Fungal Ecology 24 (2016) 15–20

Contents lists available at ScienceDirect

Fungal Ecology

journal homepage: www.elsevier.com/locate/funeco

Selective elimination of microfungi in leaf-cutting ant gardens

Kimberly Mighell [a, *], Sunshine A. Van Bael [a, b]

[a] Department of Ecology and Evolutionary Biology, Tulane University, 6823 St. Charles Avenue, New Orleans, LA, 70118, United States
[b] Smithsonian Tropical Research Institute, Apartado, 0843-03092, Panama

ARTICLE INFO

Article history:
Received 3 December 2015
Received in revised form
1 July 2016
Accepted 20 August 2016

Corresponding Editor: Peter Biedermann

Index descriptors:
Leaf-cutting ants
Endophytes
Symbiosis
Pathogens
Xylaria

ABSTRACT

Leaf-cutting ants encounter many fungi in their environment that may occur as parasites, entomopathogens, saprotrophs, or neutral/beneficial symbionts. The source of these microfungi may be the surrounding soil or the plant material brought to the nest by the ants. Whether the ants' hygienic behavior toward these microfungi is generalized or specific to different fungal species is unknown. We isolated microfungi from leaf-cutting ant gardens and forage material, and then tested the response of the worker ants to these fungal cultures. We found large variation in the rate that ants removed microfungi from their garden chamber. Some strains, including strains of the genera *Trichoderma*, *Escovopsis* and *Xylaria*, were removed at higher rates than others. Our data suggest that the worker ants moderate their behavior in a species-specific rather than generalized fashion when responding to different types of microfungi.

1. Introduction

Leaf-cutting ants (tribe: Attini) have domesticated the fungus *Leucoagaricus gongylophorus* (Basidiomycota: Agaricales: Agaricaceae) that they tend as a monoculture garden in underground chambers, superficial gardens, or within natural shelters such as rotten logs (Mueller et al., 2010; Sosa-Calvo et al., 2015). Fungiculture practiced by the genus *Atta* involves the decomposition of fresh plant substratum, which is harvested, processed and planted in a fungal garden by the ants. The fungal garden provides a reliable nutrition source, particularly for the ant larvae, and is high in proteins and carbohydrates (Quinlan and Cherrett, 1979). Leaf-cutting ants in the tropics encounter a diverse range of fungi through their natural history. Fungi are present in the soil, on and within the plant tissue they cut, and in the air as spores (Rodrigues et al., 2008; Van Bael et al., 2009). The monoculture garden fungus is subject to disease caused by non-garden fungi, and the ants are pressured to limit the presence and growth/germination of external fungi. Modifications to the leaf-cutting ants' behavior and biology include many chemical and mechanical barriers to limit this external fungal growth. These include the cleaning of leaf fragments, application of antimicrobial secretions, weeding and grooming of the garden, control of humidity in chambers, and culturing of antibiotic-producing bacteria (Currie and Stuart, 2001; Mueller et al., 2005; Pagnocca et al., 2012).

The tropics are a biodiversity hotspot for many groups, and fungal diversity follows this trend (Hawksworth, 2012). Endophytes (microfungi that live asymptomatically within plant tissue) are also believed to be hyper-diverse in the tropics (Arnold and Lutzoni, 2007). Endophytes enter the garden through the leaf fragments carried by the ants. Leaf-cutting ants have been shown to preferentially cut leaves with low levels of endophytes, increase their processing time for endophyte-rich plants, and reduce the amount of endophytes in leaf pieces after processing them to plant in their garden (Van Bael et al., 2009, 2012a; Bittleston et al., 2011). Incipient colonies fed a diet of endophyte-rich leaves grew significantly slower in their establishment period compared to colonies fed a diet of leaves with low levels of endophytes (Van Bael et al., 2012b). Likewise, leaf-cutting ant colonies infected with *Escovopsis* sp., an obligate fungal pathogen of the fungal garden of leaf-cutting ants, have slower development, with fewer workers and less garden accumulation than pathogen-free colonies (Currie, 2001). The hygienic behavioral response of the ants to *Escovopsis* spores is much more labor intensive than their response to spores of the generalist pathogen *Trichoderma* (Currie and Stuart, 2001). Leaf-cutting ants are able to detect other microfungi and react to them. Previous research found that *Acromyrmex* leaf-cutting ants exhibit strong,

* Corresponding author.
E-mail address: kimberlymighell@gmail.com (K. Mighell).

http://dx.doi.org/10.1016/j.funeco.2016.08.009
1754-5048/

negative behavioral responses to both specialist and generalist fungal pathogens in similar degrees (Tranter et al., 2014). An emerging question in attine research is how leaf-cutting ants and their garden interact with and are affected by the diversity of microbes in the environment (Mueller, 2012; Estrada et al., 2014; Rocha et al., 2014).

