

SHORT COMMUNICATION

Endophytic fungi increase the processing rate of leaves by leaf-cutting ants (*Atta*)

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Abstract. 1. Fungal endophytes are microfungi that reside asymptotically inside of leaf tissues, increasing in density and diversity through time after leaves flush. Previous studies have suggested that the presence of fungal endophytes in the harvest material of leaf-cutting ants (*Atta colombica*, Guérin-Méneville) may negatively affect the ants and their fungal cultivar.

2. In the present study, it was tested whether the presence and diversity of fungal endophytes affected the amount of time necessary for leaf-cutter ants to cut, process, and plant leaf material in their fungal garden. It was found that ants took 30–43% longer to cut, carry, clean, and plant leaf tissue with high relative to low endophyte abundance, and that the ants responded similarly to leaf tissue with high or low endophyte diversity.

3. It was further investigated whether the fungal cultivars' colonisation rate was greater on leaf material without fungal endophytes. No difference in the ants' cultivar colonisation rate on leaf tissue with high or low endophyte abundance was observed.

Key words. *Atta colombica*, Attini, foliar endophytes, insect agriculture, symbiosis.

Introduction

Leaf-cutter ants (*Atta*; Attini) maintain an obligate symbiosis with a fungal cultivar that they tend in underground gardens. The fungal cultivar depends on the ants to bring and prepare plant material for its nourishment, and in return the cultivar produces food for the ants (Hölldobler & Wilson, 2010). Both worker ants and their fungal cultivar interact with many bacteria and microfungi during the leaf-harvesting and gardening process. In particular, several previous studies have isolated strains living inside of the ants' fungal garden that are also known to be fungal endophytes (Fisher *et al.*, 1996; Rodrigues *et al.*, 2008; Urriola *et al.*, 2011). Fungal endophytes live within above-ground plant parts without causing signs of disease (Wilson, 1995). They are abundant and diverse, especially in tropical leaves (Arnold & Lutzoni, 2007).

The interaction between leaf-cutting ants and endophytic fungi is only just beginning to be explored. Previous work

has explored the hypothesis that foliar endophytes reduce defoliation to tropical plants that host them by negatively affecting leaf-cutting ant colonies and their fungal mutualists (Van Bael *et al.*, 2009; Bittleston *et al.*, 2011; Van Bael *et al.*, 2011). When given a choice between plants with high and low endophyte densities, *Atta colombica* workers harvested nearly 50% more leaf material from plants with low relative to high endophyte densities (Bittleston *et al.*, 2011). In a separate study where workers were not given a choice, *A. colombica* workers took longer to cut and remove leaves with relatively high endophyte loads (Van Bael *et al.*, 2009). That study, however, measured only cutting rates and did not follow the ants through the entire gardening process. Here we use observations and time-lapse photography of laboratory colonies to study leaf-cutting ant behaviour and the fungal cultivar's growth in the presence and absence of endophytic fungi. Furthermore, we compare ant behaviour when their forage material contains a single strain or multiple strains of endophytes. We predicted that the cutting, cleaning, and planting time required by leaf-cutting ant workers would increase for leaves with high relative to low endophyte abundance and diversity. With respect to fungal growth, we predicted that negative fungal–fungal interactions would occur (Van Bael *et al.*, 2011), and that the

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cultivar would be slower in colonising leaf material with high relative to low endophyte abundance.

Methods

Study site and study species

All study organisms were observed or collected at the Gamboa field station (9°07'N, 79°42'W) of the Smithsonian Tropical Research Institute (STRI) in the Republic of Panama. We collected *A. colombica* Guérin-Méneville queens during their natal flight and 1-year-old colonies and brought them to the Gamboa laboratory. Laboratory colonies were kept in fluon-lined plastic containers and were used for our experiments when the colonies were between 12 and 18 months old. We collected *Merremia umbellata* L. cuttings from plants along forest borders, transplanted them in pots and grew them in growth chambers (Percival Scientific Biological Incubator Model I-36LL, Perry, Iowa, USA) to produce plants with very low fungal endophyte abundance.

Gardening rate experiments

We used laboratory colonies to test whether the abundance and diversity of foliar endophytes increased the processing time by leaf-cutting ants, with endophyte abundance and diversity tested on different colonies in two separate experiments. In each experiment, we used a within-subjects crossover design and offered each colony food with high or low endophyte treatments in consecutive trials (detailed below).

Initially, all plants were maintained with low endophyte abundance by planting cuttings in growth chambers and removing old leaves that had received endophytes from the environment. We manipulated endophyte abundance and diversity of *M. umbellata* leaves using two different techniques. First, laboratory inoculations involved growing pure cultures of *Colletotrichum tropicale* conidia in liquid medium, concentrating conidia in sterile water, and applying them to *M. umbellata* leaves (detailed in supporting information). As we only manipulated the abundance of one endophyte strain in leaves, we refer to this as the 'single strain' experiment. Second, forest inoculations involved moving a subset of potted plants from the greenhouse to the forest during the night-time only. The plants obtained the natural complement of endophyte spore fall by night, but the daytime conditions in the growth chamber remained the same for inoculated and control plants (detailed in supporting information). As the treatment resulted in a greater diversity of endophytes in leaf material, we refer to this as the 'multiple strain' experiment.

