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Soluble Compounds of Filamentous Fungi Harm the Symbiotic Fungus of Leafcutter Ants

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Abstract
Chemical compounds are key to understand symbiotic interactions. In the leafcutter ant-microbe symbiosis a plethora of filamentous fungi continuously gain access the ant colonies through plant substrate collected by workers. Many filamentous fungi are considered transient in attine ant colonies, however, their real ecological role in this environment still remains unclear. A possible role of these microorganisms is the antagonism towards *Leucoagaricus gongylophorus*, the mutualistic fungus that serve as food for several leafcutter ant species. Here, we showed the antagonism of filamentous fungi isolated from different sources, and the negative impacts of their metabolites on the growth of the ant-fungal cultivar. Our results demonstrate that the chemical compounds produced by filamentous fungi can harm the mutualistic fungus of leafcutter ants.

Introduction

Symbiotic interactions are mediated by secondary metabolites which indicate their harmonic or inharmonic interface [1–3]. These metabolites translate the chemical complexity of symbioses, and are key components to establish the relations between organisms and the environment. An example of a complex interaction is the mutualism maintained by fungus-growing ants (Hymenoptera: Attini: Attina, hereafter named "attine ants") [4].

These insects cultivate a mutualistic fungus as food source for their colonies; in turn, the ants disperse, protect, and feed the fungal cultivar [5, 6]. Among the 40 described leafcutter ant species [7], *Atta sexdens* is generally considered an agricultural pest [8], due to the large amounts of fresh leaves and flowers they forage to nourish the mutualistic partner, *Leucoagaricus gongylophorus* (Basidiomycota: Agaricales: Agaricaceae). This fungus clusters in clade A in the phylogeny of the ant-fungal cultivars along with clade B fungi cultivated by other leafcutter ant species [9].

Several micro-organisms have been described as symbionts on colonies of leafcutter ants. The mycotrophic fungus genus *Escovopsis* [10, 11] is thought to be a parasite that presents chemical recognition and mechanisms towards the fungal cultivar [12–15]. Antibiotic-producing actinobacteria provide chemical defenses against this parasite [16, 17]. In addition, black yeasts found on the ant cuticles are thought to be antagonist due to the inhibitory compounds against the helper actinobacteria [18, 19].

On the other hand, in this multipartite symbiosis there are other microorganisms whose functions are still not clear. This is the case of diverse filamentous fungi that gain access of the colonies through plant substrate [20]. Therefore, non-symbiotic fungi can also be prevalent in fungus gardens [21], and become a threat to the system [22]. Ants developed a plethora of defensive behaviors and chemical traits over their evolution, to protect the mutualistic fungus, such as grooming and weeding of fungus gardens, metapleural and mandibular gland secretions [23–25], indicating the undesirable nature of these filamentous fungi. Due to this complex defensive system, these filamentous fungi are currently considered transient in such system [20, 26, 27].

Nevertheless, the real impact and modes of action of the invading filamentous fungi against the ant cultivar are still elusive. Considering fungus–fungus interactions, several studies showed growth inhibition of *L. gongylophorus* by filamentous fungi [14, 28–32]. Such interactions can be

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understood as interference and/or exploitation competition [33]. Here, we determined the inharmonic nature of filamentous fungi towards *L. gongylophorus* and how their antagonism is mediated by soluble inhibitory metabolites.

Materials and Methods

Filamentous Fungi

A total of ten filamentous fungi (Table 1) were used to evaluate their antagonistic potential towards *L. gongylophorus*. These fungi are maintained in the collection of the Laboratory of Fungal Ecology and Systematics (LESF) as conidia suspensions in glycerol 10% at -80 °C. All fungi were revived on potato dextrose agar medium (PDA, Acumedia, pH 5.6 ± 0.2) and incubated at 25 °C, for 7 days in darkness. Because invading filamentous fungi have diverse sources we used isolates obtained from: (i) fungus gardens (*Escovopsi*s sp., *Escovopsioides nivea, Fusarium oxysporum, Syncephalastrum* sp., and *Trichoderma hamatum*); (ii) soil (*Trichoderma afroharzianum,* and *Trichoderma spirale*); and (iii) as endophytes in leaves of *Theobroma cacao* (*Clonostachys* sp., *Fusarium equiseti*, and *Verticillium* sp.). These fungi were previously reported from leaf-cutting ant gardens [21, 34, 35].

