DÉBORA MELLO FURTADO DE MENDONÇA

VIRULENCE OF THE FUNGI *Escovopsis* AND *Escovopsioides* TO THE LEAFCUTTER ANT-FUNGUS SYMBIOSIS

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Magister Scientiae*.

VIÇOSA
MINAS GERAIS - BRASIL
2018

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T
M539v
2018

Mendonça, Débora Mello Furtado de, 1993-
Virulence of the fungi *Escovopsis* and *Escovopsioides* to the leafcutter ant-fungus symbiosis / Débora Mello Furtado de Mendonça. – Viçosa, MG, 2018.
ix, 52 f. : il. (algumas color.) ; 29 cm.

Texto em inglês.
Inclui apêndices.
Orientador: Simon Luke Elliot.
Dissertação (mestrado) - Universidade Federal de Viçosa.
Referências bibliográficas: f. 39-45.

1. *Atta*. 2. *Acromyrmex*. 3. Parasitismo. 4. Simbiose. I. Universidade Federal de Viçosa. Departamento de Entomologia. Programa de Pós-Graduação em Entomologia. II. Título.

CDD 22. ed. 595.796

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APROVADA: 27 de julho de 2018.

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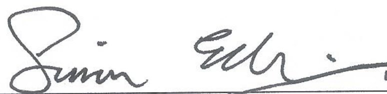
Simon Luke Elliot
(Orientador)

“É preciso força pra sonhar e perceber que a estrada vai além do que se vê.”

Los Hermanos

AGRADECIMENTOS

Em primeiro lugar, agradeço a Deus, pois sei que Ele está sempre ao meu lado me dando forças, sabedoria e serenidade.

Agradeço aos meus amados pais Penha e Sérgio por sempre terem me incentivado a estudar e me apoiado durante todo o mestrado, vocês são meus maiores exemplos. Também agradeço aos meus irmãos Sérgio Thiago, Carlos Eduardo e Dario por serem meus grandes amigos e companheiros.

À Universidade Federal de Viçosa e ao Programa de Pós-Graduação em Entomologia pela oportunidade.

À FAPEMIG pelo apoio financeiro.

À CAPES pelo suporte e apoio financeiro através do Programa Pesquisador Visitante Especial 2014 #88881.068090/2014-01.

Ao professor Simon Luke Elliot por ter me concedido a oportunidade de ser sua orientada de mestrado. Obrigada pela confiança e parceria. Sem dúvida foram dois anos de muitos desafios e aprendizado.

Aos meus queridos amigos do Laboratório de Interações Inseto-Microrganismo, pelas excelentes discussões sobre o trabalho, auxílio nos experimentos, troca de conhecimentos, correções e especialmente pela ótima convivência.

À técnica Verônica Fialho por sempre manter nosso ambiente de trabalho organizado, pelo auxílio no registro do projeto e demais assuntos relacionados à rotina de trabalho do laboratório.

Ao Sr. Manoel por todo o auxílio no campo, pelos ensinamentos e amizade. Agradeço também ao Cristian Oliveira por nos auxiliar nas coletas em campo e pela amizade.

À Marcela Caixeta por ter se tornado minha parceira nesse trabalho. Obrigada pelo apoio, discussões, auxílio durante a avaliação dos experimentos, correções, dicas e amizade.

À Camila Moreira pela coorientação, parceria e confiança. Obrigada por todas as discussões, auxílio na montagem e avaliação dos experimentos, correções, ensinamentos e amizade.

À Talitta Simões pela coorientação. Obrigada pelas discussões, sugestões, e correções.

Às secretárias da entomologia pelo auxílio nos assuntos burocráticos.

Ao professor Arne pelas sugestões sobre as análises estatísticas do trabalho.

Ao Thiago Kloss por sempre ter sido muito solícito, pelas sugestões e auxílio na realização das análises estatísticas.

Ao Marcos Mattos pelo auxílio no desenvolvimento das figuras ilustrativas referentes ao desenho experimental e os gráficos do trabalho. Obrigada também por sua amizade e apoio.

Ao querido estagiário, Gabriel Leite pelas discussões, auxílio na execução do experimento e excelente convivência.

À Silma Rocha pelas excelentes discussões, sugestões, correções e especialmente por todo apoio e amizade.

Ao professor André Rodrigues, à Camila Moreira e Thiago Kloss por terem aceitado o convite de participar como membros da banca de defesa deste trabalho.

Ao Inácio Fernandes por todo apoio e incentivo.

À Ana e a Fran, minhas queridas amigas e irmãs que Viçosa me deu de presente. Obrigada pelo apoio, companheirismo e ótima convivência.

A todos os meus queridos amigos de Viçosa e Visconde do Rio Branco. Vocês são muito importantes na minha vida, obrigada por me apoiarem e tornarem meus dias mais felizes e leves.

A todos meus familiares pelo apoio, incentivo e carinho.

A todas as pessoas que conviveram comigo durante este período e que contribuíram de algum modo com o desenvolvimento desse trabalho. Muito obrigada!

ABSTRACT

MENDONÇA, Débora Mello Furtado de, M.Sc., Universidade Federal de Viçosa, July, 2018. **Virulence of the fungi *Escovopsis* and *Escovopsioides* to the leafcutter ant-fungus symbiosis.** Advisor: Simon Luke Elliot. Co-advisors: Camila Costa Moreira and Talitta Guimarães Simões.

