

## Field colonies of leaf-cutting ants select plant materials containing low abundances of endophytic fungi

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**Abstract.** Endophytic fungi live symbiotically in the tissues of plants. Although a large amount of evidence suggests a mutualistic role for vertically transmitted endophytic fungi in agronomic grasses, the role of horizontally transmitted endophytic fungi as mutualists has been challenged. Recent studies, however, have shown that horizontally transmitted endophytic fungi can act as mutualists to their plant hosts by providing defense against pathogens and defoliators. In particular, several experimental studies have shown that endophytic fungi interact negatively with leaf-cutting ants and their fungal cultivar, but these studies were performed under laboratory conditions. Using field colonies of *Atta colombica* in Central Panama, we measured the fungal endophyte content in the forage material of leaf-cutting ants and compared it to ambient endophyte levels. We then used the collected data to model the area of plant material containing endophytes that enters a mature colony daily. We found that mature colonies collect leaf material that is 20% lower in endophyte abundance than surrounding leaves. A similar pattern was observed for newly emerged colonies. Our model suggests that via ant foraging preferences, leaf-cutting ants reduce the possible area of material containing endophytes entering the colony by 33%. Our results provide further evidence for a negative interaction between leaf-cutting ants and horizontally transmitted endophytes, suggesting that fungal endophytes have a defensive role in tropical plants by influencing leaf-cutting ant foraging preferences.

**Key words:** *Atta colombica*; fungal endophytes; interactions; mutualism; Neotropics; Panama; symbiosis.

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### INTRODUCTION

A pervasive symbiosis in terrestrial environments occurs between foliar endophytic fungi (hereafter, 'endophytes') and plants. Endophytes are fungi that spend most or part of their life cycle inside plant tissues without causing symptoms of disease (Wilson 1995a). An ever growing body of evidence suggests that these fungal endophytes are found in all plants and are abundant and diverse in plant tissues (Arnold et al. 2000, Arnold and Lutzoni 2007). The role

that endophytes play in plant ecology is still a matter of debate, and endophytes may have a wide-variety of relationships with host plants ranging from detrimental to beneficial (for review see Saikkonen et al. 1998).

Most studies of endophytes as plant mutualists have been performed on grasses in the temperate zone. Evidence from agronomic grass species suggests that endophytes increase plant vigor and provide a defense against herbivory (for review see Clay 1988), although this concept has been challenged in native grasses (Faeth 2002).

For instance, endophytes are responsible for some toxic syndromes in cattle and have been found to reduce leaf palatability for invertebrate herbivores in tall fescue and perennial ryegrass (e.g., Bacon et al. 1977, Rowan and Gaynor 1986, Prestidge and Gallagher 1988, Breen 1994), but studies on native grass species have shown negative, neutral, and positive effects on herbivores (Tibbets and Faeth 1999, Saikkonen et al. 1999, Koh and Hik 2007). The most widely studied endophytes, those in tall fescue and perennial ryegrass, are systemic, vertically transmitted (from mother to seed), and therefore are predicted to form strong mutualisms with their hosts. The majority of endophytes, however, are horizontally transmitted (through spore rain) and are predicted to have either a neutral, slight positive, or slight negative interaction with their plant hosts (Saikkonen et al. 1998).

Contrary to this prediction, studies have shown beneficial effects of horizontally transmitted endophytes in host plants. Horizontally transmitted endophytes have been shown to increase host plant resistance to disease (Redman et al. 2001, Arnold et al. 2003, Mejía et al. 2008), and other studies have suggested that horizontally transmitted endophytes may also provide resistance to herbivory for their hosts. Various studies on gall forming insects have shown that the insects prefer to oviposit on leaf areas that do not contain endophytes (Wilson 1995b, Wilson and Carroll 1997, Wilson and Faeth 2001). Van Bael et al. (2009) found decreased reproductive success in a beetle fed leaf material with a high abundance of endophytes. This was the first experimental study on endophytes as a defense against herbivory in the tropics where endophytes are especially diverse and abundant in plant tissue (Arnold and Lutzoni 2007).