The mutualistic fungus *L. gongylophorus* (FF2006) was isolated from a mature laboratory colony of *A. sexdens*. This fungus is kept by periodic transfers every 15–20 days on culture medium supplemented with oatmeal extract (g L^{-1}: 50 oat flakes, 5 NaCl, 10 glucose, 5 peptone, 10 malt extract, and 10 Agar) at 25 °C in darkness [43].

Dual Culture Bioassays

To evaluate the interaction between filamentous fungi and *L. gongylophorus* we carried out in vitro experiments. Fifteen days-old mycelium fragments of FF2006 (8 mm in diameter) were inoculated on PDA at a distance of 1.5 cm from the plate edge. Plates were incubated for 17 days at 25 °C in darkness. Then, mycelium fragments (8 mm in diameter) of each filamentous fungus previously grown on malt agar 2% (MA2%) were inoculated on the opposite side of the fungal cultivar at distance of 1.5 cm from the plate edge [31]. This setup was incubated for 14 days at 25 °C in darkness, and the colony diameter (in cm) of both fungi was measured. Each experimental combination and control groups were performed with six plates. Two control groups were set up: the first group consisted on PDA plates containing only the mutualistic fungus. To account for the natural growth variations of FF2006, five set of control plates were prepared. The second set of control plates were inoculated only with the filamentous fungi.

Filamentous fungi growth was compared daily with the respective control (fungi growing in the absence of FF2006) by a Mann–Whitney *U* test with an alpha threshold of 0.05. These comparisons were carried out separately for each day, not accounting the time (days) as a dependent variable. For the mutualistic fungus, we first used one-way analysis of variance to ensure absence of differences between all control

Table 1 Filamentous fungi used in the bioassays

Fungal ID	Fungi	Isolation source	City/state[b]	Ecological role[c]	References
LESF 130	*Syncephalastrum* sp.	Fungus garden of *Atta sexdens*	Corumbataí/SP	Pathogen of the ant cultivar	[22]
LESF 178	*Escovopsis* sp.	Fungus garden of *Atta sexdens*	Corumbataí/SP	Parasite of the ant cultivar	[10]
LESF 288	*Fusarium oxysporum* species complex[a]	Fungus garden of *Acromyrmex ambiguus*	Nova Petrópolis/RS	Plant pathogen	[36]
LESF 330	*Trichoderma hamatum*	Fungus garden of *Acromyrmex* sp.	Camacan/BA	Mycoparasite	[37]
LESF 412	*Verticillium* sp.	Leaves from *Theobroma cacao*	Ilhéus/BA	Plant pathogen	[38]
LESF 422	*Clonostachys* sp.	Leaves from *Theobroma cacao*	Ilhéus/BA	Mycoparasite (some species)	[39]
LESF 424	*Fusarium equiseti* species complex	Leaves from *Theobroma cacao*	Ilhéus/BA	Endophyte	[40]
LESF 542	*Trichoderma afroharzianum*	Soil	Botucatu/SP	Saprotroph/mycoparasite[d]	[41, 42]
LESF 549	*Trichoderma spirale*	Soil	Botucatu/SP	Saprotroph/mycoparasite[d]	[41, 42]
LESF 592	*Escovopsioides nivea*	Fungus garden of *Acromyrmex* sp.	Camacan/BA	Antagonist of ant cultivar	[32]

[a]Species complex: species groups that present similar morphologies but show differences in their phylogenies

