

Attractivity or Repellence: Relation between the Endophytic Fungi of *Acalypha*, *Colocasia* and the Leaf-Cutting Ants—*Atta sexdens*

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Abstract

Symbiotic relations are present in the nature and can contribute to the success of the organisms involved. Endophytic fungi live within the leaf tissues without causing any harm to the host plant, and some of them can be a defense mechanism against the attack by the leaf-cutting ants. Ants of the genus *Atta* are known as leaf-cutting ants and have an obligatory association with the fungus *Leucoagaricus gongylophorus*, cutting pieces of leaves and bringing them back to the colony to the fungus. The present study aimed to find out the endophytic fungi community of an attractive plant (*Acalypha wilkesiana*) and a less attractive plant (*Colocasia esculenta*) to the ants *Atta sexdens*. We found out that the communities are different in quantity and in composition and 73% of the isolated fungi were from *A. wilkesiana*, which has fungi known as attractive to leaf-cutting ants, such as genus *Colletotrichum*, *Pestalotiopsis*, *Phomopsis* and *Xylaria*. On the other hand, in *C. esculenta*, there was found the genus *Fusarium*, known to be reject by the leaf-cutting ants, and less fungal diversity than in the attractive plant *A. wilkesiana*. Therefore, our data suggest that attractivity or repellence of a plant to the leaf-cutting ants could be related to presence or absence of determined fungi more than the quantity of fungi present in a leaf.

Keywords

Attine, Musaica, Marginata, Interaction Plant-Ant, Leaf-Cutting Ants

1. Introduction

Symbiotic relations are present among many organisms in nature, contributing

to the success of the involved. Plants associate with many microorganisms, such as fungi, bacteria and virus, varying from mutualisms to parasitisms [1] [2]. The microorganisms associated with plants develop competition relations between themselves, for resources or predation and by the release of volatiles and antimicrobial compounds, having consequences in the structure and stability of the microbial community and in its homeostasis with the host plant [3].

The endophytic fungi are found between the plant's leaf tissues and establish a mutualistic relation with the host, without causing symptoms [4]. These fungi are abundant in the tropical regions [5] and have a pathogenic potential if the host plant goes through a stressful situation, for natural causes or for the use of products in its maintenance, which can disequilibrate the endophytic mycobiota and potentialize these fungi's pathogenicity [6].

Van Bael and collaborators [7] suggested that the endophytic fungi have a defense function in the plant by altering the leaf's morphology and chemistry. In addition, plants can use directly the endophytic fungi as a defense against these ants [8], as they answer in different ways to each fungus's signals [9]. The diversity of endophytic fungi that are introduced in the nest by the ant's foraging activity is not yet widely known, as well as the symbiotic interactions between these endophytic, the ants and their fungal garden [10]. In one of the few studies on the subtopic Van Bael and collaborators [7] verified a tendency for a greater fungi mass per worker in colonies fed with low endophytic leaves and that a high quantity of endophytic fungi of *Cucumis sativus* and *Manihot esculenta* leaves transported to new nests of *Atta colombica* (Guérin-Méneville, 1844) could affect its development by limiting the colony's productivity, while the high endophytic load was beneficial to old colonies, by increasing their defenses against parasitic fungi.

Ants of the genus *Atta* (Fabricius, 1804) and *Acromyrmex* (Mayr, 1865), classified as Attine: Attina, are popularly known as leaf-cutting ants and fungus growing ants [11]. Some species are considered plagues in the neotropical region due to the intense cutting of leaves of important agricultural cultures [12]. Another important and obligatory symbiotic relation is the association between the ants of the *Atta* genus and the fungus *Leucoagaricus gongylophorus*, which produces biomass, enzymes and nutrients to the colony [13]. The ants execute a quality control of the leaf material that is transported, selecting those which will be incorporated to the fungal garden and removing the unwanted ones [14]. The leaf-cutting ants have a hygiene behavior specific to the different species of endophytic fungi [9], important because filamentous fungi can have antagonistic actions to the attine ant's cultivar [15]. The mutualist fungus *L. gongylophorus*, such as the workers, also has defense mechanisms against parasite fungi [10], as the *Trichoderma* genus fungi [8].

