

Review Article

Fungal-Fungal Interactions in Leaf-Cutting Ant Agriculture

Sunshine A. Van Bael,^{1,2} Catalina Estrada,¹ and William T. Wcislo¹

¹ *Smithsonian Tropical Research Institute, P.O. 0843-03092, Balboa, Panama*

² *Department of Ecology and Evolutionary Biology, Tulane University, 400 Lindy Boggs, New Orleans, LA 70118, USA*

Correspondence should be addressed to Sunshine A. Van Bael, svanbael@tulane.edu and Catalina Estrada, estradac@si.edu

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Many organisms participate in symbiotic relationships with other organisms, yet studies of symbioses typically have focused on the reciprocal costs and benefits within a particular host-symbiont pair. Recent studies indicate that many ecological interactions involve alliances of symbionts acting together as mutualistic consortia against other consortia. Such interacting consortia are likely to be widespread in nature, even if the interactions often occur in a cryptic fashion. Little theory and empirical data exist concerning how these complex interactions shape ecological outcomes in nature. Here, we review recent work on fungal-fungal interactions between two consortia: (i) leaf-cutting ants and their symbiotic fungi (the latter grown as a food crop by the former) and (ii) tropical plants and their foliar endophytes (the cryptic symbiotic fungi within leaves of the former). Plant characteristics (e.g., secondary compounds or leaf physical properties of leaves) are involved in leaf-cutting ant preferences, and a synthesis of published information suggests that these plant traits could be modified by fungal presence. We discuss potential mechanisms for how fungal-fungal interactions proceed in the leaf-cutting ant agriculture and suggest themes for future research.

1. Introduction

Symbiosis has been a major engine for evolutionary innovation, at multiple levels of biological organization [1–3]. Most organisms are involved in symbioses, and many symbionts are essential for host survival and reproduction. Fungi, for example, are involved in a myriad of symbiotic relationships and often live symbiotically within their hosts. They are recognized for their important role in mediating ecological interactions among plants and animals [4, 5]. For example, fungal antibiotics fight animal pathogens [6], mycorrhizae allow plants to access nutrients and thereby affect plant-insect interactions [7], and fungal pathogens result in million dollar losses to agriculture, and have triggered massive social upheaval, such as that happened with the potato famine in the 19th century in Ireland [8].

Previous studies of symbiosis have almost exclusively focused on reciprocal benefits and costs shared by members of a particular host-symbiont association or a symbiont effects on a host abiotic or biotic stressors (but see [9]). The near-ubiquity of symbiosis means that real-world ecological

interactions will rarely be dominated by such single-pair symbioses and instead involve consortia of single-pair symbioses, acting in alliance with, or antagonistic to, other consortia of symbionts. Despite their ubiquity in nature, their potential importance for ecological and evolutionary dynamics, and their economic impact, very few fungal-fungal interactions have been worked out in detail, and few studies have addressed whether direct interactions among multiple symbionts can influence the success of their respective hosts [10, 11].

Here we review recent work from leaf-cutting ant agriculture that underscores the ecological and evolutionary significance of fungal-fungal interactions. Specifically, we focus on the endophytic fungi that are present in the leaf material that leaf-cutting ants bring back to their fungal gardens. We begin by outlining the natural history of each symbiotic pair and then consider how the two symbioses interact, with particular attention to the chemical ecology of fungal-fungal interactions. We conclude with a discussion of potential mechanisms for fungal-fungal interactions and suggest areas for future research.

2. Leaf-Cutting Ants and Their Fungal Cultivar

All genera of fungus-growing ants (Myrmicinae: Attini) cultivate a symbiotic fungus as food for their young, and in most genera the worker ants gather organic detritus (e.g., dead insect parts and feces) as the nutritional base for their gardens (e.g., [52–54]). One of the major transitions in the evolution of attine agriculture involved a shift from using organic detritus as garden substrate to cutting and harvesting pieces of leaves and other plant parts, which occurred in the common ancestor of *Acromyrmex* and *Atta*, the “leaf-cutter ants” (*arrieras*) [55, 56]. Leaf cutters are most abundant and diverse in Neotropical ecosystems, though two species reach the southern USA [52, 57]. This evolutionary shift to leaf cutting was associated with a spectacular increase in colony size, social structure, and ecological footprint [56].