For both the single strain and multiple strain experiments, we placed 1-year-old *A. colombica* colonies ($n = 18$ colonies per experiment) in glass boxes ($\sim 10 \text{ cm}^3$) in order to be viewed or photographed without distortion (Fig. 1). For each type of experiment, we conducted our trials within individual colonies on two separate days within 3–10 days of one another. We rotated so that half of the colonies received an E_{low} leaf first and the other half received an E_{high} leaf first. Each leaf

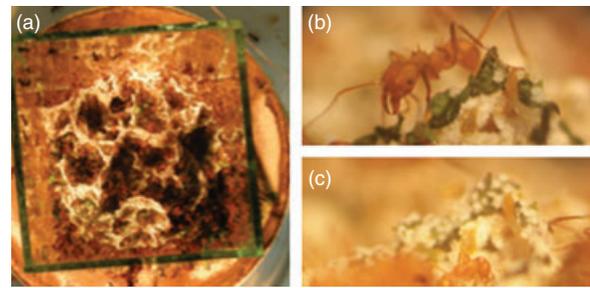


Fig. 1. Laboratory *Atta colombica* colonies were placed in clear glass boxes to observe via time-lapse photography from two angles; (a) measurements of all colony activity to calculate rates of harvesting and gardening, (b) the colonisation of freshly planted leaf material by an ant, and (c) the ants' cultivar after 3 h (the cultivar is the white material overgrowing the dark leaf material).

allotment was preceded by one starvation day, when ants were not given leaf material. The single and multiple strain experiments were conducted in two different years (2009 and 2011, respectively), with different ant colonies used for each experiment.

Each *M. umbellata* leaf was rinsed in tap water and had a small portion removed to re-isolate endophytes and assess their abundance and diversity (see details in File S1). We then scanned the leaf to measure leaf area, and presented the leaf to the colonies. We used observations and time-lapse photography to measure on a per unit leaf area basis: (i) the lapsed time between discovery of the leaf by the ants and when they began cutting it; (ii) the total time needed to cut and remove the whole leaf from the presentation area; (iii) the approximate number of workers recruited for cutting; (iv) the time duration from discovery to when the last piece was planted in the garden; and (v) for the single strain experiment only, the growth rate of fungi in a given area where new leaf material was planted. We used two cameras, one placed above to measure (i)–(iv), and one placed directly at new leaves being planted to measure (v). We further used the photographs to assess the number of workers recruited to the area where leaves were cut by ants, for the single strain experiment only. Paired t -tests (paired by colony) on \log_{10} transformed data were used throughout, as the measurements on each specific colony were not independent. According to our prediction that ants would take longer to process E_{high} leaf material, we used one-tailed t -tests. For the recruitment data, however, we used a two-tailed test, as we did not predict whether endophyte load would positively or negatively affect the number of workers recruited for cutting.

Fungal colonisation rate experiment

For the single strain experiment only, we used time-lapse photography to measure the cultivars' growth on the leaf pieces that were planted in the garden by the ants. We focussed a camera with a macro-lens on a section where leaf material had just been planted into the garden, and took one photo with a ruler for scale (Fig. 1). We then continued to take photographs every minute for 3 h, to observe how rapidly the ants' cultivar

spread over the freshly planted leaf material. We measured growth rates 30 times for 15 colonies, once for each of their E_{low} and E_{high} trials. We detail the analysis of fungal growth in the File S1.

Results and discussion

Our treatments resulted in significantly greater endophyte abundance and diversity in E_{high} relative to E_{low} leaves (detailed in File S1). *Atta colombica* colonies generally took between 2 and 4 h to cut, carry, clean, and plant one leaf from *M. umbellata*. After the colonies were presented with leaves and the ants were first observed to discover the leaf material, there was a period of ‘exploration’ when ants would pass their antennae over the leaf area before cutting. This period between discovery and cutting was similar (~9 min) for the E_{high} and E_{low} leaves in the single-strain experiment, but was significantly shorter for E_{low} leaves in the multiple-strain experiment (Table 1). In the single-strain experiment, the mean and maximum number of ants recruited to the leaf cutting area was similar for E_{high} and E_{low} leaves (mean \pm SE, $E_{\text{low}} = 12.9 \pm 1.6$, $E_{\text{high}} = 12.4 \pm 1.4$, $t_{13} = 0.27$, $P = 0.79$).