Leaf-cutting ants have a preference for certain plants, like *Ligustrum* spp., *Psidium guajava*, *Hyparrhenia rufa* and *Acalypha* sp. [16], which is used for maintenance of colonies in laboratories [17]. *Acalypha* is the most diverse genus of

the Euphorbiaceae family with mostly arboreal species [18]. The species *Acalypha wilkesiana* (Müll.) Arg. is widely used for ornamental purposes and is a potential source of bioactive compounds for cancer's treatment [19]. This species is very attractive to the leaf-cutting ants, since it can complement the colony's nutrition by liquids extracted directly from the leaves [16] [20]. Among its many varieties, *Acalypha wilkesiana* has the "Marginata" variety and the "Musaica" variety. The cultivar "Marginata" has coppery green leaves with a light pink variant border and is known to be more attractive to the leaf-cutting ants when compared with the cultivar "Musaica", which has green, red and orange spotted leaves [21].

On the other hand, the leaves of Taro (*Colocasia esculenta* (L.) Schott var. *esculenta*) are less attractive to the leaf-cutting ants, classified as "moderately accepted" [22]. Previous works have shown that *C. esculenta* lectins have an anti-insect potential [23] and the ability of blocking cysteine proteases of fungal mycelium [24]. The lectins present in this plant are considered insecticidal agents [25] and a compound of its roots (2, 3-Dimethylmaleic anhydride) is biofumigant [26]. The *Colocasia esculenta* (L.) Schott is the main species of the Araceae family, has rhizomes rich in starch and is used as human food [27]. *Atta sexdens* workers are few attracted for this plant, and its younger leaves are more cut compared to senescent leaves [28]. Preliminary tests in laboratory showed that the leaf-cutting ants *Atta sexdens* are more attracted to *A. wilkesiana* and less attracted to *C. esculenta* (*pers. obs.*). However, endophytic diversity can contribute to the choice of material foraged by leaf-cutting ants as demonstrated by Rocha *et al.* (2017) [8].

Therefore, in this study we aimed to verify how the presence of endophytic fungi could influence the foraging by the leaf-cutting ants in plants (Figure 1). For this, two vegetal species were used, one highly attractive and other less attractive and the following items were done: to 1) isolate and identify the fungi communities present in each cultivar of *A. wilkesiana* and in young and senescent leaves of *C. esculenta*, 2) compare the endophytic communities present in each plant, to verify a possible relation with the attractiveness of *A. wilkesiana* and less attractiveness of *C. esculenta* to the *Atta sexdens* ants.

2. Material and Methods

2.1. Collect, Isolation and Achievement of Pure Cultures

The study was carried out at the Center of Studies of Social Insects, in São Paulo State University (UNESP), Institute of Bioscience, Campus of Rio Claro, from June of 2018 to December of 2019. Individuals of *Acalypha wilkesiana* "Marginata", *Acalypha wilkesiana* "Musaica" and *Colocasia esculenta* variety *esculenta* were collected at Rio Claro, SP (22°24'48"S, 47°34'11"W) and kept in laboratory in 20-liter pots for two weeks. Three branches were collected from 4 individuals from each plant. After collection, the plant material was washed under running water. The external sterilization of the material was done by sequential immersion