[b]BA: Bahia; RS: Rio Grande do Sul; SP: São Paulo

[c]Ecological role was assigned according to the cited references

[d]General ecological role attributed to the genus *Trichoderma*. Although we assumed these ecological roles for both species, further studies are necessary to confirm these assumptions

groups (cultivar growing in the absence of filamentous fungi) with an alpha threshold of 0.05 (Figure S1). Then, we selected the distribution with the largest interquartile range. Using the selected control group, we compared it to the growth in the presence of filamentous fungi by a two sample *t* test (Welch's *t* test) with an alpha threshold of 0.05, on the 10th day of incubation. To assess inhibition differences between filamentous fungi, we carried out one-way analysis of variance and Scott–Knott test with an alpha threshold of 0.05 [44]. The analyses were performed in R v.3.3.3 [45].

Assays Using Metabolic Compounds

To evaluate the inhibition of *L. gongylophorus* by metabolic compounds, culture filtrates were obtained from all filamentous fungi used in the dual culture bioassays. Each fungus was cultured on MA2% for 7 days at 25 °C in darkness. After incubation, conidia suspensions were prepared and standardized in a Neubauer chamber to 10^6 conidia mL^{-1}. The suspensions were inoculated into Erlenmeyer flasks containing 90 mL of malt extract broth 2%. Flasks were incubated at 25 °C for 8 days at 120 rpm. Because the majority of the filamentous fungi used in the assays are fast-growing, they sustained considerable biomass in these conditions.

After incubation, the culture medium was filtered (Whatman filter paper, grade 1) and centrifuged at 23.478×*g* for 15 min. The supernatant was recovered and filtered in 0.45 µm membrane. Aliquots of 5 mL of filtrate were added in 5 mL of culture medium [43] (double strengthen) supplemented with oatmeal extract. This medium was used for the experiments based on the growth stability of the mutualistic fungus [43, 46]. Plates were inoculated at the center with a mycelium fragment (8 mm in diameter) of the mutualistic fungus. Six plates were performed for each filtrate, and their control was performed with 5 mL of liquid malt 2% instead of the filtrate. Differences between the final growth diameter of the mutualistic fungus in the presence of each filtrate and the control after 14 days were assessed using two sample *t* test (Welch's *t* test) with an alpha threshold of 0.05 in R [45].

Results

Absence of Cultivar Defenses Against Filamentous Fungi

In dual cultures, 9 out of 10 isolates inhibited the mutualistic fungus at least 1.1 times higher than the control (two sample *t* test, $P < 0.01$ for all combinations; Fig. 1, Table S1). After 10 days of incubation, only *Clonostachys* sp. did not inhibit the growth of the mutualistic fungus (two sample *t* test, $P = 0.745$). We observed differences in inhibition of the fungal cultivar by the different filamentous fungi