Eusocial insects interact with a diversity of parasites that can threaten their survival and reproduction. The amount of harm these parasites cause to their hosts (i.e. their virulence) can be influenced by numerous factors, such as the ecological context in which the parasite and its host are inserted. Leafcutter ants (genera *Atta* and *Acromyrmex*, Attini: Formicidae) are an example of a eusocial insect whose colonies are constantly threatened by parasites. These ants cultivate and use the fungus *Leucocoprinus gongylophorus* (Basidiomycota: Agaricales) as the colony main food source. Leafcutter ants also interact with the fungi *Escovopsis* and *Escovopsioides* (Ascomycota: Hypocreales), which are considered a highly virulent parasite and an antagonist to their fungal cultivar, respectively. Since both fungi are common inhabitants of healthy colonies, which remain growing and foraging, we hypothesized that they are of low virulence. However, this virulence could vary depending on the ecological context that the colonies are inserted. We therefore tested two hypotheses: (i) *Escovopsis* and *Escovopsioides* are of low virulence to colonies; (ii) virulence increases with decreasing of colony complexity. For this, we used three levels of complexity: queenright colonies (fungus garden with queen and workers), queenless colonies (fungus garden and workers, without queen) and fungus gardens (without any ants). Each level was exposed to conidial suspensions of *Escovopsis moelleri*, *Escovopsioides nivea*, *Trichoderma longibrachiatum* or to a control (water with Tween 80 ® + saline solution). *Trichoderma longibrachiatum* was used for comparison with *E. moelleri* and *E. nivea* since it is also a fungus commonly found in the colonies of leafcutter ants. The parameters evaluated were weight, midden production, amount of leaves cut by ants and survival of each colony complexity level. We also evaluated the *in vitro* interactions between these fungi with *L. gongylophorus* through a paired culture bioassay. Our results showed that, in general, these fungi were of low virulence to queenright colonies, while the queenless colonies and fungus gardens were suppressed. Moreover, *E. nivea* and *T. longibrachiatum* seems to be less aggressive than *E. moelleri*, this was observed in both, *in vivo* and *in vitro* experiments. The results highlight the importance of each element (queen, workers and fungus garden) in the leafcutter ants-

fungus symbiosis. Furthermore, we showed that *Escovopsis* and *Escovopsioides* may be not virulent to healthy colonies yet, depending on colony condition, the virulence level of *Escovopsis* can increase.

RESUMO

MENDONÇA, Débora Mello Furtado de, M.Sc., Universidade Federal de Viçosa, julho de 2018. **Virulência dos fungos *Escovopsis* e *Escovopsioides* na simbiose formigas cortadeiras-fungo.** Orientador: Simon Luke Elliot. Coorientadoras: Camila Costa Moreira e Talitta Guimarães Simões.

Os insetos eussociais interagem com uma diversidade de parasitas que podem ameaçar sua sobrevivência e reprodução. A quantidade de dano que esses parasitas causam ao seus hospedeiros (ou seja, sua virulência) pode ser influenciada por inúmeros fatores, como o contexto ecológico em que o parasita e seu hospedeiro estão inseridos. Formigas cortadeiras (gêneros *Atta* e *Acromyrmex*, Attini: Formicidae) constituem um exemplo de inseto eussocial cujas colônias são constantemente ameaçadas por parasitas. Estas formigas cultivam e utilizam o fungo *Leucocoprinus gongylophorus* (Basidiomycota: Agaricales) como a principal fonte de alimento da colônia. As formigas cortadeiras também interagem com os fungos *Escovopsis* e *Escovopsioides* (Ascomycota: Hypocreales), que são considerados um parasita altamente virulento e um antagonista do fungo que elas cultivam, respectivamente. Como ambos os fungos são habitantes comuns de colônias saudáveis, que permanecem crescendo e forrageando, nós hipotetizamos que eles são de baixa virulência. No entanto, esta virulência pode variar dependendo do contexto ecológico em que as colônias estão inseridas. Portanto, testamos duas hipóteses: (i) *Escovopsis* e *Escovopsioides* são de baixa virulência para colônias; (ii) a virulência aumenta com a diminuição da complexidade da colônia. Para isso, utilizamos três níveis de complexidade: colônias (jardim de fungo com rainha e operárias), colônias sem rainha (jardim de fungo e operárias, sem rainha) e jardins de fungo (sem formigas). Cada nível foi exposto a suspensões conidiais de *Escovopsis moelleri*, *Escovopsioides nivea*, *Trichoderma longibrachiatum* ou ao controle (água com Tween 80 ® + solução salina). *Trichoderma longibrachiatum* foi utilizado para comparação com *E. moelleri* e *E. nivea*, pois também é um fungo comumente encontrado nas colônias de formigas cortadeiras. Os parâmetros avaliados foram peso, produção de lixo, quantidade de folhas cortadas pelas formigas e sobrevivência de cada nível de complexidade da colônia. Nós também avaliamos as interações *in vitro* entre estes fungos com *L. gongylophorus* através de um bioensaio de cultura pareada. Nossos resultados mostraram que, em geral, esses fungos foram de baixa virulência para colônias, enquanto as colônias sem rainha e os jardins de fungo foram suprimidos. Além disso, *E. nivea* e *T. longibrachiatum* parecem ser menos

agressivos do que *E. moelleri*, e isto foi observado em ambos experimentos, *in vivo* e *in vitro*. Os resultados destacam a importância de cada elemento (rainha, operária e jardim de fungo) na simbiose formigas cortadeiras-fungo. Além disso, mostramos que *Escovopsis* e *Escovopsioides* podem não ser virulentos para colônias saudáveis, no entanto, dependendo da condição da colônia, o nível de virulência de *Escovopsis* pode aumentar.

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INTRODUCTION

Parasites can play an important role in many aspects of their hosts' life, threatening their survival and reproduction. The harm that parasites cause to their hosts, referred to as virulence (Frank, 1996), can be influenced by numerous factors, involving traits related to the parasite and to its host, in addition to the context in which both are to be found.