Recently, several studies have explored the interactions between horizontally transmitted endophytes in plant tissue and leaf-cutting ants. Leaf-cutting ants (genera *Acromyrmex* and *Atta*) are major defoliators in the Neotropics, removing at least 2% of annual leaf production and impacting the distribution of resources in habitats where they occur (Farji-Brener 2001, Herz et al. 2007a, Corrêa et al. 2010). The ants collect tissue from a diverse group of plants and therefore interact with a vast number of endophytes. The ants are not technically herbivores,

but rather the ants use plant material as a substrate on which they grow a fungal cultivar. The fungal cultivar in turn provides the ants and their larvae with a food source (Mueller et al. 2005). Van Bael et al. (2009) showed that (1) leaf-cutting ants spend more time cutting leaves with high endophyte abundances relative to leaves with low endophyte abundances, (2) workers reduce the amount of endophytes in leaves before planting them in the fungal garden, and (3) some endophytes show decreased growth rates when grown in culture with the ants' fungal cultivar. Furthermore, Bittleston et al. (2010) showed that ants cut more leaf area from seedlings with low endophyte abundances relative to seedlings with high endophyte abundances. There is also evidence that suggests colonies newly emerging after the queen's natal flight may have slower growth rates when fed high endophyte diets (Van Bael et al. 2012). Because emergent colonies have relatively few workers to assist with nest and cultivar contamination, they may be more susceptible to antagonistic effects of endophytes than mature colonies. These results further suggest that horizontally transmitted tropical endophytes serve a role in protecting tropical plants from leaf-cutting ant defoliation, although all of these studies have been performed in the laboratory.

Here we test the hypothesis that field leaf-cutting ant colonies select leaves containing lower abundances of endophytes than surrounding leaves. We also test the hypothesis that incipient colonies just emerging from the queen's natal flight collect leaf material with lower abundances of endophytes than mature ant colonies. Lastly, we model the amount of plant material containing endophytes that enters a mature ant colony per day under conditions measured in the field during the beginning of the wet season in Panama.

## MATERIALS AND METHODS

### Study sites

We sampled *Atta colombica* colonies from the village of Gamboa, Ancon, Panama (9°17' N, 79°7' W) and the Pipeline Road region of Soberania National Park, Panama (9°7' N, 79°42' W) from June to July 2012, the beginning of the wet season. The sites are characterized by moist

lowland tropical rainforest and are primarily secondary growth. Colonies sampled from Gamboa were within village limits and were characterized by high levels of human disturbance, while those from Pipeline Road were in forested areas with relatively little disturbance.

### Field methods

*Mature colonies.*—We sampled 24 mature colonies (12 in Gamboa and 12 on Pipeline Road). We define mature colonies as those that have multiple entrances and cover an area greater than 1 m<sup>2</sup>. The colonies sampled covered a size range from approximately 2 m<sup>2</sup> to 10 m<sup>2</sup>. Each sampling consisted of data collection on rates of refuse deposition, types of materials being carried by ants, and a collection of ant-carried material to estimate endophyte abundance. To determine the rate of refuse deposition, we counted the rate at which ants carrying refuse left the exit hole of the trail leading to the refuse pile for five minutes following the methods of Herz et al. (2007a). We also performed two five minute surveys of foraging trails entering the colony to determine the proportion of leaves, stipules, flowers, and fruits the colony was collecting. To assess endophyte abundance in ant-carried material, we followed one foraging trail with ants carrying leaf fragments to the source of the leaf material and collected 20 leaf fragments directly from the ants as they descended from the canopy. We sampled leaf fragments near one focal source in order to compare ant-collected leaves with nearby ambient leaves (see below). Further, we collected 5–20 fragments of each non-leaf material (e.g., flowers, fruits, leaf stipules) being carried by removing them from the ants just before they reached the colony entrance. The colony entrance was used because non-leaf material was very rare (Table 1) and could only be sampled in sufficient numbers at the colony entrance. Finally, we collected canopy-height leaves from within 10m of the source of the leaf fragments being collected by ants (hereafter, ‘ambient leaves’), using a rock propelled by a slingshot or thrown by hand. We collected 10–20 ambient leaves from one to three tree species. From the 10–20 leaves collected, we selected the three leaves that most closely resembled those collected by the ants in toughness and age. We continued to sample leaves