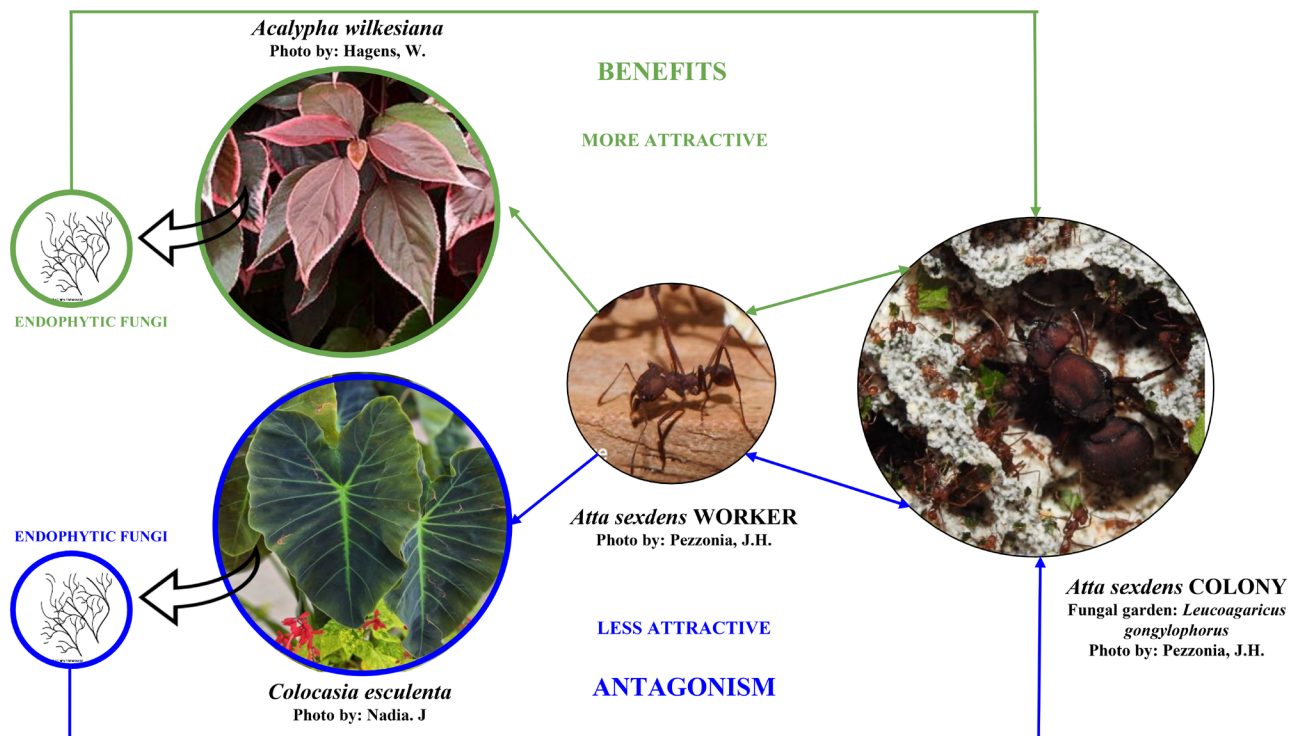


Figure 1. The schemes show the relation between the leaves, its endophytic fungi community, and the *Atta sexdens* colony. The ant's colony is composed by its fungus, queen, and workers. The endophytic fungi establish a mutualism with their host plant and can be beneficial or antagonist to the ant's cultivar *L. gongylophorus*. If beneficial, the ants could be more attracted to the host plant and, if it has an antagonist relationship with the ant's fungi cultivar, the host plant could be less attractive to the workers.

in 70% (v/v) ethanol for 5 minutes, hypochlorite (3% - 5% active chlorine) for 5 minutes, 70% (v/v) ethanol for 30 seconds and twice in deionized sterile water (1 minute each). After removal of water excess, the leaves were cut into 0.5 cm squares, which were transferred to Petri Plaques containing Potato Dextrose Agar (PDA) culture medium. In each plaque, 4 leaf squares were placed. The plaques were incubated in B.O.D. in 25°C and observed daily. Once detected the endophytic fungus growth, samples were taken from the growing hyphae to obtain axenic cultures. To confirm that the sterilization process was successful, aliquots of deionized water, used in the final wash, were plated by the plate spreading method, in PDA medium and incubated in 25°C [29] (**Figure 2**).

2.2. DNA Extraction, PCR, Purification, Sequencing and Identification

DNA extraction was made following the CTAB method for filamentous fungi adapted from Möller *et al.* [30] and Gerardo *et al.* [31]. Each morphologic group was molecularly identified by the amplification and sequencing of the internal transcribed spacer region (ITS) of the ribosomal nuclear DNA.

For the amplification of the ITS region, it was used 1 µL (10 uM) of primers ITS1-F (forward: 5'-CTTGGTCATTTAGAGGAAGTAA-3') [32], and ITS4 (reverse: 5'-TCCTCCGCTTATTGATATGC-3') [33], 2 µL of DNA (1:49), 5 µL of GoTaq 5× Reaction Buffer, 4 µL of dNTPs (1.25 mM each), 0.2 µL of GoTaq