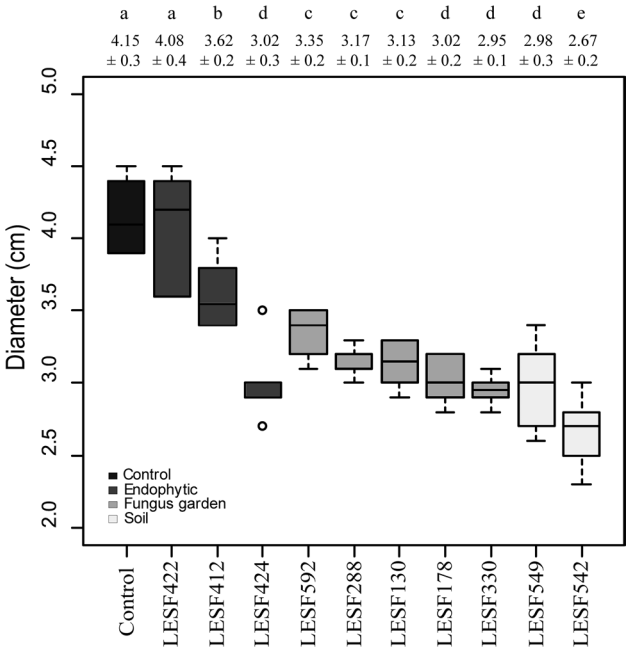

Fig. 1 Mutualistic fungi in dual culture assays. *Leucoagaricus gongylophorus* growth on PDA after 10 days in the presence of filamentous fungi and in their absence (control). Means (± SD) of the *L. gongylophorus* colony diameter (in cm) are indicate on the top of boxplots. Figures followed by different letters indicate significant differences (Scott–Knott at 5%)

(one-way analysis of variance, $P < 0.01$). *Trichoderma afroharzianum* presented the highest inhibition, while the lowest was observed for *Verticillium* sp. (Fig. 1). No association between isolation source and antagonism pattern was observed (Fig. 1). Moreover, *T. hamatum, F. equiseti*, and *Trichoderma spirale*, did not differ in their inhibitory activity with *Escovopsis* sp., the well-known mycotrophic fungus associated attine ant colonies.

Select Strains had Maximal Mycelial Growth in the Presence of the Ant Cultivar

Two filamentous fungi showed a maximized growth in the presence of mutualistic fungus (Table 2). *Escovopsis* sp. and *T. hamatum* exhibited mycelium growth 1.1 times higher in the 2nd and 3rd days in the presence of FF2006 compared to the respective controls (Mann–Whitney test, $P = 0.003$ for both fungi, Fig. 2). On the other hand, *Escovopsioides nivea* was the only fungus that showed the lowest growth in dual culture assays in the 2nd and 3rd days (Mann–Whitney test, $P = 0.005$ and 0.019, respectively), corresponding in a reduction of colony mycelium 2.1 and 1.4 times, respectively. After the 7th day of culture, no statistical differences were observed for all filamentous fungi compared to their respective controls (Fig. 2).

Table 2 Growth of filamentous fungi in the presence (dual culture) and absence (control) of the ant mutualistic fungus, *Leucoagaricus gongylophorus*

Fungal ID	2 Days		3 Days	
	Dual culture	Control	Dual culture	Control
LESF130	6.22 ± 0.72	5.97 ± 0.30	6.97 ± 0.08	7.00 ± 0.00
LESF178	**7.00 ± 0.00**	**3.78 ± 0.61**	**7.00 ± 0.00**	**5.15 ± 0.76**
LESF288	2.67 ± 0.33	2.55 ± 0.19	3.95 ± 0.28	3.63 ± 0.36
LESF330	**7.00 ± 0.00**	**5.67 ± 0.46**	**7.00 ± 0.00**	**6.43 ± 0.40**
LESF412	1.67 ± 0.21	1.63 ± 0.19	2.45 ± 0.32	2.67 ± 0.27
LESF422	1.77 ± 0.20	1.65 ± 0.19	2.47 ± 0.29	2.40 ± 0.33
LESF424	2.38 ± 0.38	2.28 ± 0.26	3.38 ± 0.39	3.47 ± 0.30
LESF542	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00
LESF549	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00	7.00 ± 0.00
LESF592	**1.92 ± 0.45**	**3.98 ± 0.71**	**3.83 ± 0.68**	**5.23 ± 0.72**

Figures indicate average diameter (in cm) and standard deviations of six plates. Means in bold indicate significant differences between filamentous fungi and their respective control in pairwise comparisons by Mann–Whitney test at 5%. No differences were observed on the 7th day

Antagonism Mediated by Soluble Compounds

The metabolites of eight out of ten filamentous fungi inhibited the growth of *L. gongylophorus* (Fig. 3a). After 14 days of experiment, only the culture filtrates of *Escovopsis* sp. and *F. equiseti* did not inhibit the growth of FF2006 (two sample *t* test, $P = 0.171$ and 0.288, respectively; Table S1). We observed changes in colony morphology of FF2006 when filtrates of *E. nivea, Syncephalastrum* sp., and *T. spirale* were present (Fig. 3b). A darkening of the center of the colony was observed probably due to the production of soluble metabolites. Curiously, similar morphological changes were detected in the dual culture assays using these fungi. No changes in *L. gongylophorus* colonies were observed when cultured in the presence of *Clonostachys* sp. filtrate (Fig. 3b).

