue of all major lineages of land plants [3]. They are extremely diverse, particularly in the tropics where estimates suggest that a single leaf may contain up to 17 species [4]. Little is known about tropical endophyte ecology, but available evidence indicates that endophytes can reduce plant

pathogen damage [5], and negatively affect the fecundity of a herbivorous beetle [6].

Leaf-cutting ants are one of the dominant Neotropical herbivores and they defoliate diverse plants [7]. Previous research indicates that *Atta colombica* leaf-cutting ants actively decrease endophytes in their fungal gardens [2]. The ants reduced the levels of one endophyte strain from leaves before using them as fungal substrate; more time was spent cutting leaves with high rather than low endophyte loads; and the *in vitro* interactions between the fungal cultivar and the different endophyte species were largely antagonistic [2]. Here, we describe a novel method to inoculate plants with natural endophyte loads, which we employ to examine whether ants respond similarly to the natural diversity of endophytes that they encounter on a daily basis, and whether ants exhibit a preference for leaves with low endophyte diversity and density. Additionally, we assess the intraspecific plant characteristics (leaf toughness and thickness) that predict how much leaf material ants will remove. We then discuss what larger effects endophyte diversity may have on tropical forests.

2. MATERIAL AND METHODS

(a) Experimental plants

Cordia alliodora plants were grown in sterilized soil from seeds collected in Gamboa, Panama (9°07' N, 79°42' W), and then transplanted into potting soil in plastic cups. All seedlings were grown in 0.5 × 1 × 1 m plastic enclosures within a greenhouse in order to exclude fungal spores. The enclosures were opened only for daily watering and plant removal. To produce seedlings with high densities and diversities of foliar endophytic fungi (E_{high}), plants were taken outside five nights per week, two to four weeks prior to choice trials. The plants were sprayed with rainwater and left overnight in fine mesh cages (to protect from insect damage) near the rainforest edge, then returned to the greenhouse early the following morning. Low endophyte seedlings (E_{low}) remained in the greenhouse overnight. Thus, all plants were maintained on the same greenhouse benches during daylight growing hours, but E_{high} plants were placed near the forest at night when spore-fall is greatest [8]. Our methods for assessing leaf toughness, thickness, and endophyte density and diversity are detailed in the electronic supplementary material.

(b) Ant colonies

We collected incipient colonies of *A. colombica* leaf-cutting ants in Gamboa, as soon as the first foragers emerged in July 2009. The nest chambers were dug up and maintained in small plastic containers. The colonies were supplied with oats, cornmeal and leaves of plant species other than *C. alliodora* until used for the choice trials.

(c) Choice trials

For each choice trial, a colony was chosen randomly along with one E_{high} and one E_{low} seedling of similar sizes. The chosen colony was not supplied with leaves for one day prior to the trial. All seedlings were rinsed in filtered water to reduce surface microbes (see the electronic supplementary material) and a few leaves were removed from each plant to obtain similar leaf areas at comparable heights. All leaves were traced to assess leaf area. Each plant-base was covered with a plastic skirt, and plastic ramps were attached to the base of each plant and to the edge of the colony's container (figure 1c). Ants were allowed access to both plants for 4.5 h. The start time (t_0), initial discovery time and first cut time were recorded for each plant, as well as spot counts of the number of individuals on each plant for 20 min after initial discovery. Final leaf areas were recorded in order to calculate the amount cut by ants. We conducted 30 independent trials as replicates (30 colonies with 60 plants total).

(d) Statistical analysis

We tested for effects of our inoculation technique by assessing whether E_{high} and E_{low} plants differed in endophyte density and morphospecies diversity (Kruskal–Wallis), leaf toughness and specific leaf area (SLA) (*t*-tests).

We assessed whether individual colonies cut more area from E_{high} or E_{low} plants using the non-parametric Wilcoxon–signed ranks test for paired data. Of the 30 trials, we restricted our analysis to

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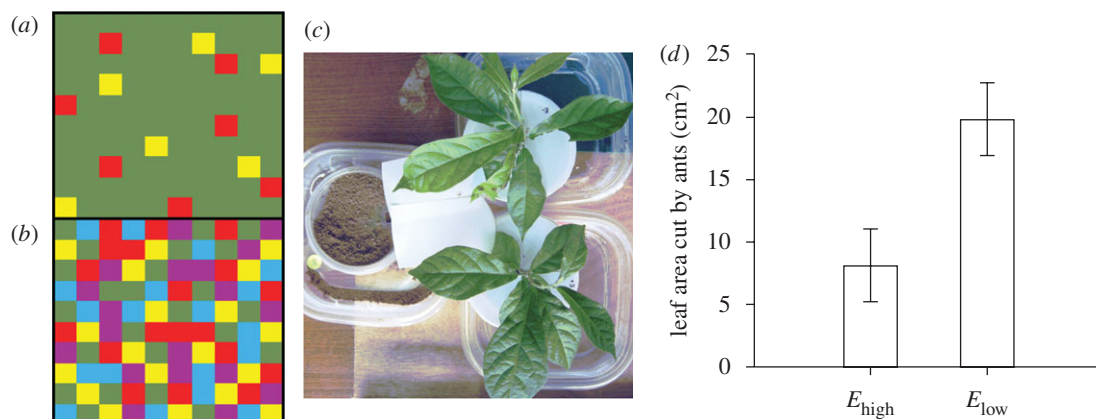


Figure 1. The presence of endophytic fungi in leaves of *Cordia alliodora* seedlings decreased the likelihood of leaf removal by *Atta colombica* ants in laboratory choice trials. Schematic describing how the average leaf tissue encountered by ants in the choice trials contained (a) a low density and diversity of endophyte morphospecies or (b) a high density and diversity of endophyte morphospecies (see text for mean values). Each square in (a) and (b) represents a 2 mm^2 leaf section from which endophytes were cultured. Green squares represent sections that did not contain endophytes, and all other colours represent unique endophyte morphospecies. (c) Experimental set-up for testing the preference of small *A. colombica* colonies for *C. alliodora* seedlings with high (E_{high}) and low (E_{low}) endophyte densities. (d) Mean ± 1 s.e. for the amount of leaf area removed by *A. colombica* workers ($n = 23$ colonies).