Although the leaf-cutting ants cultivate their mutualistic fungus as an apparent 'monoculture', a wide diversity of filamentous microfungi is commonly found inside of the garden (Rodrigues et al., 2011; Pagnocca et al., 2012). These microfungi are generally referred to as 'weeds' or 'pathogens' because they will quickly overgrow the garden if left unattended by workers. It is unknown whether leaf-cutting ants act in a specific or general manner toward the wide diversity of microfungi in their garden or toward endophytes entering in leaf forage material. We hypothesized that leaf-cutting ants would be able to differentiate among different species of microfungi, and predicted that ants would selectively remove certain groups, especially pathogens or parasites, from their garden. We tested this by isolating microfungi from ant gardens and forage material, and assessing the ants' hygienic behavior toward different species of microfungi growing in pure culture.

2. Methods

2.1. Study site

This research was conducted in the lowland tropical forest of Panama during June, July and August of 2012, within and around Gamboa, Panama. Laboratory assays were conducted at the Smithsonian Tropical Research Institute (STRI) at Gamboa, while DNA extraction and sequencing took place at STRI's molecular facilities in Panama City.

2.2. Collection of endophytes

We collected endophytes from leaf pieces that three different *Atta colombica* colonies had freshly cut and were carrying back to their nest. Two ant colonies sampled were actively foraging within Soberanía National Park, on Pipeline Road. One of these colonies was actively foraging on an *Ocotea* sp. tree, while the other was foraging on a liana growing on a *Miconia* sp. The third colony was within disturbed habitat in Gamboa and actively foraging on *Mangifera indica*. We collected eight healthy-looking leaf pieces from each trail, placed them in a sterile Petri dish and returned them to the lab. We then isolated endophytes according to established protocol (Van Bael et al., 2009). We cut the leaf pieces carried by each colony into 2 mm^2 sections, surface sterilized them for 2 min in 70% ethanol and 3 min in 10% Clorox, and then plated 80 sections per ant colony onto five 2% malt extract agar (2% MEA: 20 g of Difco Malt Extract and 20 g of Difco Agar per L of deionized water) plates with sixteen sections per plate. The endophytes were isolated into pure culture on 2% MEA plates. We grouped the endophytes according to morphotype after growth in pure culture for 10–14 d. We sequenced the commonly isolated morphotypes for putative identification and used them for the bioassay with ants.

2.3. Collection of garden microfungi

To isolate filamentous microfungi from fungal gardens, seven entire *A. colombica* colonies were collected from Gamboa, Panama. The ants were given 36 h to rebuild their garden in laboratory containers. For each colony, we sampled their garden cultivar using sterilized forceps, and plated pieces onto potato dextrose agar (PDA) plates in a sterile environment. In the following days, we isolated non-symbiont microfungi that grew out of the garden cultivar, and grew them in pure culture on PDA. One of the fungal strains, *Purpureocillium lilacinum*, was isolated from the body of a dead *A. colombica* queen of a laboratory colony. We sequenced pure cultures of all of the microfungi in the same manner as the endophytes for putative identification.

2.4. Sequencing

Freshly growing mycelium of the fungi used in this experiment was isolated for DNA extraction, amplification, and sequencing. The molecular analysis was conducted at Naos (Smithsonian Tropical Research Institute). With a Gentra Puregene Tissue Kit, we extracted DNA, and analyzed the DNA extract for purity and concentration with NanoDrop. The DNA was then amplified via PCR with ITS4 and ITS5 primers, and cleaned with ExoSAP-IT. We conducted Sanger cycle sequencing reactions in both directions with BigDye V3.1 and cleaned with BigDye X-Terminator. We proofread and edited the resulting chromatograms in Sequencher. The final sequences were identified with BLAST in GenBank and named tentatively as species or genera with at least 96% sequence similarity. Only one strain (*Xylaria* sp. 2) was identified solely by morphology, as the PCR resulted in low amplification. After identification, we categorized the garden contaminants based on how previous ecological studies of how the same genera and/or species interact with leaf-cutting ants. On this merit, we classified the fungi as saprotrophs or parasites. All fungi isolated from healthy leaf pieces were classified as endophytes, with unknown ecology within leaf-cutting ant nests. All sequences were submitted to the NCBI GenBank with accession numbers KX766626-KX766639. All cultures are preserved in glycerol vouchers and are available from the authors upon request.