Although they are not strict herbivores, leaf-cutting ants are arguably the most important defoliators in the Neotropics as they cut from a wide diversity of plants and can harvest 2–17% of the annual leaf production of forest and savanna woody plants [58–60]. They are major ecosystem engineers and have significant effects on local flora [58], seedling recruitment [61], distributions of soil nutrients [62], and human agriculture [63] (for reviews see [56, 64]).

Leaf-cutting ants maintain an obligate symbiosis with their fungal cultivar (*Leucocoprinus* (= *Leucoagaricus*) *gongylophorus* (Hoyt), Lepiotaceae, Basidiomycota) [52]. The worker ants cut leaves, carry them to the nest, clean and process them, and use them as the substrate on which they cultivate a fungus in underground chambers [54]. In turn, the fungal cultivar partially degrades the leaf material, converting leaf biomass to fungal food for the worker ants and their developing larvae. This ancient mutualism depends on the ant hygienic behaviors and an array of “public health” strategies, which range from weeding and grooming behaviors to the deployment of a diverse arsenal of antimicrobial compounds [9, 54, 65–68]. In brief, the fungal cultivar cannot persist without ants actively tending their gardens, and, conversely, the ants cannot persist without a healthy fungal cultivar.

3. Symbioses between Plants and Endophytic Fungi

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/or other above-ground plant tissues without causing any apparent signs of disease [69]. Previous studies suggest that leaves are flushed endophyte-free and that endophytes are acquired by horizontal transmission, from spores in the environment [70]. Decaying litter is likely to be the source of endophyte inocula, as reproductive structures have not been observed in live leaves [71, 72]. Endophytes can be extremely diverse in the leaves of tropical plants [73], with endophyte communities that conservatively range from 10 to 20 species per host plant and generally exhibit low similarity among hosts [72, 74].

Previous work in temperate areas has demonstrated that some endophytes defend their host plants by making their leaves less palatable to insect herbivores [75–78]. Such studies highlight some of the problems associated with studying symbioses as pairwise interactions. Particular endophyte-plant combinations, for example, can reduce the survival of one species of herbivorous insect but have no effect on a closely related species [76]. Some fungi can drastically reduce herbivory in one host plant, but they might have no effect whatsoever in another host plant attacked by the same herbivore [75, 79]. Furthermore, intraspecific variation may also shape the outcomes of these interactions. The endophyte *Neotyphodium lolii* (Latch, Christensen, and Samuels), for example, is common in perennial ryegrass, which is attacked by the Argentine stem weevil, *Listronotus bonariensis* (Kuschel) [80]. Different endophyte strains differ in their potential impact on a third trophic level, involving parasitoid wasps, *Microctonus hyperodae* Loan (Braconidae), with some strains slowing parasitoid developmental rate or survivorship, while others had no effect. Tropical host-endophyte-herbivore interactions are only beginning to be studied [10, 81], and endophyte functional ecology in general is poorly understood in tropical plants [82], but even limited data demonstrate the importance of focusing on interacting symbioses, as discussed in the next section.

4. Consortia of Interacting Symbionts

Given the spectacular diversity of endophytic fungi, coupled to the equally spectacular tropical plant diversity, generalist herbivores, such as leaf-cutting ants, potentially interact with many hundreds of foliar endophyte species. While the ant-cultivar symbiosis is relatively well studied (see [54]), the extensive interactions among the ant cultivar and fungal endophytes have only recently received attention. For example, a number of known fungal endophyte species were isolated from nests of *Acromyrmex* sp. in Brazil [92], and other studies have shown that the composition of endophytes in ant cultivars varies with different conditions, such as a change of plant species used for substrate [93]. Other than documenting their cooccurrence, little is known about the consequences of multiple interactions among ants, their fungal cultivar, and endophytic fungi.