Theory about virulence evolution predicts that there is a relationship between parasites' virulence and their mode of transmission to new hosts (Ewald, 1987; Alizon et al., 2009). It has been suggest that vertically transmitted parasites tend to be less virulent in relation to horizontally transmitted parasites, because their fitness depends on their host´s reproductive success (Clayton & Tompkins, 1994). This has been demonstrated in some empirical studies (Bull et al., 1991; Clayton &Tompkins, 1994; Tompkins et al., 1996; Agnew & Koella, 1997; Stewart et al., 2005; Pagán et al., 2014) although it cannot be taken as a general rule, especially as many parasites can present both modes of transmission (Ebert, 2013; Cressler et al., 2015). Moreover, virulence is a result of the host-parasite interactions and can be influenced by more than one factor. For example, host lifespan can be an important factor in understanding the evolution of a parasite's (Watson, 2013). It is predicted that hosts with a shorter lifespan reduce the future opportunities of parasite transmission, thus parasites should grow faster within the host and be transmitted earlier, which reflects in a higher virulence (Nidelet et al., 2009). In addition, some parasites may present a virulence dependent on the context in which they are inserted, for example increasing their virulence when their hosts are under stressful conditions (Brown et al., 2000; Brown et al., 2003; Jokela et al., 2005; Manley et al., 2017).

As with all living organisms, eusocial insects such as ants, termites and some bees and wasps may be exposed to a diversity of parasites. These parasites include viruses, bacteria, fungi, among others (Schmid-Hempel, 1998) and the manner in which sociality could affect their evolution has been discussed in the literature. Some authors argue that long-lived colonies of insect societies represent a buffered and homeostatic environment, which tends to lead to a reduction in parasite's virulence (e.g. Hughes et al., 2008). Additionally, highly genetically diverse colonies of some eusocial insects, due to polyandrous queens (i.e multiple mating queen with several males), have been associated with a lower incidence of disease and better resistance to parasite infection (Hughes & Boomsma, 2004; Tarpy & Seeley, 2006; Seeley & Tarpy, 2007). Furthermore, the level of a parasite´s virulence can increase in some stressful stages of eusocial insects' life history, such as the colony-founding period (Brown et al., 2003).

Among eusocial insects, the leafcutter ants (genera *Atta* and *Acromyrmex*, Attini: Formicidae) are well-known to interact with a diversity of microorganisms that are present in their colonies, including parasites and mutualists (Pagnocca et al., 2012). These ants cultivate the fungus *Leucocoprinus gongylophorus* (Basidiomycota: Leucocoprini, Agaricales) (Mueller et al., 2017), using it as a food, while providing nutrients, protection and dispersion in return. The fungal cultivar of leafcutter ants is farmed in subterraneous chambers that shelter the "fungus garden". This structure is composed of the fungal cultivar mycelium and fragments of leaves and flowers that serve as a substrate to the fungus' growth. It is important to note that fungus gardens can also harbor a range of bacteria, yeasts and filamentous fungi (Carreiro et al., 1997; Currie et al., 1999a; Rodrigues et al. 2005; Rodrigues et al., 2008; Suen et al., 2010). Fungi of the genera *Escovopsioides* and

Escovopsis (Ascomycota: Hypocreales) are examples of microorganisms that are commonly found in the fungus gardens (Augustin et al., 2013; Reis et al., 2015).

The genus *Escovopsioides* presents only one species described to date, *Escovopsioides nivea* (Augustin et al., 2013) and its role in colonies of leafcutter ants is as yet poorly understood. A study performed by Varanda-Haifig et al. (2017) demonstrated that this fungus is an antagonist of *L. gongylophorus* capable of inhibiting the growth of the fungal cultivar in culture medium. The genus *Escovopsis* is phylogenetically related to *Escovopsioides* (Augustin et al., 2013) and is considered a specialized parasite of the fungal cultivar of leafcutter ants (Currie et al., 1999a). Some early studies suggested that this parasite is highly virulent to its host, capable of causing the death of infected colonies and reducing the fungus garden biomass as well as the production of new ant individuals (Currie et al., 1999a; Currie, 2001). Based on the results of these studies, it has become disseminated in the literature that this fungus actually represents a highly virulent parasite (Currie et al., 1999a; Currie, 2001; Currie & Stuart, 2001; Currie et al., 2003a, b; Stearns & Hoekstra, 2005; Hölldobler & Wilson 2009; Farji-Brener et al., 2016; Verza et al., 2017). However, it is important to discuss some points related to this. Firstly, *Escovopsis* is often found in the fungus garden of healthy colonies that remain foraging and growing (Augustin, 2011; D.M.F.M., personal observation). This indicates that *Escovopsis* may not always present a high virulence as has been suggested. Second, one of the pioneering studies that investigated the impact of *Escovopsis* on the ant-fungus symbiosis was conducted using newly-founded colonies of 10-12 weeks old (Currie, 2001). Thus, it is possible these colonies were still fragile, and in this condition, could suffer a negative impact because of this parasite. Third, some studies investigated the mode

of *Escovopsis* transmission between colonies of leafcutter ants and suggested that the parasite seems to be horizontally transmitted (Currie et al., 1999a; Moreira et al., 2015; Augustin et al., 2017) which has been taken spuriously as evidence of high virulence. Nevertheless, the results that indicated horizontal transmission of *Escovopsis* cannot exclude the possibility of vertical transmission, which could support a low virulence consistent with theory of virulence evolution. In addition, horizontal transmission does not automatically indicate high virulence as in the case of the common cold of humans for example. Finally, another important issue is that, like other parasites, the level of virulence presented by *Escovopsis* may also vary depending on the ecological context in which it is inserted.