2.5. Creation of micro-colonies

We created micro-colonies from 15 young (1–3 y old) *A. colombica* colonies found in the same habitat in Gamboa. We excavated partial colonies, including collection of garden cultivar, workers (minims, minor, mediae), larvae, and trash from the rubbish pile. The colonies were allowed to settle in the laboratory and rebuild the garden for 24–48 h. After this period, we subsampled garden and ants from each colony to make six micro-colonies from each colony. These micro-colonies were constructed in a four-chambered Petri dish (Fig. 1). One chamber received a spoon full of garden cultivar, with larvae, minima, minor and mediae ants. Topical assessment ensured that each micro-colony received 25 to 30 ants. The adjacent chambers included one section with a water/humidity source (an Eppendorf tube with the top removed and filled with water and cotton) and another chamber that was left empty. The section directly across from the garden section received a small spoon full of the ants' trash pile. We allowed the ants 1 h to adjust to their new environment and to correct minor damages sustained by the garden during its relocation.

2.6. Bioassays

We tested every putative fungal species once with a newly collected colony for a total of 15 bioassay trials. Each colony was divided into six replicates, or micro-colonies. Within one colony, three of the micro-colonies received a plug of agar with replicates of the same fungal species, and three received a plug of agar without any fungus. Each fungal species and fungal-free control was introduced to a unique, subdivided ant colony. The sterile lids of microtubes served as platforms for moving plugs of agar and fungi. The garden-isolated fungi were re-grown on 2% MEA to

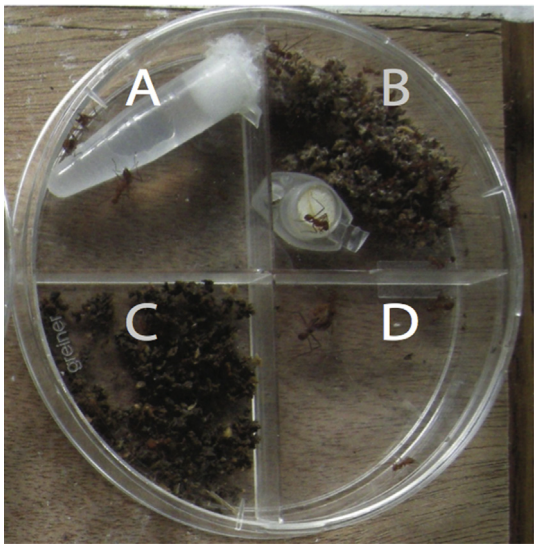

Fig. 1. Each micro-colony was constructed in a four-chambered Petri dish. Chamber A contained a water source, chamber B contained garden fungus and larvae, chamber C contained a sample of the ants' trash, and chamber D was left empty. The plug of filamentous fungus or the control plug was placed in the garden chamber (B). This micro-colony was replicated six times for each colony, with three replicates receiving a fungal plug of one strain and three receiving a control plug.

control for media type in the bioassays. We cut the plugs from the border of an actively growing fungus in pure culture in 2% MEA, or from pure 2% MEA without fungi (control). The mass of the cap was measured before and after the addition of the plug. The cap and plug, with mycelia on the top, were introduced to the colonies and ants were allowed to interact with the fungi or control plugs. Then we removed and measured the cap and plug mass after 1 h, and after 3 h since the beginning of the bioassay. Some weight loss was expected to have occurred via evaporation, but the use of the control plug mitigates this effect. Time-lapse photography captured the presence of the ants in each micro-colony at intervals of 1 min for the first hour, and intervals of 5 min for the ensuing 2 h. Ants' actions were observed throughout the experiment. Ant response to the external filamentous fungi was recorded as % weight loss of the plug due to ants physically cutting the fungal plug and carrying the pieces to their trash pile.

2.7. Statistical analysis

The % weight loss of the plugs from each micro-colony after 3 h was used to determine the effect size of each fungal strain on the ants' removal behavior. Glass's Δ was calculated as "Glass's $\Delta = \frac{\bar{\mu}_{Fungi} - \bar{\mu}_{Control}}{\sigma_{Control}}$", giving each fungal strain a single effect size variable. Glass's Δ was calculated as the mean weight loss of the fungal plugs ($\bar{\mu}_{Fungi}$) minus the mean weight loss of the control plugs ($\bar{\mu}_{Control}$) divided by the standard deviation of the control ($\sigma_{Control}$). A high effect size signified a lot of fungal removal by the ants, while a low effect size occurred when the ants did not remove the strain. We used Glass's Δ for effect size since the control group was representative of the ant colony used in all six bioassays per colony. A Mann-Whitney U tested whether the effect size differed by isolation source for that strain (fungal garden/dead queen or leaf pieces entering the garden). This test aimed to see whether the ants' responses toward filamentous microfungi (removal effect sizes) were general or specific to the isolation source of the microfungi.