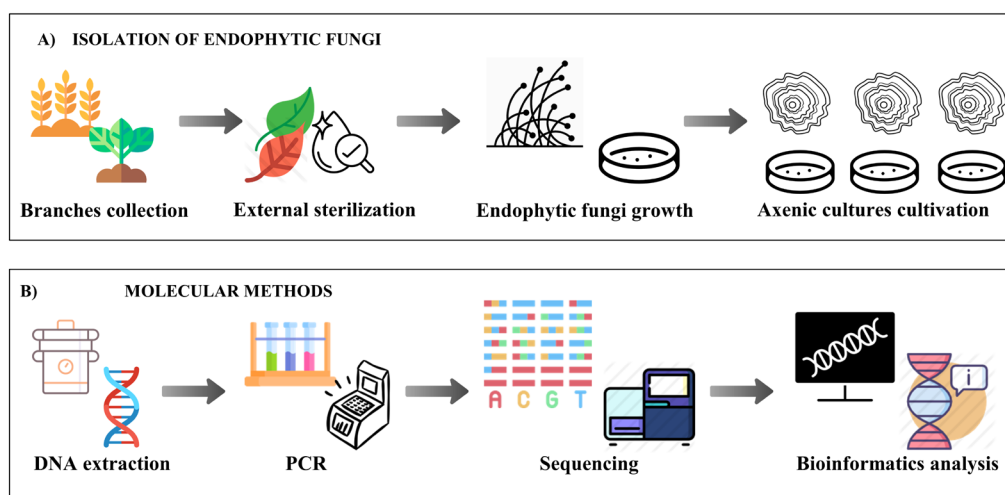


Figure 2. Method design, (A) Isolation of endophytes fungi: isolation and axenic culture. (B) Molecular methods: DNA extraction, PCR, Sanger Sequencing, and bioinformatics analysis.

DNA polymerase (5 U/ μ L), 2 μ L of $MgCl_2$ (25 mM), 1 μ L of BSA (10 mg/mL) and adjusting the final reaction volume to 25 μ L with sterile Milli-Q[®] water. The reaction was conducted in thermocirculator Veriti™ 96 Well of Applied Biosystems according to the following program: 94°C for 10 min, followed by 30 cycles of 94°C for 1 min, annealing at 52°C for 1 min and 72°C for 2 min, and final extension at 72°C for 10 min. The PCR amplified products were analyzed by electrophoresis in agarose gel 0.81%. The amplified fragments presented around 500 base pairs (**Figure 2**).

The PCR product's purification was made with the illustra™ PCR DNA and Gel Band Purification Kit (GE Healthcare), according to the manufacturer's protocol. The purified samples were quantified in the NanoDrop 2000 Thermo Scientific—Uniscience. The sequencing reactions were made with the BigDye Terminator kit, according to the manufacturer's orientation. Sequences were obtained using the automatic sequencer 3130 Genetic Analyzer (Applied Biosystems) [34]. Forward and reverse sequences were edited in the Bioedit Sequence Alignment Editor [35] and aligned with the Clustal W tool [36] (**Figure 2**). With the Blastn tool from NCBI, the consensus sequences were compared to those with the higher similarity deposited on the GenBank. Those comparisons varied from 95% to 100% of similarity rate with E.value equal to 0.0. Only the sequences with more than 95% of similarity rate were considered in the comparisons. The communities of *A. wilkesiana* varieties were compared as one endophytic community with the communities of the *C. esculenta* leaf ages, which were considered as one community as well.

3. Results

From a total of 112 isolates, 82 endophytic fungi were isolated from the attractive plant *A. wilkesiana*, which represents 73% of the total of isolates, being 32% from “Marginata” and 43% from “Musaica”. After a morphological grouping of

A. wilkesiana isolates, it was obtained 50 morphotypes. Some of the isolates are shown in **Figure 3**.

The varieties of *A. wilkesiana* presented a bigger endophytic community, with 82 isolates, when compared to the *C. esculenta* community, with 30 isolates. In *A. wilkesiana*, nine isolates, being eight from the variety “Marginata” and 1 from the variety “Musaica”, could not be genetically identified due to impossibility of the ITS region sequencing or because of a low rate of similarity (<95%) with the data available at the GenBank. The identified isolates of the attractive plant (*A. wilkesiana*) are listed in **Table 1**, in which is evident that there are differences in the fungal composition of the endophytic communities of the *Acalypha wilkesiana* varieties, “Marginata” and “Musaica”. In the variety “Marginata”, the genus *Alternaria* sp. and *Diaporthe* sp. were the most frequent (with four isolates each) and in the variety “Musaica”, the genus *Colletotrichum* sp. (with four isolates), *Alternaria* sp. and *Nigrospora* sp. (with three isolates each) were the most found in the community.

Thirty endophytic fungi were isolated from the leaves of *C. esculenta*, from which five were recovered from young leaves and thirteen from the senescent ones. Three isolates from young leaves and one from the older leaves could not be identified because, although the ITS amplification was successful, the sequencer was not able to sequence the TV6, TN2, TN4 and TN5 samples, and we were not able to obtain consensus sequences for comparison to GenBank.

In **Table 2** are listed the identified genus from the isolates of young and old leaves of the less attractive plant *C. esculenta*. In the table, it is observable the difference in quantity and composition of the endophytic communities of the two leaf ages.

Comparison of the fungal communities.