It is also important to consider that colonies of leafcutter ants function as superorganisms. All the components of the colonies, besides the fungus gardens, as the reproductive and non-reproductive castes engage in different functions, such as reproduction, defence and foraging. These individuals act similarly to the germ and somatic cells in the body of multicellular organisms (Cremer & Sixt, 2009). Thus, all elements that comprise the colonies act as a cooperative unit (Detrain & Deneubourg, 2006) and its components cannot survive and reproduce without one another (Cremer et al., 2017). In this sense, studies that investigate the impact of *Escovopsis* and *Escovopsioides* to the fungal cultivar of leafcutter ants must be conducted considering the role of the ants in the symbiosis as well as the other elements that comprise the colonies. Although this is important to consider, some studies that evaluated the impact of *Escovopsis* were performed using *in vitro* assays, colonies without queens (referred to as queenless colonies) or the fungus gardens without ants (Folgarait et al., 2011a, b; Wallace et al., 2014; Marfetán et al., 2015). These studies are very important to understand the antagonism of

Escovopsis with *L. gongylophorus* and how this may affect leafcutter ant colonies. However, since the virulence of parasites can be influenced by several factors, it may be that the use of only parts of the superorganism to conduct experiments also influences the level of virulence presented by *Escovopsis*.

In this context, our objective was to test two hypotheses: First, *Escovopsis* and *Escovopsioides* are of low virulence to healthy colonies of leafcutter ants; second, the virulence of these fungi varies according to the level of complexity that a colony presents. Here, we considered that a low virulent parasite can cause some negative impact to its host, however not enough to compromise its survival. On the other hand, a high virulent parasite is capable to lead its host dead. In addition, we also considered that a higher level of colony complexity involves the interaction between the queen and workers as well the fungus gardens (which include the fungal cultivar and the other microorganisms that are also present). As we disregard one or more components of this symbiosis, the level of complexity of this interaction decreases. The fungal cultivar *L. gongylophorus* cultivated in culture medium (*in vitro*) was considered the simplest level of the interaction since it excludes all the other elements that compromise a colony. Therefore, we considered those colonies, queenless colonies, fungus gardens and only *L. gongylophorus in vitro* represent decreasing levels of complexity.

To test our hypotheses, we evaluated the impact of the parasite *Escovopsis* and the antagonist *Escovopsioides in vivo* and *in vitro* (experiments I and II, respectively). In the first experiment, we divided the leafcutter ant colonies in three different complexity levels: (i) queenright colonies (fungus garden with queen and workers), (ii) queenless colonies (fungus garden and workers, without queen) and (iii) fungus gardens (fungus gardens without any ants). Each one of these

complexity levels was exposed to conidial suspensions of *Escovopsis* and *Escovopsioides*. The second experiment was conducted through a paired culture bioassay, between these two fungi with *L. gongylophorus*. Our expectation was that *Escovopsis* and *Escovopsioides* would not represent a threat to queenright colonies, however, the virulence of these fungi would increase as the complexity levels of the colonies decreases.

MATERIAL AND METHODS

Experimental approach

We conducted two experiments to investigate the impact of the parasite *Escovopsis* and the antagonist *Escovopsioides* on the leafcutter ant-fungus symbiosis. In the first experiment (Experiment I), we evaluated the impact of these fungi, *in vivo*, on leafcutter ants colonies in three different complexity levels: (i) queenright colonies (fungus garden + queen + workers; Fig.1A), (ii) queenless colonies (fungus garden + workers, without queen; Fig.1B) and (iii) fungus gardens (fungus gardens without any ants; Fig.1C). For this, we exposed the different levels of colonies to the following treatments: (a) conidial suspensions of *Escovopsis moelleri* and (b) *Escovopsioides nivea*. In the second experiment (Experiment II), we evaluated the interaction of *Escovopsis* and *Escovopsioides* with the fungal cultivar, *in vitro*, through a paired culture bioassay (Fig.1D). The purpose of this experiment was to exclude most of the elements that comprise a colony resulting in the simplest level of complexity, composed of only the fungal cultivar, *L. gongylophorus*. Thus, from these two experiments, we tested the hypotheses that *Escovopsis* and *Escovopsioides* are of low virulence for colonies, however, the virulence can increase depending on the level of colony complexity.

