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Limited persistence of endophytic fungi in leaf-cutting ant gardens

Persistência limitada de fungos endofíticos em jardins de formigas cortadeiras

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Abstract

Fungi that are known foliar endophytes have often been isolated from leaf-cutting ant fungal gardens. Recent *in vitro* growth trials showed that endophytic fungal growth was suppressed by the Lepiotaceous fungi cultivated by leaf-cutting ants. Here we conducted experiments with laboratory ant colonies to assess how long one strain of a common endophytic fungus persisted in the ants' fungal garden after incorporation by worker ants. We observed that after 72 hours our focal strain could no longer be cultured from the incorporated leaf material or surrounding garden tissues. Moreover, we were unable to culture our focal strain from the ants' garbage dumps. The limited persistence of an endophyte in ant fungal gardens may be due to ant hygiene behaviors and/or antagonism from the ants' fungal cultivar.

Key words: *Atta colombica*, *Colletotrichum tropicale,* endophyte, *Leucocoprinus gongylophorus,* mutualism.

Resumo

Fungos endofíticos foliares têm sido frequentemente isolados em jardins de formigas cortadeiras. Recentes observações de crescimento *in vitro* mostraram que fungos endofíticos foram suprimidos pelos fungos Lepiotaceous cultivados pelas formigas cortadeiras. Neste trabalho nós conduzimos experimentos usando colônias de formigas cultivadas em laboratório para avaliar quanto tempo a cepa de um fungo endofítico comum persistiu em jardins de fungos de formigas após a introdução de formigas operárias. Nós observamos que após 72 horas, a principal cepa introduzida não podia ser mais cultivada com material de folhas introduzidas ou com tecidos de jardins vizinhos. Também não foi possível cultivar a cepa principal a partir de materiais descartados pelas formigas. A persistência limitada de endófitos em jardins de fungos cultivados pelas formigas pode ocorrer devido ao comportamento higiênico das formigas e/ou antagonismo dos cultivares de fungos das formigas.

Palavras-chave: *Atta colombica*, *Colletotrichum tropicale,* endófito, *Leucocoprinus gongylophorus,* mutualismo.

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Introduction

Foliar endophytic fungi (hereafter "endophytes") are cryptic microorganisms that form symbiotic associations with plants, and live most of their life cycle within plant leaves and other above-ground plant tissues without causing any apparent signs of disease (Wilson, 1995). Previous work in temperate areas has demonstrated that some endophytes defend their host plants by making their leaves less palatable to insect herbivores (Clay, 1990; Wilson and Carroll, 1994; Wilson and Faeth, 2001). In contrast, tropical host-endophyte-herbivore interactions are only beginning to be studied and endophyte functional ecology in general is poorly understood in tropical plants (Herre *et al.*, 2007). Previous studies suggest that leaves are flushed endophyte-free, and that endophytes are acquired by horizontal transmission, from spores in the environment (Arnold and Herre, 2003).Endophytes can be extremely diverse in the leaves of tropical plants (Arnold *et al.*, 2000), with endophyte communities that conservatively range from 10-20 species per host plant and generally exhibit low similarity among hosts (Van Bael *et al.*, 2005; Arnold and Lutzoni, 2007). Given this diversity, coupled to high levels of tropical plant diversity, generalist herbivores, such as leaf-cutting ants, potentially interact with hundreds of foliar endophyte species.

Leaf-cutting ants maintain a symbiosis with a Lepiotaceous fungal cultivar; the ants provide their fungal cultivar with plant material as substrate, and their cultivar provides food for workers and their offspring (Hölldobler and Wilson, 1990). While the fungal cultivar has been traditionally considered a monoculture, many researchers have isolated other yeasts, bacteria and fungi from nests, and these organisms are also found inside and on the surfaces of the leaf material brought in by the ants (Fisher *et al.*, 1996; Rodrigues *et al*., 2008, Sen *et al*., 2009).Whether these microorganisms are active invaders or simply passing through probably differs for different organisms. Understanding their persistence time in the nest may yield clues to their role, if any, in the symbiosis between leaf-cutting ants and their fungal cultivar.

Endophytes may have negative effects for leaf-cutting ant colonies (Tibbets and Faeth, 1999; Van Bael *et al.*, 2009). Ants appear to actively remove some portion of the endophytes from the leaf material before planting it in their fungal gardens. Moreover, several endophyte strains showed slower growth when they were together with the ants' fungal cultivar, suggesting that the cultivar is antagonising or competing with other fungal strains (Van Bael *et al.*, 2009). Since these observations were based on *in vitro* studies on plates, we assessed endophyte persistence in live fungal gardens. We experimentally infected leaves with high densities of one endophyte strain and compared the culturability over time of our focal endophyte in leaf tissue that was or was not processed by ants.