Figure 1 shows the endophytic diversity of *Acalypha wilkesiana* (of both cultivars

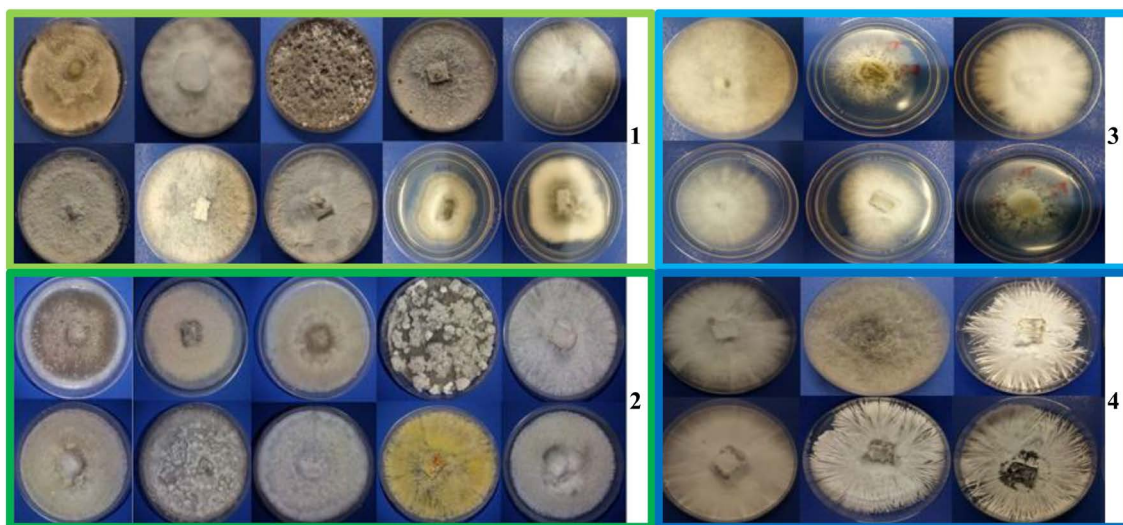


Figure 3. Diversity of the morphotypes isolated from the varieties “Marginata” (Group 1) and “Musaica” (Group 2) of *A. wilkesiana* and from the different ages of *C. esculenta* leaves. Group 3 contain the isolated from young leaves, and Group 4 the isolates from senescent leaves.

Table 1. Identified genus-taxa for each of the attractive plant's cultivar (*A. wilkesiana*) cultivar, with similarity higher than 95% and E. value=0.0.

Cultivar	Isolate	GenBank code	Similarity	Identification
Marginata	MA1	MN458518.1	99.67%	<i>Colletotrichum</i> sp.
	MA2	JQ341087.1	97.70%	<i>Xylaria</i> sp.
	MA3	JQ936257.1	99.66%	<i>Diaporthe</i> sp.
	MA4	FJ799941.1	98.47%	<i>Diaporthe</i> sp.
	MA5	HG779020.1	99.83%	<i>Curvularia</i> sp.
	MA6	MN540457.1	100%	<i>Colletotrichum</i> sp.
	MA7	MN458523.1	100%	<i>Diaporthe</i> sp.
	MA8	KX925567.1	98.59%	<i>Alternaria</i> sp.
	MA9	KP133219.1	99.82%	<i>Nemania</i> sp.
	MA10	MF380823.1	96.93%	<i>Colletotrichum</i> sp.
	MA11	KY568987.1	98.97%	<i>Coniothyrium</i> sp.
	MA12	KR093853.1	95.83%	<i>Phomopsis</i> sp.
	MA13	MG747466.1	97.48%	<i>Diaporthe</i> sp.
	MA14	MN685225.1	100%	<i>Alternaria</i> sp.
	MA17	MN044802.1	99.83%	<i>Alternaria</i> sp.
	MA19	MG753547.1	99.80%	<i>Preussia</i> sp.
	MA20	KX397027.1	98.85%	<i>Phomopsis</i> sp.
	MA23	MF927538.1	98.02%	<i>Xylaria</i> sp.
	MA25	MN622992.1	99.66%	<i>Alternaria</i> sp.
	MA28	KM979832.1	97.59%	<i>Phomopsis</i> sp.
	MA29	JN153062.1	99.30%	<i>Phomopsis</i> sp.
Musaica	MU1	KM979832.1	99.54%	<i>Phomopsis</i> sp.
	MU2	MF070235.1	99.58%	<i>Diaporthe</i> sp.
	MU3	MF375899.1	99.33%	<i>Pestalotiopsis</i> sp.
	MU5	DQ235676.1	98.97%	<i>Phomopsis</i> sp.
	MU6	MN686286.1	99.79%	<i>Colletotrichum</i> sp.
	MU7	MF375899.1	99.78%	<i>Pestalotiopsis</i> sp.
	MU8	KR093890.1	99.55%	<i>Curvularia</i> sp.
	MU9	MN639709.1	100%	<i>Nigrospora</i> sp.
	MU10	KR093890.1	99.60%	<i>Curvularia</i> sp.
	MU11	JQ936101.1	99.59%	<i>Cochliobolus</i> sp.
	MU12	MN636331.1	99.45%	<i>Alternaria</i> sp.
	MU13	MK881738.1	97.87%	<i>Colletotrichum</i> sp.
	MU14	MG976425.1	97.49%	<i>Nigrospora</i> sp.
	MU15	MF076603.1	99.84%	<i>Colletotrichum</i> sp.