When the processing times were controlled for leaf area, we found that *A. colombica* colonies took significantly longer to cut, clean, and plant leaf material with high relative to low endophyte abundance (Table 1). This increase of time to process E_{high} leaf material was not as a result of the cutting time alone, but was evident only when the whole cutting, processing, and planting behaviour was taken into account (Table 1). In contrast, there was no significant effect of inoculation type (single vs. many strains) on harvesting and gardening rates. For leaves with single strains, the ants took 43% more time to cut, carry, clean, and plant E_{high} relative to E_{low} tissue. There was a 30% increase in time for leaves inoculated with multiple strains. While the abundance

Table 1. Cutting, preparation, and planting time for endophyte-high (E_{high}) and endophyte-low (E_{low}) leaves by *Atta colombica* laboratory colonies.

	E_{low} mean \pm SE	E_{high} mean \pm SE	Paired- <i>t</i> , d.f.	$P_{(1 \text{ tail})}$
Single strain experiment ($n = 18$ colonies)				
Time before cutting (min)	9.7 \pm 2.4	9.3 \pm 2.1	0.19,17	0.57
Cutting time (min/cm ² leaf area)	1.0 \pm 0.14	2.8 \pm 0.86	-1.6,6	0.08
Time from first cut to last plant in the garden (min/cm ² leaf area)	6.5 \pm 0.7	9.2 \pm 1.3	-2.0,17	0.033
Multiple strain experiment ($n = 18$ colonies)				
Time before cutting (min)	5.4 \pm 0.9	13.7 \pm 2.4	-2.6,17	0.01
Cutting time (min/cm ² leaf area)	3.3 \pm 0.76	4.9 \pm 1.2	-0.48,13	0.318
Time from first cut to last plant in the garden (min/cm ² leaf area)	7.1 \pm 1.0	9.2 \pm 1.4	-1.9,17	0.035

of endophytes played a significant role in how the ants processed leaf material, the ‘diversity’ of endophytes did not. This suggests that the techniques used by the ants to clean endophytes out of the leaf material are likely to be generalised rather than specific to different endophyte strains or species. The only difference between the experiments was a greater ‘exploration’ time (time lapse between discovery of the leaf and cutting it) of the ants when they were presented with E_{high} relative to E_{low} leaf material from the multiple strain experiment (Table 1).

Contrary to our prediction, the rate of new cultivar growth on leaf material planted in the garden did not differ between E_{low} and E_{high} leaf material (Fig. 1). The cultivar colonisation rate (mm²/h) was not significantly higher on E_{low} relative to E_{high} leaf material (paired $t_8 = 0.93$, $P = 0.19$). The mean \pm SE fungal colonisation rate was 0.20 ± 0.08 mm²/h on E_{low} leaves compared with 0.12 ± 0.04 mm²/h on E_{high} leaves, thus there was a trend in the direction of negative effects on cultivar growth rate. As a result of difficulties in the method (see File S1) our final sample size was small. Furthermore, we did not differentiate whether ant behaviour (more inoculation events) or the actual growth rate of the cultivar was responsible for the fungal growth patterns observed in our experiments. For example, previous work has shown that worker ants encourage early-stage fungal growth by pruning (Bass & Cherrett, 1994). We suggest that further *in vitro* and *in vivo* experiments that exclude ants are necessary to better understand the fungal–fungal interactions between endophytes and the ants’ cultivar.

For decades, researchers have investigated the factors that influence leaf-cutting ant foraging decisions, including the effects of secondary metabolites, nutritional value, leaf age, and toughness (reviewed in Van Bael *et al.*, 2011). In general, ants prefer to cut leaf tissue that is younger and not fully lignified (Cherrett, 1972; Nichols-Orians & Schultz, 1989). In nature, this is also the type of leaf tissue that is least likely to contain high abundance or diversity of fungal endophytes, because leaves obtain endophytes via sporefall after flushing (Arnold & Herre, 2003). Whether ants choose younger leaves for their ease of cutting, their lack of fungal endophytes, their nutritional quality, or all of these factors combined is difficult to assess. The results presented here suggest that the presence of endophytes may present costs to leaf-cutting ant colonies in terms of slowing down the work tempo of the colony. Further work that includes examination of overall colony development will be necessary to fully understand such costs.

Acknowledgements

We thank H. Fernández-Marín, H. Herz, and two anonymous reviewers for helpful advice. For help in the field and laboratory, we thank J. Urriola, K. Schaffer, E. Rojas, C. Estrada, T. Tascon, M. Sosa, and F. Santos. For logistical support we thank R. Urriola and A. Ruiz. This work was funded by NSF DEB-0949602 to S.A.V. and W.T.W., and SENACYT FID10-091 to S.A.V. and W.T.W., the Smithsonian Institute and the Smithsonian Tropical Research Institute. Permission

was granted to do this research by Panama's Authority on the Environment (ANAM).

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference:

10.1111/j.1365-2311.2012.01364.x

File S1

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Accepted 10 April 2012