23 trials where both plants were discovered early in the trial (within the first hour). We interpreted the seven discarded trials as colonies that were disinterested in feeding during the trial, or colonies in which ‘choice’ did not occur because the colony was satiated with the first plant type that it discovered and cut (see the electronic supplementary material). We compared ant recruitment to E_{high} and E_{low} plants using a paired t -test on square root-transformed recruitment counts.

Finally, to assess which plant characteristics best predicted leaf removal, we used a general linear model with endophyte density, leaf toughness and SLA as dependent variables and leaf area removed by ants as the independent variable. We first ran a test with all two-way interactions included, and as none were significant we removed them for the final model. All p -values reflect two-sided probabilities and all tests were performed in SYSTAT (v. 11).

3. RESULTS

(a) Novel inoculation technique

Plants that were inoculated by night-time spore-fall contained approximately 5.5 times higher endophyte density than those that did not (mean \pm s.e. for E_{high} $76.4 \pm 4.1\%$, $n = 30$; for E_{low} $13.7 \pm 2.1\%$, $n = 30$; Mann–Whitney U -test statistic = 885, $\chi^2 = 41.5$, 1 d.f., $p < 0.001$).

The mean number of endophyte morpho-species that an ant would encounter in a given area of leaf tissue was higher for E_{high} relative to E_{low} plants (figure 1a,b; mean \pm s.e. for E_{high} 4.15 ± 0.24 morphospecies, $n = 30$; for E_{low} 1.85 ± 0.25 morpho-species, $n = 30$; Mann–Whitney U -test statistic = 821, $\chi^2 = 26.7$, 1 d.f., $p < 0.001$). The plants did not differ in leaf toughness ($t = 0.072$, 58 d.f., $p = 0.943$) or thickness (SLA, $t = -0.79$, 48 d.f., $p = 0.433$).

(b) Ant preference

Ants that discovered both plant types removed approximately 2.5 times more leaf area from E_{low} plants relative to E_{high} plants (figure 1d, Wilcoxon signed-rank test, $z = -2.34$, $p = 0.019$, $n = 23$ colonies). Recruitment of workers after plant discovery was greater on E_{low} relative to E_{high} plants, but this trend was only marginally significant (paired $t = 1.91$, 21 d.f., $p = 0.07$).

(c) Leaf traits

Ants removed significantly more leaf area from plants with leaves that were weaker, thinner, and contained lower endophyte densities (toughness, $F_{1,56} = 14.51$, $p < 0.001$; SLA, $F_{1,56} = 41.7$, $p < 0.001$; endophyte density, $F_{1,56} = 7.19$, $p = 0.01$). These three leaf traits explained 51 per cent of the variation in leaf material removed ($r^2 = 0.509$).

4. DISCUSSION

In past studies, leaf-cutting ants have been shown to lick clean leaf surfaces [9], decrease the endophyte load in cut leaves [2] and weed unwanted fungi from their gardens [10]. Here, we report that when given a choice, *A. colombica* leaf-cutting ants prefer plants with low rather than high endophyte loads. These findings suggest that endophytes are antagonistic to the ants’ fungal symbiont, and that cutting and processing leaves with endophytes may be costly to the ants. Endophytes produce a broad array of chemicals, some of which have antagonistic activity towards bacteria and other fungi [11]. Moreover, endophytes and the chemicals they produce may be toxic or distasteful to insects [12], pathogenic to insects [13] and/or could decrease insect fitness [6]. Continued research on the impacts of endophytes on leaf-cutting ant colonies and their cultivar may reveal similar effects.

In our study, the E_{high} plants contained over five times the endophyte density of E_{low} plants, and there was no correlation between endophyte content and leaf toughness or thickness. The ants cut more than twice the amount of leaf material from E_{low} than E_{high} plants, and had a non-significant tendency to recruit more workers to the E_{low} plants. *Atta colombica* have been shown to cut more quickly from E_{low} plants than E_{high} plants [2], and it is possible that the ants are cutting their preferred resource at a faster rate. On each plant, the ants were more likely to remove material from weaker, thinner leaves, and these leaf traits explained a greater proportion of the variation

in damage than endophytes alone. However, the endophyte treatments did not differ significantly in these traits, indicating that leaf-cutting ants are not slowed or deterred solely by physical attributes.

Leaf-cutting ants use a large variety of plants from the surrounding environment. On Barro Colorado Island, *A. colombica* colonies were found to cut material from 126 plant species, although fewer were extensively attacked [7]. If endophytes significantly slow leaf-cutting ants, they could reduce defoliation of their host plants by negatively impacting a main herbivore. For plants, endophytes probably have a low cost, and if they reduce the quantity of material removed, the benefit to hosts may be substantial. Furthermore, as internal symbionts growing within their hosts, it is probable that defence mechanisms against pathogens and herbivores have evolved for endophytes to protect themselves from destruction along with host tissue. Endophytes appear to act as an acquired plant immune system: filling space and outcompeting pathogens that could otherwise attack the host while slowing or discouraging herbivores [14]. Endophytes may have a considerable influence on the composition of a tropical forest; further investigation into the biology of tropical endophytes and their interspecific interactions is necessary to fully appreciate the extent of their impact.

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