Due to their relationship with a fungal cultivar, the interactions between leaf-cutting ants and endophytes may be fundamentally different than those between other insect herbivores and endophytes. Since most insect herbivores ingest and digest the plant material they remove, the effect of the endophyte-plant consortia is likely to be direct (e.g., fungal infections, toxicity, and nutritional quality of food) and thus insect physiological response to the consortia will be direct (e.g., detoxification of plant or endophyte metabolites). For leaf-cutting ants, however, the effects of the endophyte-plant consortia could be direct and/or indirect, if targeting ant fungal cultivar. Endophytes may act as pathogens toward the ants [94] or their cultivar, may be beneficial to either or both, or may be neutral commensals. Similarly, control over

TABLE 1: A review of studies that correlate plant secondary metabolites with ant foraging preferences, with additional observations of whether these key families of compounds have antifungal properties, change as a result of plant-fungal interactions, or are known to be produced in *in vitro* endophyte cultures.

Plant secondary metabolites	Correlation with ant host plant preferences (– repellent, + attractant, 0 neutral)	Anti-fungal properties*	Fungal-induced changes in plants [§]	Endophyte secondary metabolites
(1) Nonpolar compounds	– [12–15]			Aromatic compounds and nonanoic acid [16–19]
<i>Terpenoids</i>		Yes [20]	P infection triggers plant production of terpenoid phytoalexins [‡] [21]	
Monoterpenoids (e.g., <i>trans</i> - β -ocimene)	– [22]	Yes [23]	REs trigger increase of α -terpinene, but not other monoterpenes [24]	
Sesquiterpenoids (e.g., caryophyllene epoxy, caryophyllene, nerolidol, lasidiol, guaiacol, and spathulenol)	– [25–29]	Yes (cultivar) [25–27]	REs trigger increase of <i>trans</i> - β -caryophyllene but not other sesquiterpenoids [24] AM increase [30] or do not affect [31] emissions	Caryophyllene and derivatives, bulnesene, valencene, cuparene, heptedilic acid, hydroheptelidic acid [16, 32–35]
Diterpenoids (e.g., kolavenol)	– [26, 27]	No (cultivar) [27]		Taxol, guanacastepene, and subglutinols [34, 36]
Triterpenoids (e.g., lupeol, 3 α -hydroxyolean-12-en-27-oic acid derivatives)	– [37, 38]		AM induce accumulation of triterpenoids in roots [39]	
<i>Cuticular waxes</i>	– [15, 37, 38]	Yes [17]	P infection triggers production of alkene [23]	
(2) Polar compounds (e.g., glycosides, and alkaloids, phenolics)	– [14]	Yes (e.g., lignants on cultivar) [40]	P infection triggers plant production of polar phytoalexins [‡] (e.g., phenolics, alkaloids, and flavonoids) [21, 23, 41]	Phenolic acids, rugulosin, lignans, ergosterol, and steroid volatile alcohols [32, 34, 35, 42]
<i>Alkaloids</i>	0 [12, 13]	Yes [20, 43]		Cytochalasin compounds, ergot, loline alkaloids, lolitrems, and peramine [16, 34, 44]
<i>Tannins</i>		Yes [20]		
Hydrolyzable	+ [12, 13], – [45–47]			
Condensed	– [46, 48], + [45], 0 [12, 13, 47]			
<i>Glycosides</i> (e.g., saponins and anthocyanins)	– [12, 49, 50]	Yes [20]	AM induce accumulation of glycosylated cyclohexenone derivatives [30]	Petaloside and triclin [16, 34]

* General antibiotic effect against a common variety of tested fungi or, in particular, toward leaf-cutting ant cultivar (cultivar).

[§]P: fungal pathogens, AM: arbuscular mycorrhizae, and RE: root endophytes.

[‡]Phytoalexins: low-molecular-weight antibiotic compounds produced *de novo* by plants in response to microbial stimulation [51].

which endophytes can remain in the garden may involve ant hygienic behavior, the physiological properties of the cultivar, or both [10]. One example of a direct effect of endophytes on leaf-cutting ants comes from a previous experiment with grasses in the temperate zone. Evidence of

endophyte toxicity toward leaf-cutting ant queens and workers for some grass-endophyte combinations was found, suggesting a defensive mutualism between the grass and endophyte [11]. The *Neotyphodium* endophytes involved in this study, however, differ from those found in tropical plants

due to their vertical transmission (from mother to seed), lack of diversity within hosts, and restriction to grass hosts [79].