3. Results

3.1. Isolation of endophytes and garden contaminants

We isolated over 51 fungal endophytes from *M. indica*, 75 endophytes from the liana, and 89 endophytes from the *Ocotea* sp. These were collectively grouped into 76 morphotypes, and the eight most abundant morphotypes (10.5% of the total morphotypes, representing 22% [n = 47] of the total isolated endophytes) were used in the bioassay. We isolated 35 different strains of garden contaminants, which were grouped into seven morphotypes. Every morphotype isolated as a garden contaminant was used in the bioassay, although one fungus was excluded from the analysis due to a distinct hygienic behavior observed (see discussion). There were no fungi that appeared as both endophytes and garden contaminants in this study.

3.2. Bioassays

The ants in the bioassays removed at least some part of every fungal plug and placed the removed pieces in the garbage section. In the act of removing agar plugs, the ants used their mandibles to cut away the agar and fungus, sometimes stopping to clean themselves with their metapleural glands in the process. After one piece of the agar/fungus was cut, the ants carried the segment to the trash section in their mandibles. Before returning to the agar plug, the ants stopped at least once to clean themselves. If returning to the fungal garden rather than the nest, the ants often cleaned themselves multiple times. This behavior was observed for all microcolonies for which Glass's Δ was used. The average weight removed for all fungal plugs was 0.0976 ± 0.0163 g (mean ± standard error). When converted to percent of the plug removed, the ants removed an average of 58.75 ± 10.22% of all of the fungal plugs. While the ants mostly ignored the control plugs, there was a small degree of removal, often occurring when the plug was initially placed within the microcolony. The average weight removed from control plugs was 0.0163 ± 0.0031 g. When converted to percent of plug removed, this represented 7.69 ± 1.37%. All of the fungal strains except one (*Syncephalastrum*) had a positive effect size, meaning that the ants removed more of the fungus than the control without fungus. Although the spores were partially scraped off the plugs of *Syncephalastrum* sp., the ants did not remove much agar, and the control ants of this colony partially removed the agar plugs. When looked at individually, the fourteen fungi had a range of removal effect sizes (Table 1). Comparing the removal effect size based on isolation source yielded no significant differences between the fungi isolated from leaves versus the fungi isolated from ant fungal garden/colony (Mann Whitney U: df = 1, $p = 0.775$). This seems to be driven by the variation of effect size within the endophytic and garden contaminants category. From within the garden contaminants, the parasitic species had higher effect size than the saprotrophs. Roughly half of the endophytic fungi elicited strong responses from the ants comparable to parasitic strains, and half of the endophytic fungi elicited low-level responses comparable with saprotrophic fungi.

4. Discussion

In the past, *Escovopsis* has been stressed as a major evolutionary selective pressure on leaf-cutting ants' hygienic behavior and an ecological pressure on colony success (Currie et al., 1999; Currie, 2001; Currie and Stuart, 2001; Little et al., 2006). However, recent evidence supports the hypothesis that other types of external filamentous fungi, including endophytes, can significantly alter ant hygienic behavior and influence colony success (Carlos et al., 2009;

Table 1
Removal rates and effect sizes for fungal cultures assayed. A high effect size signified a lot of fungal removal by the ants while a low effect size occurred when the ants did not remove the strain.