until we were satisfied that our ambient collected leaves were a similar age and toughness to the leaves which the ants were collecting. Since we sampled in the beginning of the wet season when most trees were flushing new leaves, both the ambient leaves and the ant-collected leaves were immature (<1 month old), which are easily distinguishable from older leaves by color, texture, resistance to bending, and thickness. We also took care to collect leaves from a height approximate to that at which the ants were cutting leaf fragments. Overall, we collected leaves representing a mix of species surrounding the trees the ants were collecting leaves from. We placed the collected materials in separate plastic petri dishes and then into a cooler with ice until they were returned to the lab. After returning to the lab, we transferred the samples to a refrigerator until they were processed (see methods below).

*Emergent colonies.*—We sampled 24 emergent colonies (all in Gamboa). Our definition of an emergent colony was a newly founded colony in which new workers had begun to forage outside of the nest within the previous three weeks. Due to extremely low foraging activity for the emergent colonies, we did not take surveys of deposition rates or the proportions of materials that the emergent colonies collected. We observed each colony until workers cut leaf fragments from nearby vegetation. We then collected 20 leaf fragments directly from the ants to estimate endophyte abundance. The leaf fragments were placed into a sealed plastic petri dish and then returned to the lab. After returning to the lab, we transferred the samples to a refrigerator until they were processed (see methods below).

### Laboratory methods

We processed all samples for both the mature and emergent colonies in the laboratory the same day in which the samples were collected from the field. For each mature colony we took pictures of five representative fragments of each type of material that the colony was collecting. Using ImageJ (v. 1.45s, Rasband 2012), we calculated the area of the fragments from the pictures. Next, we rinsed all of the collected material (including the fragments used for area measurements above) with tap water, and then cut the material

Table 1. Estimates of proportions of plant material types and their endophyte abundances collected from 24 *Atta colombica* colonies during the wet season in Central Panama (mean with SE in parentheses).

Material	Proportions of	
	Total material collected by ants	Leaf material with fungal endophytes
Leaves	0.845 (0.05)	0.516 (0.07)
Stipules	0.070 (0.03)	0.102 (0.03)
Flowers	0.058 (0.02)	0.254 (0.07)
Fruits	0.027 (0.02)	0.300 (0.12)

into  $2 \times 2$  mm squares. For ant-collected and ambient leaf fragments, this resulted in  $\sim 50$  leaf squares per fragment, while the ant-collected fruit and flower fragments were usually smaller and produced fewer squares. We placed the squares into metal tea strainers; the materials were kept separate by type and colony throughout the process. We immersed the metal tea strainers in 70% ethanol for two minutes followed by immersion in 10% commercial bleach for three minutes to sterilize the surface of the pieces. For mature and emergent colonies, we plated 16 haphazardly chosen squares from the leaf fragments taken from the ants onto plastic petri dishes containing 2% malt extract agar in a sterile hood. For the mature colonies only, we also plated 16 haphazardly chosen squares from the ambient leaves and a minimum of six squares from each type of non-leaf material the colony was collecting onto separate petri dishes containing 2% malt extract agar, with the number of squares plated being relative to the proportion of that non-leaf material the ants were collecting. The petri dishes were sealed with Parafilm and stored in a sterile room inside of a closed plastic box at room temperature. After three days and again after seven days, the petri dishes were checked for the growth of endophytes. Any endophytes were marked and recorded; any endophytes growing after three days were removed from the plates to prevent the overgrowth of other pieces. The abundance of endophytes was calculated by dividing the number of plated squares from which endophytes grew by the total number of squares that were plated, so that abundance data are reported as proportions.