Continued

MU16	KX578788.1	99.84%	<i>Colletotrichum</i> sp.
MU17	LC474421.1	100%	<i>Colletotrichum</i> sp.
MU18	MK979377.1	98.14%	<i>Alternaria</i> sp.
MU19	MN486553.1	99.62%	<i>Nigrospora</i> sp.
MU20	MH301314.1	99.48%	<i>Alternaria</i> sp.
MU21	MH864407.1	99.51%	<i>Bipolaris</i> sp.

Table 2. Identified genus-taxa for each of the repellent plant leave's ages (*C. esculenta*), with similarity higher than 95% and E. value = 0.0.

Plant	Leaf Age	Isolate	GenBank code	Similarity	Identification
<i>Colocasia esculenta</i>	Senescent	TV1	JQ341100.1	98.27%	<i>Nemania</i> sp.
		TV2	JQ760952.1	99.79%	<i>Nemania</i> sp.
		TV3	MG976425.1	99.49%	<i>Nigrospora</i> sp.
		TV4	JQ341083.1	98.50%	<i>Xylaria</i> sp.
		TV5	FJ799949.1	99.84%	<i>Xylaria</i> sp.
		TV7	JQ341064.1	97.51%	<i>Xylaria</i> sp.
		TV8	MH370554.1	99.26%	<i>Fusarium</i> sp.
		TV9	MN082533.1	99.16%	<i>Fusarium</i> sp.
		TV10	MH370554.1	98.86%	<i>Fusarium</i> sp.
		TV11	MH370554.1	99.18%	<i>Fusarium</i> sp.
		TV12	KJ471523.1	95.91%	Xylariaceae sp.
		TV13	MK834675.1	100%	<i>Nigrospora</i> sp.
<i>Colocasia esculenta</i>	Young	TN1	KU604568.1	97.12%	<i>Hypoxylon</i> sp.
		TN3	FJ799950.1	95.94%	<i>Xylaria</i> sp.

“Marginata” and “Musaica” together) and *Colocasia esculenta* (of both leaf ages together). *A. wilkesiana* presented a larger diversity of fungi genus when compared with the less attractive plant, *C. esculenta*. This difference can be verified in **Figure 4** because of the different colors that composes the pie charts, since each color below represents a different endophytic fungus genus that was found in this work. It is also evident in **Figure 4** that the plants share some genus, represented by the colors orange, light yellow and blue.

The two endophytic communities have some genus in common. The cultivar “Marginata” shares the genus *Xylaria* sp. with the two leaf ages of Taro (*C. esculenta*) and the genus *Nemania* sp. with the senescent leaves of Taro. The cultivar “Musaica” shares the genus *Nigrospora* sp. with the senescent leaves of the less attractive plant. Although both *A. wilkesiana* cultivars have the same number of fungi genus, they share only 5 of them, which are not present in *C. esculenta* leaves, being the most frequent *Alternaria* sp. and *Colletotrichum* sp., followed by *Phomopsis* sp., *Diaporthe* sp. and *Curvularia* sp. The genus *Coniocyrtium* sp.