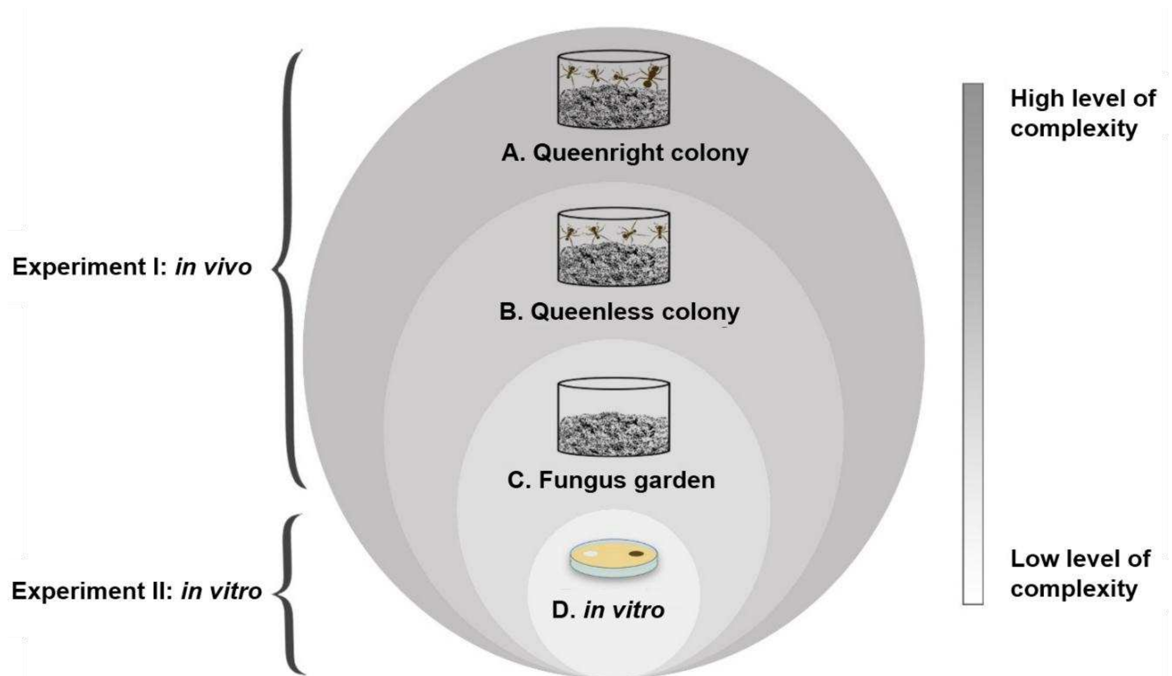


Fig.1 Schematic representation of experiments I and II showing the complexity levels of leafcutter ant-fungus symbiosis: **(A)** queenright colony; **(B)** queenless colony; **(C)** fungus garden; **(D)** fungal cultivar (*in vitro*). Note that a queenright colony is composed of the fungus garden, the queen and specialized workers with different functions. Therefore, we considered in our study that a queenright colony encompasses all these elements. On the other hand, a queenless colony do not contain an essential component of the queenright colony, the queen. As we disregard one or more organisms involved in the association between leafcutter ants and its fungal cultivar, the complexity levels of this interaction decrease. This can also be observed in fungus gardens without ants as well in the cultivation of the fungal cultivar *in vitro*, which we considered the simplest level of this interaction. From these considerations, we evaluated the impact of the fungi *Escovopsis* and *Escovopsioides* on *Acromyrmex subterraneus subterraneus* colonies and their fungal cultivar in different complexity levels.

Organisms

Twelve colonies of *Acromyrmex subterraneus subterraneus* (~1 year old) were collected in three areas on Campus of Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais, southeastern Brazil: Dendrologia (20º 46'21"S 42º52'25"W), Recanto das Cigarras (20º 45'26"S 42º51'45"W) and Horto Botânico (20º 45'25"S 42º52'23"W). The first two areas are fragments of secondary Atlantic forest while

Horto Botânico is a living plant collection comprising native and foreign plant specimens. The fungus garden of each colony was transferred to a plastic pot (500 ml) and then placed in a plastic tray (43 × 29 × 7cm). In the base of the pots, we made a 2 cm diameter exit hole to allow the passage of workers for the foraging arena in the tray. The inner sides of each tray were covered with neutral talcum powder (magnesium silicate) to prevent the ants from escaping. We offered daily, fresh leaves of *Acalypha wilkesiana* as forage for the ants. The colonies were maintained under controlled temperature (25±2°C) and humidity conditions (75±3% RU). In order to check if *Escovopsis* and *Escovopsioides* were naturally present in the collected colonies, we sampled fragments from fungus gardens and plated in growth media. The presence of these fungi was recorded and is presented in the Appendix (S1). *Escovopsis* was found in five of the 12 colonies while *Escovopsioides* was found in just one, and the colonies were considered suitable for conducting the experiment.

In both experiments, the same fungal isolates were used, these are *Escovopsis moelleri* (VIMI-10.0001), *Escovopsioides nivea* (VIMI-17.0136) and *Trichoderma longibrachiatum* (VIMI-17.0135). In the paired culture bioassay (*in vitro*) we also used a *Leucocoprinus gongylophorus* isolate (VIMI-17.0137). *Trichoderma longibrachiatum* was used in the assays for comparison with *E. moelleri* and *E. nivea*. This allowed us to evaluate if the possible effects observed in our experiments is due to *E. moelleri* and *E. nivea* or can be caused by the presence of any other fungi. The genus *Trichoderma* constitutes a close group to *Escovopsis* and *Escovopsioides*, belonging also to the family Hypocreaceae and is found in leafcutter ant colonies (Rocha et al., 2014; Montoya et al., 2016).

Trichoderma longibrachiatum isolate was collected from the fungus garden of a dead colony of *Ac. subterraneus subterraneus*.

The isolates *E. moelleri*, *E. nivea* and *T. longibrachiatum* have rapid growth in culture medium and produce large amount of conidia, facilitating experimental work. *Escovopsis moelleri* was obtained from the fungus collection of Laboratory of Insect-Microorganism-Interactions, Universidade Federal de Viçosa (LIIM-UFV). This isolate was collected from the fungus garden of *Acromyrmex subterraneus molestans* (isolated by H.C. Evans and J.O. Augustin, Augustin et al., 2013) and stored on silica gel at 5°C. *Leucocoprinus gongylophorus*, *E. nivea* and *T. longibrachiatum* (all isolated by D.M.F.M.) were collected from *Ac. subterraneus subterraneus* fungus gardens. *Leucocoprinus gongylophorus* and *E. nivea* were cultivated on MEA (20 g malt extract and 15 g agar L^{-1}), while *T. longibrachiatum* was cultivated on PDA 20% (7.9 g potato, dextrose, agar and 12 g agar L^{-1}). Posteriorly, these fungi were incubated at 25°C for seven days. After this period, they were re-isolated (ca. 2 times) until we obtained a pure culture. The isolate of *L. gongylophorus* was identified through its morphological characteristics, such as the production of gongylidia. *Escovopsioides nivea* and *T. longibrachiatum* were identified by their morphological characteristics and at the molecular level. For morphological identifications, we made slides and examined these with a Nikon (Eclipse E200) light microscope. The molecular characterization was conducted by sequencing the genomic region translation elongation factor (TEF). The obtained sequences were compared with other sequences available at GenBank through Basic Local Alignment Search (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The isolates were stored in 10% glycerol at -80°C and included in the LIIM mycological collection.