#### Model of mature colony daily endophyte load

We calculated the daily foraging rate ( $H_{24}$ ) of mature colonies from refuse deposition rates

(RDR) using the regression equation of Herz et al. (2007a),  $H_{24} = 964.4 \times \text{RDR} + 20,472$ , which was determined from a nearby population of *Atta colombica* on Barro Colorado Island, Panama ( $9^{\circ}09' \text{ N}$ ,  $79^{\circ}51' \text{ W}$ ). Using the daily foraging rate, the proportions of each plant material type collected by each colony, and the area of each plant material type found using ImageJ (v. 1.45s, Rasband 2012), we estimated the daily area of each kind of plant material collected by the colonies. We then used the endophyte abundance data from our collected materials to estimate the daily area containing endophytes for each kind of plant material collected by the colonies. Lastly, we also determined the daily area of plant material and the daily area of plant material containing endophytes under the assumption that the ants collected only ambient leaves. We obtained all averages and standard errors for the model using bootstrapping with 5,000 iterations in R (2.14.2; R Development Core Team 2012).

#### Statistical analysis

The effect of site (Gamboa versus Pipeline Road) and the site  $\times$  treatment interaction were non-significant (between-within subject ANOVA on rank transformed data; site  $F_{1,22} = 0.950$ ,  $p = 0.340$ ; site  $\times$  treatment  $F_{1,22} = 0.052$ ,  $p = 0.805$ ; Systat v.11, Systat Software 2003), so we did not consider site as a factor in our final analyses. We used nonparametric analyses because the endophyte abundance data failed to meet normality assumptions. We used a one-tailed Wilcoxon signed-ranks test to test the hypothesis that leaf fragments collected by mature colonies have a lower endophyte abundance than ambient leaves. Endophyte abundances were paired by colony so that leaf fragments collected from ants were compared to ambient leaves in the same vicinity. We used a one-tailed Wilcoxon rank-sum test to test the hypothesis that emergent colonies



collect leaf fragments with lower endophyte abundances than mature colonies. We performed the Wilcoxon rank-sum test in R (2.14.2; R Development Core Team 2012) and the Wilcoxon signed-ranks test in Systat (v. 11; Systat Software 2003).

## RESULTS

### *Atta colombica* foraging preference

The ants collected mostly leaves, followed by stipules from emerging fig leaves, flowers, and fruits (Table 1). These materials harbored different endophyte abundances with the greatest abundance in leaves, followed by fruits, flowers, and stipules (Table 1). Leaves collected by the ants had a significantly lower abundance of endophytes than the surrounding ambient leaves ( $Z = -3.209$ ,  $p < 0.001$ ,  $n = 24$  colonies; Fig. 1). Emergent colonies did not collect leaf material with endophyte abundances significantly different than those of leaf material selected by mature colonies ( $W = 280$ ,  $p = 0.5699$ ,  $n = 48$  colonies; Fig. 2).

### Model of mature colony endophytic fungal load

Our model estimates that an average mature colony forages  $9.119$  ( $SE = 0.786$ )  $m^2$  of plant material a day, of which  $4.308$  ( $0.684$ )  $m^2$  harbors endophytic fungi (Fig. 3). The model also

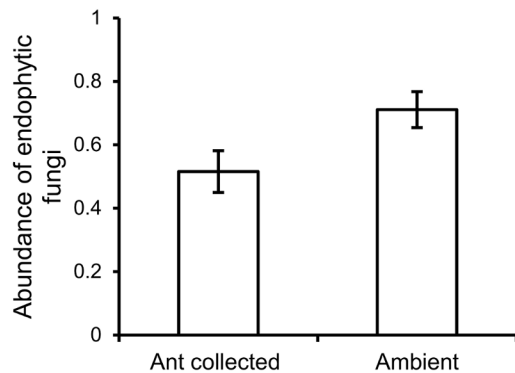


Fig. 1. Mature *Atta colombica* colonies in Central Panama harvested leaf material that contained  $\sim 20\%$  less of its area inhabited by fungal endophytes relative to ambient canopy leaves (one-tailed Wilcoxon signed-ranks test;  $Z = -3.209$ ,  $p < 0.001$ ,  $n = 24$  colonies). Bars represent data from 24 colonies, with error bars representing 1 SE.