Putative fungal identification	Mean % loss of fungal plug ± SE	Mean % loss of control plug ± SE	Glass's Δ	Isolation source	Lifestyle in ant nests from literature
Trichoderma harzianum species complex	65.85 ± 20.34	5.09 ± 0.20	178.19	Fungal garden	Known mycoparasite[a]
Xylaria sp. 1	99.90 ± 0.14	4.81 ± 0.41	132.86	Leaf	Unknown
Xylaria sp. 2*	99.40 ± 0.22	1.85 ± 0.43	130.71	Leaf	Unknown
Pestalotiopsis microspora	100.09 ± 0.02	7.91 ± 0.90	58.96	Leaf	Unknown (But see Reis et al., 2015)
Escovopsis sp.	35.10 ± 5.97	8.45 ± 0.32	48.61	Fungal garden	Known mycoparasite[a]
Hypoxylon stygium	83.25 ± 16.88	5.03 ± 1.03	43.97	Leaf	Unknown
Purpureocillium lilacinum	100.13 ± 1.66	3.66 ± 2.60	21.46	Dead *Atta* queen	Entomopathogen[b]
Bionectria ochroleuca	18.75 ± 3.91	6.41 ± 0.67	10.56	Fungal garden	Saprotroph[c]
Colletotrichum gloeosporioides species complex	25.58 ± 10.24	6.07 ± 1.84	6.11	Leaf	Unknown
Endomelanconiopsis endophytica	78.02 ± 21.67	17.30 ± 5.84	6.00	Leaf	Unknown
Xylaria adscendens	17.44 ± 5.48	7.53 ± 1.81	3.16	Leaf	Unknown
Stenella queenslandica	86.42 ± 13.77	20.63 ± 13.77	3.16	Leaf	Unknown
Rhizomucor variabilis	5.67 ± 0.79	5.57 ± 1.05	0.06	Fungal garden	Saprotroph[d]
Syncephalastrum sp.	6.94 ± 0.33	7.37 ± 0.72	−0.34	Fungal garden	Saprotroph[e]

*This species was identified by morphology only.
[a] (Currie and Stuart, 2001).
[b] (Rodrigues et al., 2010).
[c] (Freinkman et al., 2009).
[d] (Lauer et al., 2008; Patil et al., 2013).
[e] (Rodrigues et al., 2005).

Van Bael et al., 2009, 2012a, 2012b). Likewise, we know that leaf-cutting ants' gardens harbor a wide diversity of filamentous fungi. Many of these fungi are believed to act as opportunistic antagonists (nutritional competitors) in the leaf-cutting ant system yet some may have unknown functions within the symbiosis (Rodrigues et al., 2005, 2008; Pagnocca et al., 2012). With certain microfungi, growth can be limited through spot treatment with metapleural gland secretion, to which some fungal hyphae and spores are sensitive (Bot et al., 2002). 'Quality control' of leaves entering the colony may limit establishment of select endophytes in the garden as well, and *Atta laevigata* selectively rejects leaves with certain endophytic taxa more than leaves with other endophytic taxa (Rocha et al., 2014). The attine biofilm is composed of a taxonomically diverse assemblage of actinomycetes, with generalized, broad-spectrum antibiotic activities, supporting the idea that the ant microbiome defends against an array of pathogens (Mueller, 2012). Although saprotrophs and parasites that enter into the leaf-cutting ant symbiosis show no evidence of being as specialized as *Escovopsis* parasites, leaf-cutting ants are certainly aware of external fungi and take precautions to control the fungal diversity within their garden.

Three of these fungi, *Escovopsis* sp., *P. lilacinum*, and *Trichoderma harzianum* species complex, were classified as pathogens from reports in the literature. *Escovopsis* is a specialist parasite of the garden cultivar and *T. harzianum* is considered a general parasite (Currie and Stuart, 2001). The genus *Purpureocillium* contains entomopathogenic species which have been demonstrated to be vertically transmitted in *Atta* colonies via reproductive ants (Rodrigues et al., 2010). Three were classified as saprotrophs (weeds): *Bionectria ochroleuca*, *Rhizomucor variabilis*, and *Syncephalastrum* sp. Previously, *Syncephalastrum racemosum* has been considered a weed in some leaf-cutting ant nests, particularly laboratory nests (Rodrigues et al., 2009; Sen et al., 2009). *S. racemosum* may not be a disease-causing agent in field attine nests, but more often establishes in laboratory nests (Rodrigues et al., 2005). We, therefore, categorized our *Syncephalastrum* sp. as a saprotroph. *R. variabilis* has been identified as a potential human pathogen and has been isolated from dead salamander eggs (Lauer et al., 2008; Patil et al., 2013), but has never been identified as a pathogen of fungi or insects. *B. ochroleuca* has been isolated from *A. colombica* fungal gardens previously (Van Bael et al., 2012a), and *Bionectria* sp. have been isolated from other fungus farming ants *Apterostigma dentigerum* (Freinkman et al., 2009). It has also been isolated as an endophyte and from soil (Guesmi-Jouini et al., 2014), and it has been observed as an entomopathogen in Cicadellidae (Hemiptera) (Toledo et al., 2006). However, it has never been shown to act as a parasite on leaf-cutting ants or their garden. One of these endophytes assayed, *Pestalotiopsis microspora*, has been isolated from *Atta cephalotes* fungal gardens, where its lifestyle is unknown (Reis et al., 2015). This supports the idea that endophytic fungi can establish as contaminant fungi within the fungal garden of leaf-cutting ants.