Discussion

Metabolites are crucial for maintaining symbiotic interactions between social insects and microorganisms [12, 16, 47]. Interactions between attine ants and defensive microbial symbionts [16, 47] guarantee the stability of ant colonies against antagonists. Here, we showed the growth of the mutualistic fungus *L. gongylophorus* is inhibited in the presence of different filamentous fungi and their soluble metabolites. Because the genetic structure of *L. gongylophorus* suffered a long and irreversible process of domestication, this fungus is dependent upon the ants for protection [6, 48],

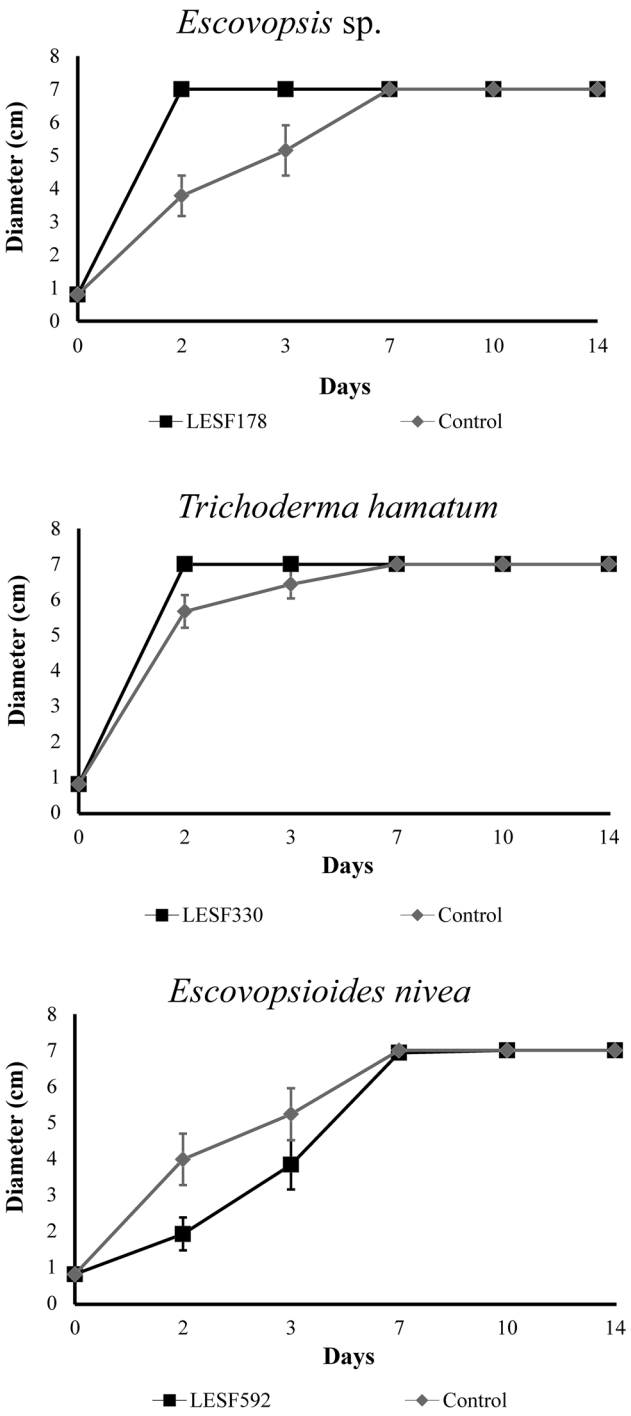

Fig. 2 Growth of *Escovopsis* sp., *Trichoderma hamatum*, and *Escovopsioides nivea*. Values indicate *Leucoagaricus gongylophorus* colony diameter (in cm) considering six plates. Black squares indicate the growth of filamentous fungi in dual culture with *L. gongylophorus*, and gray diamonds their respective control group in pure culture

particularly from harmful filamentous fungi that may threat the colonies when the worker force is depleted [21].