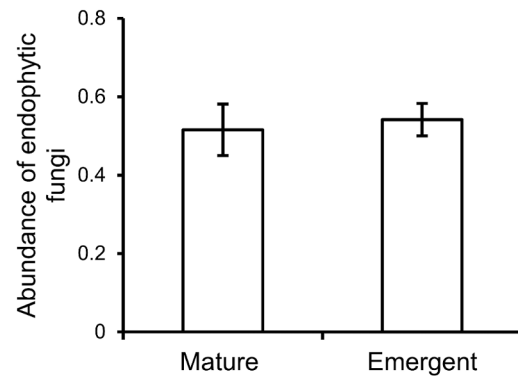


Fig. 2. Mature and emergent *Atta colombica* colonies in central Panama harvest leaf material that is not significantly different in the amount of area inhabited by endophytic fungi (one-tailed Wilcoxon rank-sum test,  $W = 280$ ,  $p = 0.5699$ ,  $n = 48$ ). Bars represent data from 24 mature and 24 emergent colonies, with error bars representing 1 SE.

estimates that a colony foraging only on ambient leaves would forage  $9.385$  ( $0.684$ )  $m^2$  of leaves a day, of which  $6.655$  ( $0.714$ )  $m^2$  would harbor endophytes (Fig. 3). Therefore, our model estimates that via their foraging preferences, ants reduce the relative area of plant material containing endophytes entering the colony by  $33\%$  (Fig. 3).

## DISCUSSION

Previous studies have shown that leaf-cutting ants take longer to cut leaf material with higher endophyte abundances and prefer to cut material from seedlings with low endophyte abundances, but these studies were all performed under laboratory conditions (Van Bael et al. 2009b, Bittleston et al. 2010). Here we show that mature field colonies of *Atta colombica* cut leaf material that is  $20\%$  lower in endophyte abundance than the surrounding leaf material. We also estimated that the ants, via foraging preferences, reduce the area of material containing endophytes entering the colony by  $33\%$  relative to ambient endophyte levels. These results support the hypothesis that the endophyte abundance of plant material influences the foraging preferences of *A. colombica* and that horizontally transferred endophytes provide a defense for plants against defoliation by leaf-cutting ants.

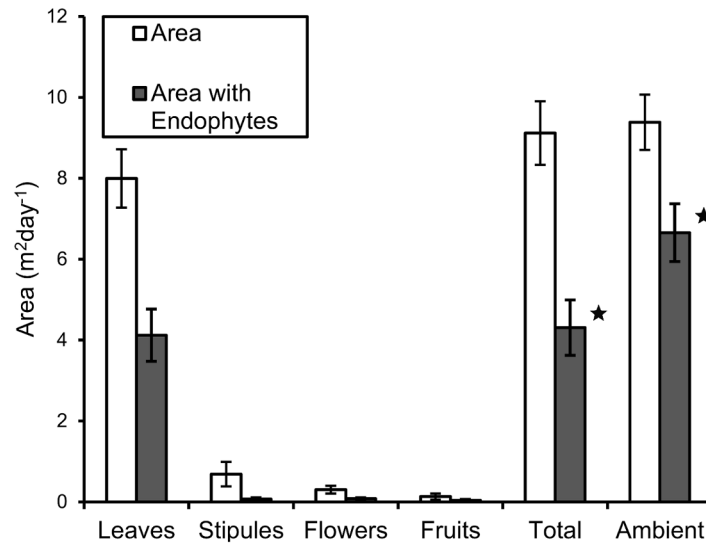


Fig. 3. Graphical depiction of a bootstrap model of the average area of plant material and the average area of plant material containing endophytic fungi collected per day by *Atta colombica* colonies during the wet season in central Panama. Bars represent results of the model based on data from 24 mature colonies both under parameters measured in the field and under the assumption that the ants collect only ambient canopy leaves. Starred bars show 33% relative reduction in the area of total plant material (leaves + fruit + flowers + stipules) containing endophytes between the field parameterized model (total) and the model assuming ants collect only ambient canopy leaves (ambient). Error bars represent 1 SE.