Our result that the removal effect size of some endophytic fungi (*Xylaria* sp. 1, *Xylaria* sp. 2, and *P. microspora*) was greater than the effect size of the well-studied parasite *Escovopsis* supports the idea that ant hygienic behavior is directed towards many different fungi and not solely driven by one species. Our comparison among isolation groups showed no significant difference in removal effect sizes. More information on the lifestyle of the endophytes within the leaf-cutting nest will allow us to study the effect of ecological groups on ant behavior. We were able to show that within isolation groups there are a variety of effect sizes. This suggests that ants respond to cues of each fungus differently. The measure of removal effect size for the different fungi could serve as an indicator for potential antagonists to the garden or the ants.

We excluded *Penicillium* from the analysis because of a unique behavior that was observed. The ants initially attempted to cut the *Penicillium*, but were perturbed by the abundance of conidia, which coated their heads and antennae. They then proceeded to cut adjacent pieces of the garden and roll them on top of the *Penicillium* plug. When the garden fragment was coated in conidia, they carried the fragment to the trash pile, cleaned themselves thoroughly with metapleural gland secretions, and repeated the process until all of the conidia from the plug were gone. This observation shows that ants are adept at dealing with diverse fungal structures and have unique behavioral responses.

Recent work has shown that endophytes can alter leaf chemistry and influence leaf-cutting ants' preferences (Estrada et al., 2013). However, we were able to show that ants responded to endophytes in pure culture, demonstrating that the ants are also using cues

from the fungus itself. We observed that all of the pathogens had relatively high rates of removal, while the saprotrophs had relatively low rates of removal. However, endophytes were roughly split between the high and low removal rates. Therefore, we hypothesize that some of the endophytes may serve an ecological role similar to those of pathogens or saprotrophs. What happens to the fungi that are not removed fully by the ants, such as *Syncephalastrum* sp.? It may be that these fungi are indeed parasitic, but have made themselves less detectable to the ants, and therefore elicit lower response rates; or they may pose little harm to the garden and are not deemed a priority by the ants. Possibly, these fungi are opportunistic antagonists with which the ants are unfamiliar or are somehow not detectable by the ants. The lower response rate of the ants to *Escovopsis* than to *Trichoderma* may be explained by increased processing time of the *Escovopsis* spores or the ability of *Escovopsis* to be poorly detected by the ants. It should be noted that the sub-colonies are also sub-gardens, and may not capture all of the players of the multi-partite interactions that happen in an intact, functioning leaf-cutting ant garden. We may be able to assign putative life style labels to the endophytic fungi we assayed based on the strength of the ant's removal effect size and future fitness experiments.

An interesting question is why certain endophytic fungi (such as two *Xylaria* sp.) elicit such strong removal rates by the ants. *Xylaria* is a cosmopolitan genus, with members found as antagonists in fungal-farming termite colonies (Visser et al., 2011). *Xylaria* as endophytes could fill a similar ecological niche in the leaf-cutting ant system. While endophytes are often cited as commensal or mutualistic symbionts within plants, host genotype and environment are vital to shaping the interaction (Hardoim et al., 2015). In fact, we know that many endophytes have the potential to be plant pathogens, or are descended from plant or insect pathogens (Rodriguez et al., 2009). However, endophytes are essential elements of plant defense against pathogens, sometimes through direct inhibition of pathogenic fungal growth (Hardoim et al., 2015). It seems likely that, as in plant tissue, endophytes play a complex role in the plant fungal gardens, with certain strains acting as antagonists, and displaying a range of symbiotic lifestyles. As the ants mediate this interaction of garden fungi and endophytes, they are an essential element in directing these symbioses and filtering out antagonists. Future studies should focus on the role that endophytes may play in the garden of leaf-cutting ants, what cues the ants are detecting from the endophytes, and how certain fungal strains affect colony success.

Acknowledgments

We would like to thank Enith Rojas, Jannette Fabiola Santos, and Kyle Coblentz for their help conducting this research. Funding was provided by Tulane University's School of Science and Engineering and NSF DEB-0949602.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funeco.2016.08.009.