Experiment I (*in vivo*)

Impact of *Escovopsis* and *Escovopsioides* on colonies of leafcutter ants at different levels of complexity

The aim of this experiment was to evaluate if the impact caused by *Escovopsis* and *Escovopsioides* in colonies of leafcutter ants depends on the level of colony complexity. For this, we exposed queenright colonies, queenless colonies and fungus gardens to *Escovopsis* and *Escovopsioides*, individually.

Conidial suspensions

To prepare our conidial suspensions, we firstly grew the isolates *E. moelleri* and *E. nivea* on plates containing MEA, while *T. longibrachiatum* was grown on PDA 20%. These fungi were incubated at 25 °C for 15 days. After this period, the colony of each grown fungus was removed from the plates and individually inserted into a Falcon tube containing sterile distilled 0.01% of Tween 80® + NaCl 0.85% solution. The suspensions were stirred for 3 min and then filtered using a sterile gauze. This procedure allowed the separation of the conidia from hyphae fragments, resulting in suspensions containing only conidia. We prepared the suspensions according to the maximum conidial concentrations we could obtain for each fungus. The concentration of the suspensions was determined using a Neubauer chamber. *Escovopsis moelleri* and *T. longibrachiatum* suspensions contained $1x10^8$ conidia ml^{-1} while *E. nivea* suspension contained $1x10^6$ ml^{-1}. All suspensions were kept at 5°C overnight.

Experimental set-up

In order to prepare the three different levels of colony complexity, we performed the following procedure: each initial colony was divided into three fragments (Fig. 2A), one fragment consisted of a queenright colony and the two remaining fragments consisted of queenless colonies, totalizing 12 queenright colonies and 24 queenless colonies (Fig. 2B). Each fragment was placed in 250 ml plastic pots (Fig. 2B). The fragments were maintained in the separate pots in the same tray for 30 days, at this time the fungus garden fragment reached the top of the 250 ml pot. To obtain the treatment that consisted only of fungus garden, one day before the start of the experiment, we carefully removed, using tweezers, the ants from 12 queenless colonies (Fig. 2C). For this, the fungus gardens were fragmented to get access to all their regions and we could remove all the ants. We observed in preliminary tests that fungus garden decreased its weight and suffered a change in its physical structure when the ants were removed, presumably due to the manipulation during the procedure. In order to apply the same conditions to all the fragments, the fungus gardens of the 12 queenright colonies and 12 queenless colonies were fragmented similarly, without damaging the queens or the workers. We opted not to use insecticides to remove the ants from fungus gardens, because some studies have shown that some insecticides may have inhibitory effects on fungal growth (Cowley & Lichtenstein, 1970; Olmert & Kenneth, 1974; Ali et al., 2012), which could interfere in our experiment. Thus, we considered mechanical removal the most adequate procedure. After this period, each pot that contained each complexity level was placed individually in a tray (343 × 200 × 66 mm). The inner sides of the trays were covered with neutral talcum powder to prevent the ants from escaping. Thus, we had in total 12 queenright colonies (fungus garden + queen + workers), 12 queenlees colonies (fungus garden + workers) and 12 pots

containing only fungus garden (Fig. 2D). To assemble the experiment, and during its accomplishment, many steps were required and are listed in Table 1.

Table 1. Summary of the days and the respective activities conducted for the assembly and evaluation of the experiment.

Summary of experiment set up days	
Day -28	Sampling of the colonies' fungus gardens; Division of each initial colony into three fragments (totalling 12 queenright colonies and 24 queenless colonies);
Day -2	Weighing of the 12 queenright colonies and 12 queenless colonies;
Day -1	Removal of ants from the other 12 queenless colonies; Weighing fungus gardens; Preparation of fungal suspensions;
Day 0	Inoculation of fungal treatments; Survival evaluation;
Day 1	Weighing and sampling of midden; Weighing of the leaves cut by ants from queenright colonies and queenless colonies;
Day 2	Sampling of queenright colonies, queenless colonies and fungus gardens;
Day 11	Last day of weighing fungus gardens;
Day 28	Last day of weighing and sampling of midden;
Day 29	Last day of weighing and sampling of queenright colonies and queenless colonies; Last day of weighing the leaves cut by the ants from queenright colonies and queenless colonies;
Day 118	End of the experiment: last day of survival evaluation.

Fungal inoculation

We inoculated 4 ml of conidial suspensions of *E. moelleri*, *E. nivea* and *T. longibrachiatum* individually, on each pot (Fig. 2E). For the control blank, we inoculated 4 ml of 0.01% Tween 80® + NaCl 0.85% solution (Fig. 2E). The queenright colonies, queenless colonies and fungus gardens that were originated

from the same initial colony were exposed to the same treatment. Each combination of fungus treatment with a colony complexity level was replicated three times. The inoculation of conidial suspensions was done using a sterile spray bottle (Fig. 2E). We sprayed the suspensions on the surface of fungus gardens until all the 4 ml were dispensed (Fig. 2E). The trays that contained each treatment were placed randomly in three shelves. Each shelf contained one repetition of each treatment. During the experiment, laboratory conditions were 25±2°C, 75±3% RU and 12 hours of photoperiod.