Theory suggests that defensive mutualisms between endophytes and their hosts are likely to be more effective for vertically than horizontally transmitted fungi [79, 95], yet empirical data provide examples of defensive mutualisms with horizontally transmitted endophytes as well [96–98]. In the tropics, where endophyte dispersal is horizontal, there is some evidence that indicates that the leaf-cutting ants are sensitive to endophytes in the host plants that the ants utilize. In one laboratory experiment, *Atta colombica* Guérin-Méneville workers took longer to cut leaf material with endophytes relative to leaves without and the workers decreased the endophyte load in leaf material before planting it in their gardens [99]. In laboratory choice-test experiments, *A. colombica* preferred to cut leaf tissue from tree seedlings with low rather than naturally high endophyte loads [100]. In contrast to the results from grass endophytes [11], no evidence of endophyte toxicity to the workers was observed in these experiments. The differential behavior of the ants toward leaf tissue with or without endophytes, however, suggests that the endophytes are antagonistic to the fungal symbiont of the ants. Furthermore, the ant cultivar must outcompete or detoxify the endophytes that are not removed by the workers [10, 101]. *In vitro* experiments of fungal-fungal interactions suggested that endophytes were inhibited by the ant fungal cultivar [99] implying that both the ants and their cultivar respond to endophytes. Whether endophytes inhibit or slow the cultivar growth, however, has yet to be tested.

5. Mechanisms Underlying Leaf-Cutter Ant Preferences

Although leaf-cutting ants are generalists with respect to the diversity of plant species they harvest (see Section 2), they are remarkably selective with respect to the plant species, the individual plant, and the leaves within a plant that they cut. Typically they prefer to cut from younger leaves than older ones, woody rather than herbaceous species, light-demanding rather than shade-tolerant species, introduced rather than native species, and lianas rather than trees, taking into account the proportional abundance of these growth forms. Overall, these patterns suggest that leaf-cutting ants search for relatively easy-to-cut, less defended leaves, with high nutritional value [102–105].

We have reviewed previous work on the chemical and physical properties of leaves that affect leaf-cutter ant preferences, comparing the properties to additional studies that describe effects of fungi *in vitro* and *in planta* (Tables 1 to 3). Most studies have focused on one or a few leaf characteristics, some of which are correlated; in the case of plant chemistry, only a handful of plant secondary compounds have been identified that affect ant selectivity (Table 1). Changes in those key leaf properties that could result from plant-fungal interactions and might modify the value of the leaves for ants include (1) volatile blends emitted by plants after tissue damage

TABLE 2: A review of studies that correlate plant nutrients with ant foraging preferences, with additional observations of whether these key plant traits change as a result of plant-fungal interactions (pathogens).

Plant nutrient content	Correlation with ant's host plant preferences (– repellant, + attractant, 0 neutral)	Fungal-induced changes in plants
Proteins	+ [13]	Increase in free and protein amino acid content [21]
Nitrogen	+ [13, 45–47, 83, 84]	Variable responses from increase to decrease of leaf N and a general increase of C/N ratio [21]
Nonstructural carbohydrates (NSC)	+ [46, 47], 0 [12, 13, 45]	Variable responses from increase to decrease of NSC (e.g., accumulation of starch) [21]
K, P, Cu	+ [83, 84]	
Al, Mn	– [84], 0 [83]	
Zn, Ca, Fe, Mg	0 [83]	

(Table 1); (2) high-molecular-weight secondary compounds (e.g., large terpenoids) (Table 1); (3) cuticular waxes (Table 1), (4) plant nutritional content (Table 2), and (5) physical properties (Table 3). The vast information about the foraging preferences of leaf-cutting ants, combined with the diversity of plant and endophyte species they encounter, provides an excellent opportunity to identify whether fungi affect the plant traits responsible for the reduction in plant damage by these insects.

Fungi, including plant endophytes, are known to produce organic compounds with antimicrobial and insect repellent effects, especially when competing with other microorganisms [106] (see Table 1). They can also transform chemical defenses produced by plants into new organic compounds [107]. Moreover, endophytes can have effects on physical properties of leaves such as drought tolerance [108] and increased lignin deposition in cell walls (Siela Maximova, unpublished). These observations, combined with the ant behavior described above, suggest that endophyte-mediated changes in leaf chemistry or leaf physical traits may play a significant role in leaf-cutter ant preferences.