As expected, *Escovopsis* sp. had higher growth in presence of *L. gongylophorus*, since it has already been

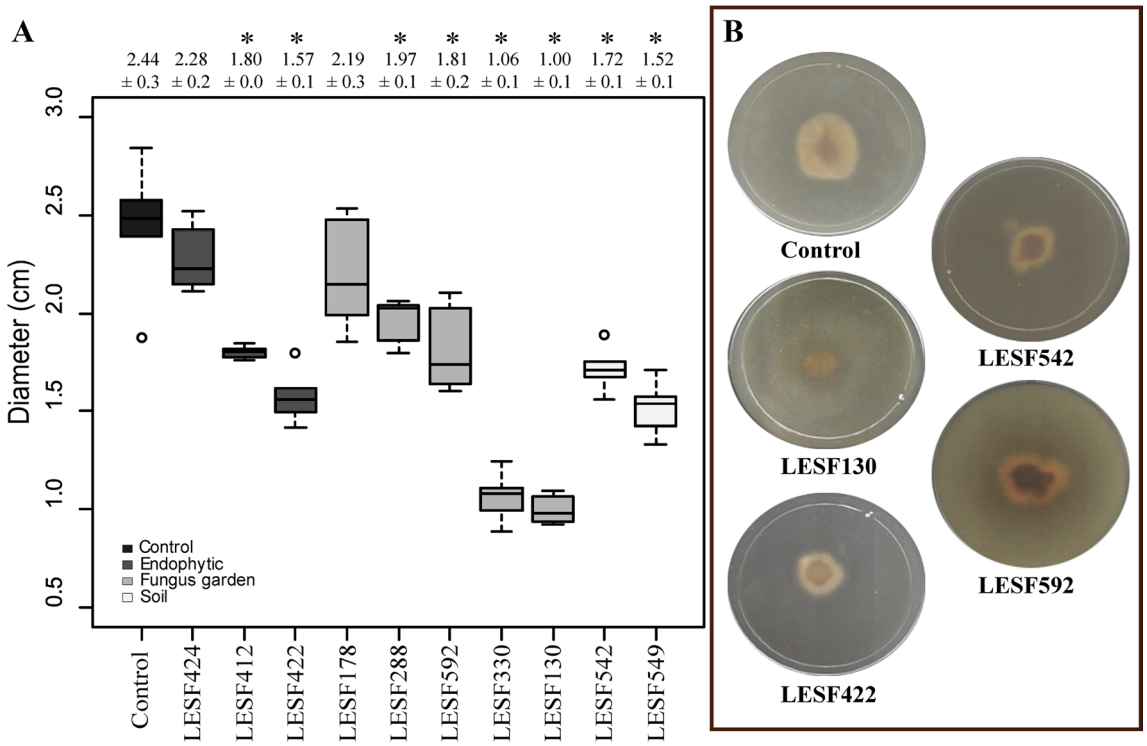

Fig. 3 Harmful nature of soluble metabolites on *Leucoagaricus gongylophorus* growth. **a** Values indicate the mean colony diameter (in cm) of six replicates after 14 days of experiment. Mean values (± S.D) followed by * indicate differences with control group (two sample *t* test at 5%). **b** Morphological aspect of *L. gongylophorus* colonies when grown in the presence of culture filtrates of different filamentous fungi. An atypical darkness as observed for cultures filtrates of *Syncephalastrum* sp. (LESF130), *Trichoderma afroharzianum* (LESF542), and *Escovopsioides nivea* (LESF592)