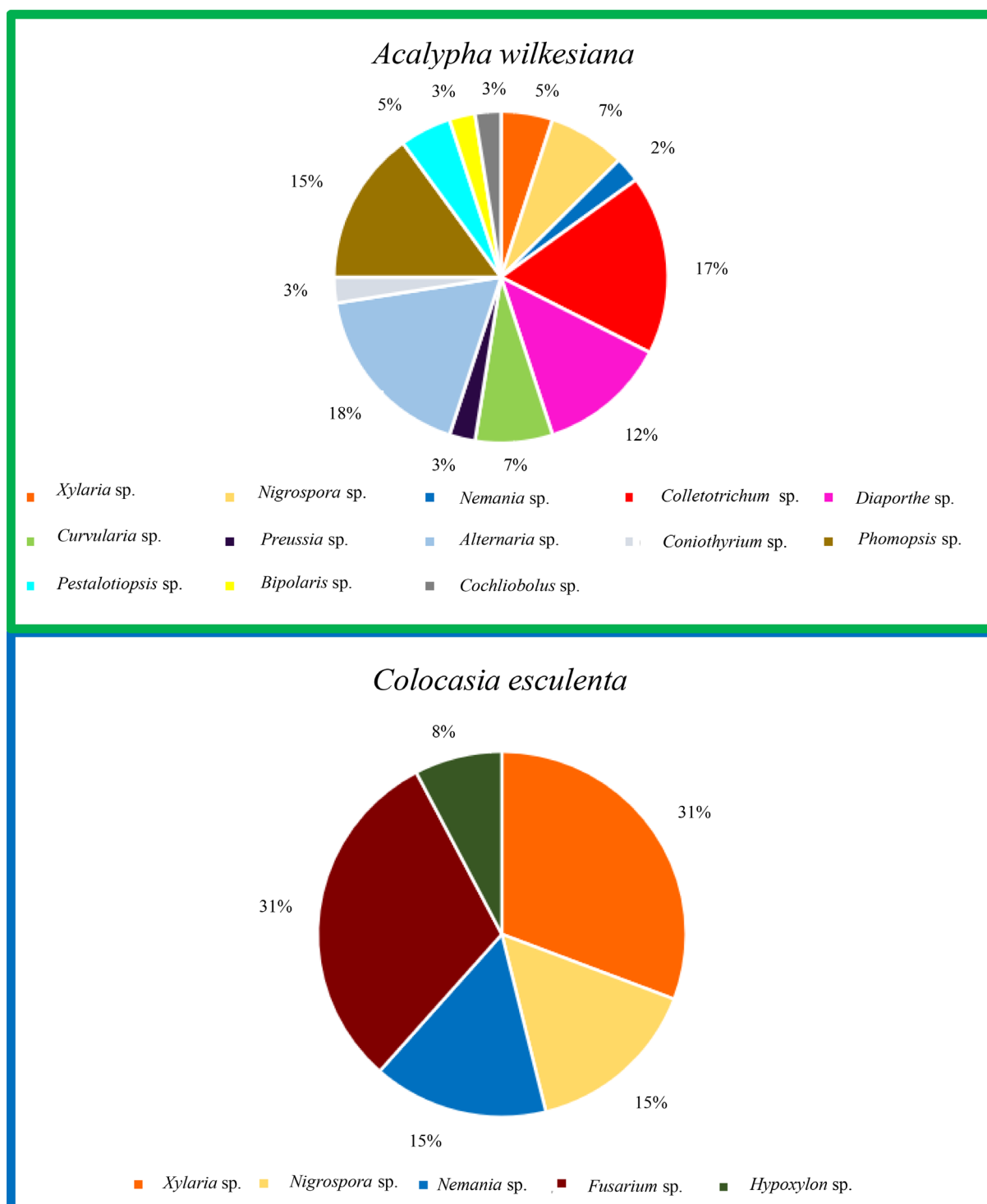


Figure 4. Community's composition of *A. wilkesiana* and of *C. esculenta*. Each identified genus is represented by a different color, putting in evidence that the endophytic communities have different compositions.

and *Preussia* sp. were found only in “Marginata” leaves, and *Bipolaris* sp., *Cochliobolus* sp. and *Pestalotiopsis* sp. were found only in “Musaica” leaves. In *C. esculenta*, the less attractive plant, the genus found exclusively in its leaves were *Fusarium* sp. in the old leaves and *Hypoxyylon* sp. in the young leaves.

4. Discussion

In the present study, it was done the analysis of the microbiota of cultivable endophytic fungi present in the plant species *A. wilkesiana* (“Marginata” and “Musaica”) and *C. esculenta* var. *esculenta* (young and senescent leaves). It was put in evidence that the endophytic fungi communities differ between the attractive plant to the leaf-cutting ants *Atta sexdens* (*A. wilkesiana*) and the less attractive plant (*C. esculenta*). Among the 300 thousand plant species described till today, each one is a host of, at least, one endophytic microorganism [37], what can constitute a type of plant defense against the leaf-cutting ant’s [7] and other pathogen microorganism’s attack, since the endophytic fungi can have an antibiotic action via its extracellular bioactive metabolites [38]. There are evidences that the leaf-cutting ants prefer entering the colony with leaves that have endophytic fungi with antifungal properties not directed to the symbiont *Leucoagaricus gongylophorus*, and by that protecting the colony from parasite fungi if necessary [14].