Materials and Methods

We collected nine *Atta colombica* - *Leucocoprinus gongylophorus* colonies that were approximately one year old in Gamboa, Panama (9°06'59"N 79°42'03"W). We kept them in a Gamboa laboratory in plastic tubs and fed them oats, rice and corn in order to produce fungal gardens that were yellow and white. This allowed us to distinguish leaf material that was incorporated during our experimental trials (Figure 1).

Approximately ten days before each trial, we inoculated leaves of *Merremia umbellata* with *Colletotrichum tropicale* (Rojas *et al.*, 2010; previously called *Glomerella cingulata* in Van Bael *et al.*, 2009) by spraying on conidia in sterile water, following methods in Van Bael *et al.* (2009). This involved filtering a conidia suspension from liquid fermentation in yeast and molasses and concentrating the conidia. We added 0.5% Tween 20 to aid in conidia dispersion. Conidia concentrations of 10^7–10^8 conidia/ml were sprayed onto leaves using a Nalgene aerosol spray bottle.

At the start of each trial, we selected one large leaf, rinsed it in tap water for one minute, and cut it into four equal sections. The individual sections were used to (i) quantify the percentage of leaf area occupied by endophytic fungi (i.e. to test the inoculation method), (ii) assess the presence of spores remaining on leaf surfaces, (iii) present to the ants, so they would cut, process and incorporate the leaf material into their fungal garden, and (iv) serve as an experimental control, as leaf material that was not cut or processed by the ants. We assumed our experimental control (iv) was similar to leaf material that had been cut by the ants but left in caches near the nest opening or underground. Ants often cache leaf pieces in piles for 2-5 days before incorporating them into the fungal garden (S. Van Bael, personal observation).

We started each trial by presenting a leaf section to the ants, and we designated time zero as the moment when the first leaf piece was incorporated into the fungal garden by ants. At time zero and 4, 24, 48, 72 and 96 h afterward, we processed leaf pieces from each of the four sections described above. From each leaf section, we cut 20 2mm^2 leaf pieces or collected ant-processed pieces that were approximately 2mm^2. After processing (described below), the 20 leaf pieces were plated on one dish for each time period, so that each ant colony yielded 24 plates (one plate per leaf section, four plates per time period). The leaf pieces were processed as follows: (i) to test the inoculation method, pieces were surface sterilized for 1 min in 70% ethanol and then 10% commercial bleach. We plated the leaf pieces in 2% malt extract agar (MEA) and assessed *C. tropicale* density eight days later. (ii) To assess presence of

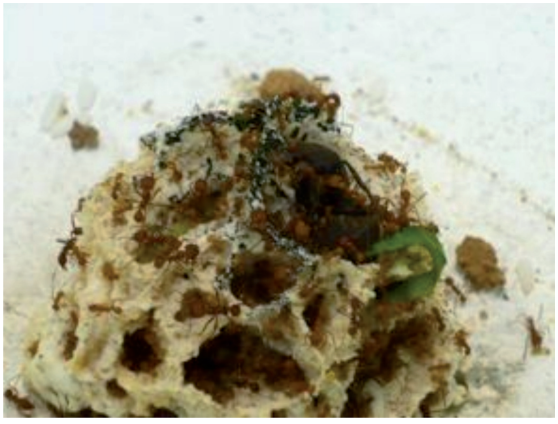

Figure 1. An *Atta colombica* laboratory colony from our experiment. By feeding the colonies oats and corn, the fungal garden was white and yellow until the ants incorporated our experimentally treated leaves. This allowed us to target the experimentally treated leaves for isolations in a time series.

spores on leaf surfaces, leaf pieces were placed on 2% MEA plates. Each leaf piece stayed on the plate for 10 minutes before it was removed using sterile forceps. We marked the site where the leaf had been pressed with a permanent marker on the plate. We assessed *C. tropicale* growth at each of the 20 sites on the plate eight days later. (iii) To quantify the effect of ant processing and garden antagonism, we removed 20 leaf pieces (each approximatley 2mm^2) from the fungal garden using sterile forceps. We plated these pieces on 2% MEA plates and assessed presence of *C. tropicale* and *L. gongylophorus* eight days later. (iv) As a control, we plated leaf pieces onto 2% MEA plates using sterile forceps, then assessed the presence of *C. tropicale* eight days later. During the 96 hours of each trial, leaf sections from (i), (ii) and (iv) above were kept in a sterile petri dish, with some sterile water droplets to maintain humidity.

At each sampling time for each colony, we took an additional sample from the ants' garbage dump. We plated 20 fresh pieces of waste dump material on 2% MEA plates and assessed *C. tropicale* growth up to eight days later. We tested the difference between treatments using a Repeated Measures analysis of variance in SYSTAT v. 11 (2004).