References

Arnold, A.E., Lutzoni, F., 2007. Diversity and host range of foliar fungal endophytes: are tropical leaves biodiversity hotspots? Ecology 88, 541–549.
Bittleston, L.S., Brockmann, F., Wcislo, W., Bael, Van, a, S., 2011. Endophytic fungi reduce leaf-cutting ant damage to seedlings. Biol. Lett. 7, 30–32.
Bot, A.N.M., Ortius-Lechner, D., Finster, K., Maile, R., Boomsma, J.J., 2002. Variable sensitivity of fungi and bacteria to compounds produced by the metapleural glands of leaf-cutting ants. Insectes Sociaux 49, 363–370.
Carlos, A.A., Forti, L.C., Rodrigues, A., Verza, S.S., Nagamoto, N.S., Camargo, R.S., 2009. Influence of fungal contamination on substrate carrying by the leafcutter Ant *Atta sexdens rubropilosa* (Hymenoptera: Formicidae). Sociobiology 53, 785–794.
Currie, C.R., 2001. Prevalence and impact of a virulent parasite on a tripartite mutualism. Oecologia 128, 99–106.
Currie, C.R., Scott, J.A., Summerbell, R.C., Malloch, D., 1999. Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Lett. Nat. 398, 701–705.
Currie, C.R., Stuart, A.E., 2001. Weeding and grooming of pathogens in agriculture by ants. Proc. R. Soc. 268, 1033–1039.
Estrada, C., Rojas, E.I., Wcislo, W.T., Van Bael, S.A., 2014. Fungal endophyte effects on leaf chemistry alter the in vitro growth rates of leaf-cutting ants' fungal mutualist, *Leucocoprinus gongylophorus*. Fungal Ecol. 8, 37–45.
Estrada, C., Wcislo, W.T., Van Bael, S.A., 2013. Symbiotic fungi alter plant chemistry that discourages leaf-cutting ants. New Phytol. 198, 241–251.
Freinkman, E., Oh, D.-C., Scott, J.J., Currie, C.R., Clardy, J., 2009. Bionectriol A, a polyketide glycoside from the fungus Bionectria sp. associated with the fungus-growing ant, *Apterostigma dentigerum*. Tetrahedron Lett. 50, 6834–6837.
Guesmi-Jouini, J., Garrido-Jurado, I., Lopez-Diaz, C., Ben Halima-Kamel, M., Quesada-Moraga, E., 2014. Establishment of fungal entomopathogens *Beauveria bassiana* and *Bionectria ochroleuca* (Ascomycota: Hypocreales) as endophytes on artichoke *Cynara scolymus*. J. Invertebr. Pathol. 119, 1–4.
Hardoim, P.R., van Overbeek, L.S., Berg, G., Pirttilä, A.M., Compant, S., Campisano, A., Döring, M., Sessitsch, A., 2015. The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiol. Mol. Biol. Rev. 79, 293–320.
Hawksworth, D.L., 2012. Global species numbers of fungi: are tropical studies and molecular approaches contributing to a more robust estimate? Biodivers. Conserv. 21, 2425–2433.
Lauer, A., Simon, M.A., Banning, J.L., Lam, B. a, Harris, R.N., 2008. Diversity of cutaneous bacteria with antifungal activity isolated from female four-toed salamanders. ISME J. 2, 145–157.
Little, A.E.F., Murakami, T., Mueller, U.G., Currie, C.R., 2006. Defending against parasites: fungus-growing ants combine specialized behaviours and microbial symbionts to protect their fungus gardens. Biol. Lett. 2, 12–16.
Mueller, U.G., 2012. Symbiont recruitment versus ant-symbiont co-evolution in the attine ant − microbe symbiosis. Curr. Opin. Microbiol. 15, 269–277.
Mueller, U.G., Gerardo, N.M., Aanen, D.K., Six, D.L., Schultz, T.R., 2005. The evolution of agriculture in insects. Annu. Rev. Ecol. Evol. Syst. 36, 563–595.
Mueller, U.G., Scott, J.J., Ishak, H.D., Cooper, M., Rodrigues, A., 2010. Monoculture of leafcutter ant gardens. PLoS One 5, 3–9.
Pagnocca, F.C., Masiulionis, V.E., Rodrigues, A., 2012. Specialized fungal parasites and opportunistic fungi in gardens of attine ants. Psyche A J. Entomol. 2012, 1–9.
Patil, A., Chandramohan, K., Shivaprakash, M., Nadgir, S., Lakshminarayana, S., 2013. *Rhizomucor variabilis*: a rare causative agent of primary cutaneous zygomycosis. Indian J. Med. Microbiol. 31, 298.