Overall, most of the fungal community isolated from *A. wilkesiana* is known by the production of bioactive compounds, including antibiotics [37] [39]-[44]. *Phomopsis* sp. is also known for having the capacity to survive the chemical and physical cleaning done by the ants before entering their colonies [45], besides being cited as a fungal genus well accepted by the attine ants, such as the genus *Colletotrichum* sp., *Pestalopsis* sp. And *Xylaria* sp. [14]. Probably, the attraction of a plant is related to the volatile compounds produced by the endophytic fungi. As these fungi can produce antibiotics that can harm the *L. gongylophorus*, the presence of determined compounds may signalize the toxicity or nutritional potential of a plant and influence in the decision of the ants to forage or not a plant.

This could indicate that the leaf preference by the *Atta* sp. ants is related to the presence of determined endophytic fungi, and not by the quantity of fungus present in a leaf, contradicting previous works that have put in evidence that these ants prefer leaves with less quantity of endophytic [46] [47], that, in this study, turned out to be *C. esculenta*, previously classified as less attractive leaves to the leaf-cutting ants. As *Hypoxyton* sp. and *Fusarium* sp. were genus found only in *C. esculenta*, they could be the ones causing the less attractiveness of the host plant to the leaf-cutting ants.

Hypoxyton sp. is a fungal genus that possess an antifungal and cytotoxic potential via its metabolic activities [48] and *Fusarium* sp., previously identified in the rejected material by the leaf-cutting ants, during the observations of Rocha and collaborators [14]. *Fusarium* sp. was found only in the senescent leaves of *C. esculenta*, which has not presented fungi of the genus *Diaporthe* sp., found in *A. wilkesiana*. These two fungal genera are suggested to have antagonist actions toward each other [49], what can explain the presence of each one in different plants and suggest a relation between the attractiveness of *Diaporthe* sp. to the leaf-cutting ants, as it is a genus present in *A. wilkesiana* and not in *C. esculenta*.

Workers of *A. sexdens* explore more the plant's apex when compared to its other regions, that being an innate behavior of the leaf-cutting ants [27]. The plant's apex is the place where most of the young leaves are, which can provide more nutrients to the ants and, in *C. esculenta*, the young leaves have a smaller diversity of endophytic fungi when compared to the old leaves of the same plant. The ant's choice for younger leaves could be related to the ease of cutting, lack of endophytes, their nutritional value or all these factors together, but it is a difficult question to be assessed [50]. Even though ants may remove more material from weaker and thinner leaves, the endophytic load does not differ within these traits, indicating that leaf-cutting ants are not influenced only by physical leaf's attributes [47] but by multiple factors which are difficult to be assessed alone [46].

Coblentz and Van Bael [46] and Bittleston *et al.* [47], suggested that the leaf-cutting ants have a preference for leaves with less quantity of endophytic fungi and Van Bael, Estrada and Wscilo [51] found out that these ants prefer to cut young leaves of plants. The presence of different fungi in the analyzed plants suggests that the composition of the plant's endophytic communities affects its attractiveness to the leaf-cutting ants, and not only the quantity of fungi as, according to Mighell and Van Bael [9], the ants respond differently to each endophytic fungi. They can alter the leaf's chemical characteristic, which is associated with the ant's preference for determined plants [52], and could explain why *A. wilkesiana* has a bigger quantity of endophytic fungi than *C. esculenta*, but a different diversity, and be more attractive to the leaf-cutting ants *A. sexdens*.

5. Conclusion

This study's data suggest that the endophytic fungal communities of the attractive plant *A. wilkesiana*, and the less attractive plant, *C. esculenta* are different in the quantity of isolated fungi and in the genus found in each one, suggesting a relation between the endophytic mycobiota and the preference of cutting by the *A. sexdens* workers. Our results suggest that the leaf-cutting ants may prefer leaves that have specific endophytic fungi, that fungi such as *Hypoxyton* sp. and *Fusarium* sp. may be responsible for the less attractiveness of a plant and that the endophytic community composition can influence the attack of those plants by the leaf-cutting ants.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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