Results

Our inoculations were successful at introducing high densities of *C. tropicale* into leaf tissue. The mean ± standard error percentage of leaf pieces with *C. tropicale* growing endophytically was 78 ± 4% at the start of trials. At 72h, 69 ± 6% of the leaf pieces had *C. tropicale* growing endophytically. Although leaf surfaces were rinsed in tapwater, some spores of *C. tropicale* persisted on leaf surfaces; 24 ± 4%and 10 ± 2% of the leaf pieces exhibited growth from where leaf pieces were pressed on plates at 0h and 72h respectively. Thus, theants in our experiment were faced with leaf pieces that had high levels of endophyte colonization as well as some surface spores.

The leaf pieces that were processed by ants and placed in the fungal garden showed a significantly lower density of *C. tropicale* relative to those not processed by ants (Figure 2). This pattern persisted over time (Repeated measures ANOVA, treatment $F_{1,96}$ = 266, $p<0.000$; time $F_{5,96}$ = 6, $p<0.000$; treatment x time $F_{5,96}$ = 2, $p=0.05$). By 72h, we were nearly unable to culture *C. tropicale* from the leaf pieces planted in the garden. We never cultured *C. tropicale* from the ants' garbage dumps.

Discussion

We observed that one common endophyte, *C. tropicale*, did not persist past 72h after its host leaf tissue was planted in the leaf-cutting ants' garden. At the time of planting, ants decreased endophytes and surface spores by 60% relative to non-processed leaf tissue, a value that is similar to a previous experiment (Van Bael *et al.*, 2009). The exact mechanism by which the ants decreased endophyte abundance is not known, but *A. colombica* is known to use antibiotic compounds from meta-pleural glands to prepare leaf substrate before planting it in their gardens (Fernández-Marín *et al.*, 2006). While the pattern of limited endophyte persistence in the fungal garden is clear, the present experiment does not describe the mechanism. For example, once tissue is planted in the garden, endophytes could be out-competed by *L. gongylophorus*. In our study we cultured *L. gongylophorus* from our leaf pieces as early as four hours after planting in the garden, and a similar result was observed by Fisher *et al*. (1996). We cannot rule out the possibility, however, that worker ants continue to process the leaf material after it is planted, as they weed and groom the garden (Currie and Stuart, 2001). Experiments that examine ant post-planting behavior are necessary to distinguish whether one or both of these mechanisms is involved.

Previous experimental work with *Atta cephalotes* colonies also suggested that the microbial communites inside of leaf-cutting ant fungal gardens may change rapidly, with rapid turnover due to changes in the food source (Fisher *et al.*, 1996). This suggests that the results presented here on the limited persistence of one endophyte strain may extend to a broad range of endophyte species. Further work will be necessary to understand the impacts of diverse endophytes on leaf-cutting ants and their cultivar.

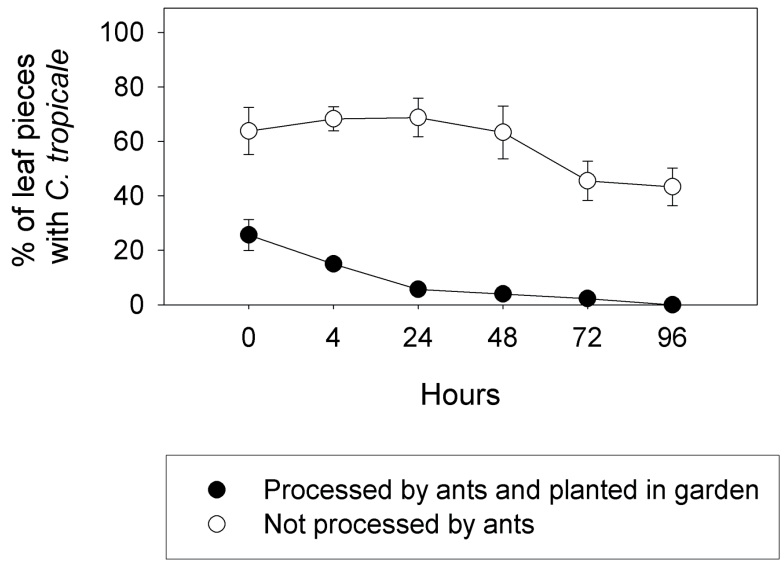

Figure 2. The persistence of the endophytic fungus, *C. tropicale* in leaf pieces that were processed or not processed by *Atta colombica* workers and planted in the fungal garden. Workers planted leaf pieces into their garden at 0h, and we removed leaf pieces from the garden and cultured them at fixed intervals afterward. This time series was performed on nine colonies.

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