described as capable of host chemical recognition (chemotaxis) [13], and mechanisms of parasitism [14, 15]. *T. hamatum*, also showed maximized growth in the presence of the ant cultivar. This may be possibly related to the use of metabolites derived from FF2006 as nutrient source in culture, since high exploitation competition is a common trait of *Trichoderma*, known as an environment opportunistic fungus [41]. On the other hand, *E. nivea* showed delayed growth in the presence of *L. gongylophorus*, this result supports its less aggressive pattern observed previously [32], as well as the minor resistance of the mutualistic fungus to some filamentous fungi, even in absence of tending ants.

Trichoderma species have been reported as potential biological control agents against leafcutter ants, due to its antagonism towards the mutualistic fungal partner [28, 30]. In addition, *Trichoderma* is a well-known biological control agent of several plant pathogens [49, 50]. Here, *T. afroharzianum* showed the best inhibition results, and the *T. hamatum* and *T. spirale* have the same inhibition potential than *Escovopsis* sp., the well-known antagonist of this system. Future studies will demonstrate the usefulness of these strains as biological control agents.

Many endophytic fungi are also considered transients in attine ant colonies. It is known these endophytes cause an increase of time workers spend to process the foraged plant material [51]. Furthermore, some of these fungi cannot overcome the barriers of the mutualistic fungus [52, 53]. Recently, some of them have been described as bodyguards for their plant hosts, resulting in more rejection of plant material by the ants [52, 54]. Here, among the endophytes, only *Clonostachys* sp. did not present antagonistic activity against the ant-fungal cultivar. In contrast, *F. equiseti* also showed similar inhibition than *Escovopsis* sp. in dual culture assays. These results indicate that the plant material may carry some undesirable fungi for the ant colony, and in the absence of protection by the ants, such fungi may be a threat to the mutualistic fungus [53].

The metabolites produced by the different filamentous fungi used in the experiments impaired the *L. gongylophorus* development. However, this was not the case of *Escovopsis* in the present study. Although it exhibited inhibition in the dual culture assays (Fig. 1), no inhibition by metabolites was observed. A similar result was verified for *F. equiseti* (Fig. 1). Some studies demonstrated the production of compounds by *Escovopsis* [12, 32]. Thus, the lack of inhibitory activity observed in the present study may be related to absence of inhibitory compounds under the growth conditions we used, or by diverse mode of action associated

with competition for nutrients and space, in the dual culture assays.

Regardless of isolation source, all *Trichoderma* species produced inhibitory soluble metabolites in vitro. This reflects the expansion of genes related to important enzymes for mycoparasitism and saprotrophic lifestyle [42]. Also, the endophytic fungi *Verticillium* sp. and *Clonostachys* sp. showed inhibition of *L. gongylophorus* by soluble metabolites, such production indicates that even fungi outside a co-evolutionary scenario exhibit some sort of antagonism, especially in the absence of ant workers [21].

Simple sugars are present in the fungus gardens due to the degradation of plant material by *L. gongylophorus* [55], promoting a substrate with readily available nutrients. Thus, nutrient uptake and interference competition for available resources by saprotrophic and mycoparasitic fungi may be important in community establishment in the fungus garden. Production of metabolites can have an important role on colony disruption [12, 56], as indicated by production of active shearinines and emodin by *Escovopsis* sp. against its fungal host, the ants and their bacterial symbionts (actinobacteria). The search for bioactive metabolites it is also important to map out biocontrol strategies against crop pests.

Filamentous fungi from different sources and diverse ecological roles have potential as antagonists of the ant-fungal mutualism when defensive barriers are overcome [21]. Here, we showed that inhibitory metabolic compounds and/or nutritional competition promoted by diverse filamentous fungi harm the ant-fungal cultivar.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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