Most work identifying compounds from endophytes has been done from pure *in vitro* cultures, with little *in vivo* work exploring whether and how endophytes interact with plant tissues to change leaf chemistry [109–111]. Nevertheless, *in vitro* results so far show that endophytes typically produce both species-specific unique molecules and common plant metabolites [107, 112]. For example, preliminary analyses with *Colletotrichum tropicale* Rojas, Rehner and Samuels [113], a common endophyte used in experiments [99], have revealed the *in vitro* production of more than 15 volatile organic compounds by this fungus,

TABLE 3: A review of studies that correlate plant physical characteristics with ant foraging preferences, with additional observations of whether these plant traits change as a result of plant-fungal interactions.

Plant physical traits	Correlation with ant host plant preferences (– repellant, + attractant, 0 neutral)	Fungal-induced changes in plants
Toughness	– [85–88], 0 [12, 49]	Pathogens and endophytes trigger lignification of plant cell walls [21, 41]
Water content “sappiness”	+ [45, 49, 85], – [89], 0 [12, 13]	Not affected by pathogen infections [21]
Density	– [85]	Pathogens trigger increase of leaf fiber content [21]
Trichomes: length	– [13]	
Latex	– [90]	
Epiphyll community	– [91]	

most of which are sesquiterpenoids typically associated with plants and trigger rejection of leaves by leaf-cutting ants (C. Estrada, unpublished). Thus, changes in chemical profiles of endophyte-infected plants could result from (1) fungal-derived compounds, (2) quantitative alterations in typical plant chemical blends triggered by the presence of fungi inside leaves (e.g., lowering or increasing overall leaf vigor), or (3) a combination of both effects. An alternative hypothesis is that infections by endophytes can prime plant responses against herbivores [114]. This implies that chemical defenses of infected and uninfected plants will be similar, but that plant responses to herbivory and thus herbivore deterrence will happen faster.

6. Summary and Future Research Directions

The scarcity of data on how endophytes affect plant chemical and physical traits has hindered our ability to understand the broad variation in effects that different fungal species, and fungal-plant interactions, have on herbivores and ultimately the role that these symbionts have in plant antiherbivore defense and plant-herbivore coevolution [115]. Leaf-cutting ants are not strictly herbivores, in that they do not eat plant material directly but use a fungal intermediary to convert plant matter to a consumable form. An important line of plant defense against herbivores involves secondary chemical compounds that are toxic to insect herbivores (e.g., [116]). If these secondary compounds are not toxic to fungi, then the use of a fungal symbiont by the ants represents a detoxification mechanism that enables the ants to circumvent plant chemical defense, analogous to the way some endosymbionts are used by insects for detoxification purposes (reviewed in [117]). Hence, leaf-cutting ants and

their fungal-driven preferences for leaves offer excellent opportunities to conduct bioassays on the types of active compounds produced by endophytes or by endophyte-mediated interactions with plants. Future work should focus on whether endophyte-mediated plant protection is due to the contribution of particular fungal traits (e.g., mycotoxins), changes in typical plant traits (e.g., quantitative changes in plant defenses or nutrient content), or the emergent properties arising from specific endophyte-plant interactions (e.g., biotransformation of plant chemical defenses). This information will help us understand the sources of the puzzling variation in the effects that endophytes have on herbivores [118], the existence of affinities between endophytes and plant species [119], and the overall impact that these symbionts might have at higher levels of ecological organization (e.g., population to ecosystem) and across multiple trophic levels [80, 120].

Moreover, understanding how endophytes influence leaf-cutting ants and their fungal cultivar will facilitate research conducive to implementing pathogen-specific, crop-specific or herbivore-specific biological control programs using endophytes (e.g., [98, 121]). A particular need for such a program is one that would reduce the economic losses caused by leaf-cutting ants in tropical agriculture and agroforestry. Plant-microbial and animal-microbial symbiotic consortia tend to be cryptic and thus are little understood components of most terrestrial and marine food webs. A growing body of empirical research indicates that cryptic consortia of symbionts may be responsible for a large part of the ecological and evolutionary patterns observed in nature [3, 82, 122] and that interactions among plants, animals, fungi, and other microbes are vastly more intricate and extensive than currently conceived.

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