We predicted that emergent colonies would collect leaf material lower in endophyte content than mature colonies due to the smaller number of workers available in emergent colonies to deal with contamination of the nest by endophytes. Contrary to that prediction we found that emergent colonies collected leaf material with similar endophyte abundances as mature colonies. The emergent colonies foraged extremely slowly (K. Coblenz, *personal observation*) and if their foraging rate is proportional to mature colonies in relation to the number of workers, then there may be no difference in the workers' capacity to deal with endophyte contamination. The foraging ecology of emergent colonies is relatively unknown and we did not directly measure foraging rates of emergent colonies, however, so any comparison in this regard was impossible. The emergent colonies had been foraging for less than three weeks and were observed to exhibit delayed rejection of leaf material (Herz et al. 2008; K. Coblenz, *personal observation*). This may be the result of the naivety of workers from emergent colonies in recognizing certain materials as detrimental to cultivar

growth or colony health. If such naivety drives delayed rejection behavior, this may have led to a discrepancy between our measurements of endophyte abundance in material entering the colony and the abundance of endophytes in material actually used as substrate for the cultivar. Future studies should examine whether this naivety exists in emergent colonies and whether it has a role in the high mortality of emergent colonies.

The proportion and leaf area estimates of our model are in concordance with two previous studies on the nearby Barro Colorado Island, Panama (Wirth et al. 1997, Herz et al. 2007b). The 9.119 m<sup>2</sup> per day of plant material entering the colony estimated by our model falls within the ranges measured by both Wirth et al. (1997) and Herz et al. (2007b). Our model also estimated that the proportion of plant material collected by the colony consisting of leaves was 0.88, which is close to the value of 0.85 measured by Wirth et al. (1997) in the wet season, but higher than the value of “three quarters” measured by Herz et al. (2007b); although the Herz et al. study was over 15 months covering the wet and dry seasons.

Overall the agreement of our model's estimates with these previous studies reinforces our confidence in the model's results.

Seasonal changes are linked to changes in the foraging of leaf-cutting ants, but because our study took place only in the beginning of the wet season we cannot directly account for how those changes may alter the relationship between endophytes and leaf-cutting ants. During the beginning of the wet season many trees flush new leaves, which allowed us to better control for leaf age and toughness because newly flushed leaves are relatively soft and are easily discernible from mature leaves. As plant materials mature the abundance of endophytes in the material increases, along with changes in other leaf characteristics such as toughness and secondary compounds (Coley 1983, Herre et al. 2007). In the dry season, there are fewer new leaves available to the ants and an increase in non-leaf plant material is seen in their diet, accounting for a third of all material collected during the dry season (Wirth et al. 1997). Non-leaf plant material is expected to have lower endophyte abundances than leaf material due to its relatively short-lived nature on the parent plant, as was found in our study. Therefore, it is possible that by collecting higher proportions of non-leaf material in the dry season, when the leaf material available is older and has a relatively high abundance of endophytes, the ants may be able to keep the amount of material containing endophytes that enters the colony relatively stable across seasons. Alternatively, the ants may be forced by resource availability to harvest material that is high in endophyte content during the dry season leading to seasonal fluctuations in the amount of material containing endophytes entering the colony. Future research will be necessary to discern which pattern occurs in the field.