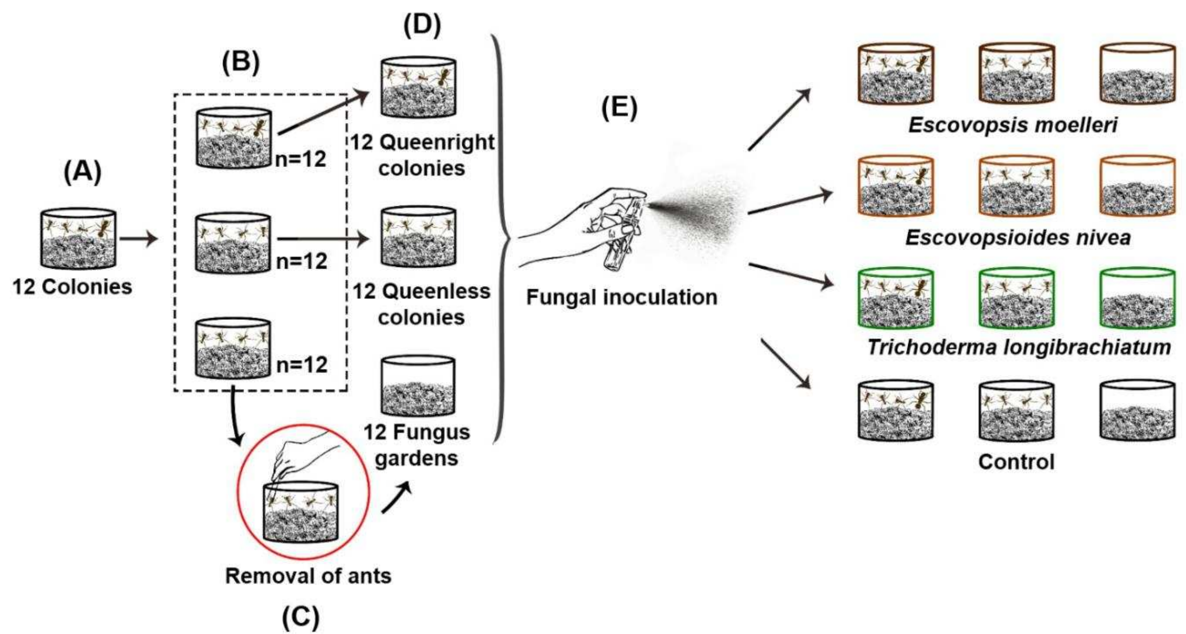


Fig. 2 Schematic representation of experimental set-up. Twelve colonies of the leafcutter ant *Acromyrmex subterraneus subterraneus* were necessary to assemble the experiment. **(A)** Each of these 12 initial colonies was separated in three fragments. **(B)** One fragment consisted of a queenright colony (fungus garden + workers + queen), and the two other fragments consisted of a queenless colony (fungus garden + workers, without queen), resulting in the total of 12 queenright colonies and 24 queenless colonies. Each queenright colony with their respective queenless colonies were placed in 250 ml individual pots and maintained together in the same tray for 30 days to allow the growth of the fungus gardens. **(C)** After this period, in order to obtain the fungus gardens without any ants, we removed using tweezers, the workers from 12 queenless colonies. **(D)** Thus, we obtained our three colonies complexity levels: queenright colony (n=12), queenless colony (n=12) and fungus garden (n=12). **(E)** We exposed each of these colonies complexity levels to one of the three fungal treatments or control: conidial

suspension of *Escovopsis moelleri* (1×10^8 conidia ml^{-1}), *Escovopsioides nivea* (1×10^6 conidia ml^{-1}), *Trichoderma longibrachiatum* (1×10^8 conidia ml^{-1}) or control (0.01% Tween 80® + NaCl 0.85% solution). The inoculation of suspensions was done through a sterile spray bottle. We sprayed 4 ml of conidial suspensions or the control on the surface of fungus gardens. The queenright colonies, queenless colonies and fungus gardens that originated from the same initial colony were exposed to the same treatment. Each treatment was replicated three times (combination of a fungus treatment + a colony complexity level).

Data collection

Survival of queenright colonies, queenless colonies and fungus gardens

The survival of queenright colonies, queenless colonies and fungus gardens, was checked every day. These records were made for 118 days, after fungi inoculation. We considered fungus gardens without ants to be dead when they were completely covered by other fungi. The queenright colonies and queenless colonies were considered dead when they did not contain any live ants, their fungus gardens presented a dry texture, almost crumbling or if they overgrow by parasite fungus. We kept the colonies for seven months after the end of the experiment to see if they remained alive.

Weights of queenright colonies, queenless colonies and fungus gardens

To evaluate if the weight of each complexity level suffered variation after being exposed to fungi treatments, the weights of queenright colonies, queenless colonies and fungus gardens from each treatment were measured. We weighed the queenright colonies and queenless colonies two days before the experiment starts while the fungus gardens were weighed one day before. We repeated this procedure 48 hours after inoculations of conidial suspensions and then every 72 hours. The initial weight of each queenright colony presented some variation and this is also

observed between the queenless colonies and among the fungus gardens. These variations are natural since each colony presents particular characteristics and thus, it is difficult to find colonies with equal weights.

Midden weights

Middens from each queenright colony and from queenless colonies were weighed to evaluate if ants produced different quantities of midden depending on fungal treatment. We weighed the midden produced 24 hours after the inoculation of the treatments. This procedure was repeated every 72 hours.

Weights of leaves cut by ants

The weights of leaves cut by ants from each queenright colony and queenless colony were determined to evaluate if ants alter the amount of food supplied to the fungal cultivar due to the possible detrimental effects performed by the fungi treatments. For this purpose, we offered, daily, 3 g of *Acalypha wilkesiana* fresh leaves and after 24 hours, we weighed the leaves that were not cut (leaves remaining in the trays). To calculate the real quantity of leaves that were cut, we considered the percentage of water loss of these leaves. Thereby, we daily maintained on each shelf that had the treatments, one tray containing only *Acalypha wilkesiana* fresh leaves (3 g) that were weighed in order to evaluate the water loss. Therefore, we calculated the weight of leaves that were cut according to the adapted formula from Antunes & Della Lucia (1999) and Gandra (2014).

$$Cr = QFi - QFf - \%PA$$

Cr= weight of cut leaves
QFi= Quantity of leaves that we offered
QFf= Quantity of leaves that were not cut by ants
%PA= Percentual weight of water loss

Sampling from fungus gardens and middens

We sampled the fungus gardens from each queenright colony, queenless colony and fungus garden without ants to verify the presence of *Escovopsis*, *Escovopsioides* or *Trichoderma*. We also sampled the middens produced by queenright colonies and queenless colonies to observe if the ants were removing these fungi from their fungus gardens (Appendix S5).