Quinlan, R.J., Cherrett, J.M., 1979. The role of fungus in the diet of the leaf-cutting ant *Atta cephalotes* (L.). Ecol. Entomol. 4, 151–160.
Reis, B.M.D.S., Silva, A., Alvarez, M.R., de Oliveira, T.B., Rodrigues, A., 2015. Fungal communities in gardens of the leafcutter ant *Atta cephalotes* in forest and cabruca agrosystems of southern Bahia State (Brazil). Fungal Biol. 119, 1170–1178.
Rocha, S.L., Jorge, V.L., Della Lucia, T.M.C., Barreto, R.W., Evans, H.C., Elliot, S.L., 2014. Quality control by leaf-cutting ants: evidence from communities of endophytic fungi in foraged and rejected vegetation. Arthropod. Plant. Interact. 8, 485–493.
Rodrigues, A., Silva, a., Bacci Jr., M., Forti, L.C., Pagnocca, F.C., 2010. Filamentous fungi found on foundress queens of leaf-cutting ants (Hymenoptera: Formicidae). J. Appl. Entomol. 134, 342–345.
Rodrigues, A., Bacci, M., Mueller, U.G., Ortiz, A., Pagnocca, F.C., 2008. Microfungal "weeds" in the leafcutter ant symbiosis. Microb. Ecol. 56, 604–614.
Rodrigues, A., Cable, R.N., Mueller, U.G., Bacci, M., Pagnocca, F.C., 2009. Antagonistic interactions between garden yeasts and microfungal garden pathogens of leaf-cutting ants. Ant. Van Leeuwenhoek 96, 331–342.
Rodrigues, A., Mueller, U.G., Ishak, H.D., Bacci, M., Pagnocca, F.C., 2011. Ecology of microfungal communities in gardens of fungus-growing ants (Hymenoptera: Formicidae): a year-long survey of three species of attine ants in Central Texas. FEMS Microbiol. Ecol. 78, 244–255.
Rodrigues, A., Pagnocca, F.C., Bacci Jr., M., Hebling, M.J.A., Bueno, O.C., Pfenning, L.H., 2005. Variability of non-mutualistic filamentous fungi associated with *Atta sexdens rubropilosa* nests. Folia Microbiol. (Praha) 50, 421–425.
Rodriguez, R.J., White Jr., J.F., Arnold, A.E., Redman, R.S., 2009. Fungal endophytes: diversity and functional roles. New Phytol. 182, 314–330.
Sen, R., Ishak, H.D., Estrada, D., Dowd, S.E., Hong, E., Mueller, U.G., 2009. Generalized antifungal activity and 454-screening of *Pseudonocardia* and *Amycolatopsis* bacteria in nests of fungus-growing ants. Proc. Natl. Acad. Sci. U. S. A. 106, 17805–17810.
Sosa-Calvo, J., Jesovnik, A., Okonski, E., Schultz, T.R., 2015. Locating, collecting, and maintaining colonies of fungus-farming ants (Hymenoptera: Formicidae: Myrmicinae: Attini). Sociobiology 62, 300–320.
Toledo, a.V., Virla, E., Humber, R. a., Paradell, S.L., Lastra, C.C.L., 2006. First record of *Clonostachys rosea* (Ascomycota: Hypocreales) as an entomopathogenic fungus of *Oncometopia tucumana* and *Sonesimia grossa* (Hemiptera: Cicadellidae) in Argentina. J. Invertebr. Pathol. 92, 7–10.
Tranter, C., LeFevre, L., Evison, S.E.F., Hughes, W.O.H., 2014. Threat detection:

contextual recognition and response to parasites by ants. Behav. Ecol. 1–10.
Van Bael, S.A., Estrada, C., Rehner, S.A., Santos, J.F., Wcislo, W.T., 2012a. Leaf endophyte load influences fungal garden development in leaf-cutting ants. BMC Ecol. 12.
Van Bael, S.A., Seid, M., Wcislo, W., 2012b. Endophytic fungi increase the processing rate of leaves by leaf-cutting ants (*Atta*). Ecol. Entomol. 37, 318–321.
Van Bael, S.A., Fernández-Marín, H., Valencia, M.C., Rojas, E.I., Wcislo, W.T., Herre, E.A., 2009. Two fungal symbioses collide: endophytic fungi are not welcome in leaf-cutting ant gardens. Proc. Biol. Sci. 276, 2419–2426.
Visser, A.A., Kooij, P.W., Debets, A.J.M., Kuyper, T.W., Aanen, D.K., 2011. *Pseudoxylaria* as stowaway of the fungus-growing termite nest: interaction asymmetry between Pseudoxylaria, Termitomyces and free-living relatives. Fungal Ecol. 4, 322–332.