Our selection of ambient leaves was critical for an accurate analysis of the influence of endophytes on leaf-cutting ant selection. Leaf-cutting ant foraging is complex and a variety of factors other than endophyte content have also been found to influence the foraging preferences of leaf-cutting ants including: leaf toughness (Nichols-Orians and Schultz 1989), secondary compounds (Hubbell et al. 1983, Howard 1988), nutrient content (Berish 1986, Howard 1988,

Farji-Brener 2001), and epiphylls (Mueller and Wolf-Mueller 1991). To further complicate the matter, many of these variables in leaf tissue are covariates. For example, all of the above factors change with leaf age (Coley 1983, Mueller and Wolf-Mueller 1991, Herre et al. 2007), and fungi, including endophytes, have been shown to induce changes in some of these factors (for review see Van Bael et al. 2011). Therefore, some generalizations such as preference for young leaves rather than old leaves can be made, but assessing the impact of a single factor in isolation on ant foraging preferences is difficult. We attempted to control for some of these factors by selecting leaves that were similar in toughness, age, and height of collection, and were collected from the immediate vicinity as leaves collected by the ants, but inevitably the leaves likely differed in some of these factors. For example, we collected ambient leaves near the height where the ants were observed foraging or where leaf-cutting ant damage was visible on leaves, but it is possible that some ants were collecting leaves from higher or lower in the canopy. However, because we collected leaves from the height where most of the leaf-cutting activity was observed, this decreased the likelihood that leaves collected from other heights were included in our sample. In laboratory experiments, where the aforementioned factors were similar, ants consistently chose to cut leaf material with lower endophyte content (Bittleston et al. 2010). Furthermore, we collected leaves from a variety of tree species growing near the trees that the ants were collecting leaves from. Ants are known to collect leaves from a large number of species and one study found that *A. colombica* sampled leaves from 77% of plant species present and preferably cut leaves from over half of the species present when new leaves were being flushed, as they were during our study period (Rockwood 1976). Therefore, we are confident that using a variety of species in comparing the ant-selected leaves to ambient leaves provided little bias to our results given that the ants preferentially defoliate the majority of species with new leaves.

Our results suggest that leaf-cutting ants preferentially select leaf material that is low in endophytes in order to use as a substrate for their fungal cultivar. Collecting leaf material with less

endophyte abundance may be advantageous for a variety of reasons. Endophytes brought into the colony may compete directly with the cultivar on the substrate, or the endophytes may produce mycotoxins which could cause decreased growth of the cultivar. Either of these interactions could lead to decreased productivity of the cultivar. There is no evidence that the endophytes themselves have direct toxic effects on the ants, but Estrada et al. (2013) demonstrated that the ants can differentiate between chemicals extracted from plants with and without endophytes. Although leaf-cutting ants reduce the endophytes that enter the colony, approximately half the area of plant material that enters still contains endophytes. Leaf-cutting ants display a variety of adaptations in order to reduce nest contamination including hygienic behaviors and the production of antimicrobial compounds (Quinlan and Cherrett 1977, Bass and Cherrett 1994, Currie et al. 1999, Currie and Stuart 2001, Fernández-Marín et al. 2006). The cultivar itself has also been shown to decrease the growth rates of some endophyte species and therefore may also assist in deterring endophyte contamination of the nest (Van Bael et al. 2009b). Through these mechanisms the ants and cultivar are likely able to limit potential contamination by endophytes in this interaction between symbioses.

Previous work suggests that horizontally transmitted endophytes, unlike vertically transmitted endophytes, should have mostly neutral interactions with their host plants (Saikkonen et al. 1998). Recent evidence has begun to challenge this idea as horizontally transmitted endophytes have been shown to act as a defense against pathogens (Redman et al. 2001, Arnold et al. 2003, Mejía et al. 2008) and herbivory (Wilson 1995b, Wilson and Carroll 1997, Wilson and Faeth 2001, Van Bael et al. 2009, Bittleston et al. 2010). Much of this work has been done in the tropics where endophytes in plant tissues are particularly abundant and diverse (Arnold and Lutzoni 2007). The extent to which the diversity or the identities of particular endophytes in plant tissues affect their benefits to plants remains unknown and is a rich prospective area for research. This study further supports the idea that horizontally transmitted, non-systemic endophytes may indeed act as mutualists in plant hosts.

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