Data analysis

We evaluated the impact of *E. moelleri*, *E. nivea* and *T. longibrachiatum* on leafcutter ant colonies in three different complexity levels. For this, we adjusted linear mixed models (LMM) with Gaussian distributions. Response variables were (i) weight of queenright colonies, queenless colonies and fungus gardens, (ii) midden weight produced by queenright colonies and queenless colonies and (iii) weight of leaves cut by ants from queenright colonies and queenless colonies. Explanatory factors consisted of two levels: the colonies complexity levels and fungi treatments. We evaluated the differences between treatments by comparing: (i) weight of queenright colonies on days -2 and 29, weight of queenless colonies on days -2 and 29 and weight of fungus gardens on days -1 and 15, (ii) Weight of midden from queenright colonies and queenless colonies on days 1 and 28, (iii) weight of leaves cut from queenright colonies and queenless colonies on days 1 and 29. We considered each sample as a repeated measure and the significance was evaluated using F test ($P < 0.05$). To evaluate the survival of each colony complexity level after exposure to one of the three fungi treatments or the control, we conducted a survival analyses with Weibull distribution. The models

significances were evaluated using Chi-squared tests ($P<0.05$). All analyses were conducted in the R statistical software (R Core Team, 2017).

Experiment II (*In vitro*)

Interaction of *Escovopsis* and *Escovopsioides* with the fungal cultivar of leafcutter ants

The aim of this experiment was to evaluate the interaction between *Escovopsis*, *Escovopsioides* and *Trichoderma* with *L. gongylophorus*, *in vitro*, that we considered the simplest level of complexity. We evaluated the growth of *Escovopsis*, *Escovopsioides* and *Trichoderma* towards the fungal cultivar through a paired culture assay (co-culture) in Petri dishes.

Paired culture bioassay

The isolates *E. moelleri*, *E. nivea* and *T. longibrachiatum* were previously grown in Petri dishes (9 cm in diameter) containing MEA and incubated at 25°C for 10 days. The isolate of *L. gongylophorus* was previously grown on MEA for 15 days, because it has slower growth. After this period, 8 mm diameter disks of *L. gongylophorus* were cut and plated 5 mm from the border of Petri dishes (90 × 15 mm) containing 20 ml of MEA. The plates were incubated at 25°C for 15 days, the period required for the growth of this fungus, as described above. Posteriorly, mycelium disks of *E. moelleri*, *E. nivea* and *T. longibrachiatum* were placed individually in plates containing a disk of *L. gongylophorus*. These fungi were inoculated on the opposite side of the fungal cultivar, also at 5 mm from the plate edge. For the control treatment, we used an 8 mm diameter disk of plain MEA instead of the fungal cultivar. Thus, we obtained the six following combinations: (i) control × *E. moelleri* (n=6), (ii) *L. gongylophorus* × *E. moelleri* (n=10), (iii)

control × *E. nivea* (n=7), (iv) *L. gongylophorus* × *E. nivea* (n=10), (v) control × *T. longibrachiatum* (n=10), (vi) *L. gongylophorus* × *T. longibrachiatum* (n=10). The different number of repetitions between treatments were due the loss of some plates by contamination with other microorganisms. During the experiment, the plates were maintained incubated at 25°C and distributed on five shelves of an incubator. Each shelf contained two blocks with one repetition of each treatment per block. The growth of fungi was evaluated every 12 hours, for 10 days. However, we conducted the analysis using the data of day 5, when the first fungal isolate reached the fungal cultivar on the opposite side of the plate. We photographed and scanned the plates using a digital camera (Nikon D2000) and a multifunction printer (HP LaserJet Pro CM1415fnw). From the images, we measured the growth of the fungi towards *L. gongylophorus* using ImageJ 1.49v software.

Data analysis

We evaluated the growth of *Escovopsis*, *Escovopsioides* and *Trichoderma* toward the fungal cultivar of leafcutter ant, *L. gongylophorus*. We adjusted generalized linear models (GLM) with Gaussian distributions. The models were adjusted separately for each fungus. Response variables were the growth of the fungi (cm). Explanatory variables consisted of each fungus treatment in the presence or absence of *L. gongylophorus*. The significance was evaluated using F tests ($P<0.05$). All analyses were conducted in the R statistical software (R Core Team, 2017).

RESULTS

Experiment I (*in vivo*)

Impact of *Escovopsis* and *Escovopsioides* on colonies of leafcutter ants at different levels of complexity

Survival of queenright colonies, queenless colonies and fungus gardens

All queenright colonies remained alive during the 118 days evaluated (Fig.3A). Seven months after the end of the experiment, the queenright colonies were still alive and at this juncture were discarded. The survival time of queenless colonies did not differ between control and *E. nivea* treatment (χ^2_1 = 1.557, P= 0.212; Fig.3B). However, queenless colonies submitted to *E. moelleri* died faster than queenless colonies from control, *E. nivea* and *T. longibrachiatum* (χ^2_1 = 10.065, P = 0.002; Fig.3B). The survival of queenless colonies exposed to *T. longibrachiatum* did not differ from queenless colonies exposed to control and *E. nivea* (χ^2_1 = 1.649, P = 0.199; Fig.3B). On the other hand, all fungus gardens died 11 days after the inoculation of fungi treatments (Fig.3C).

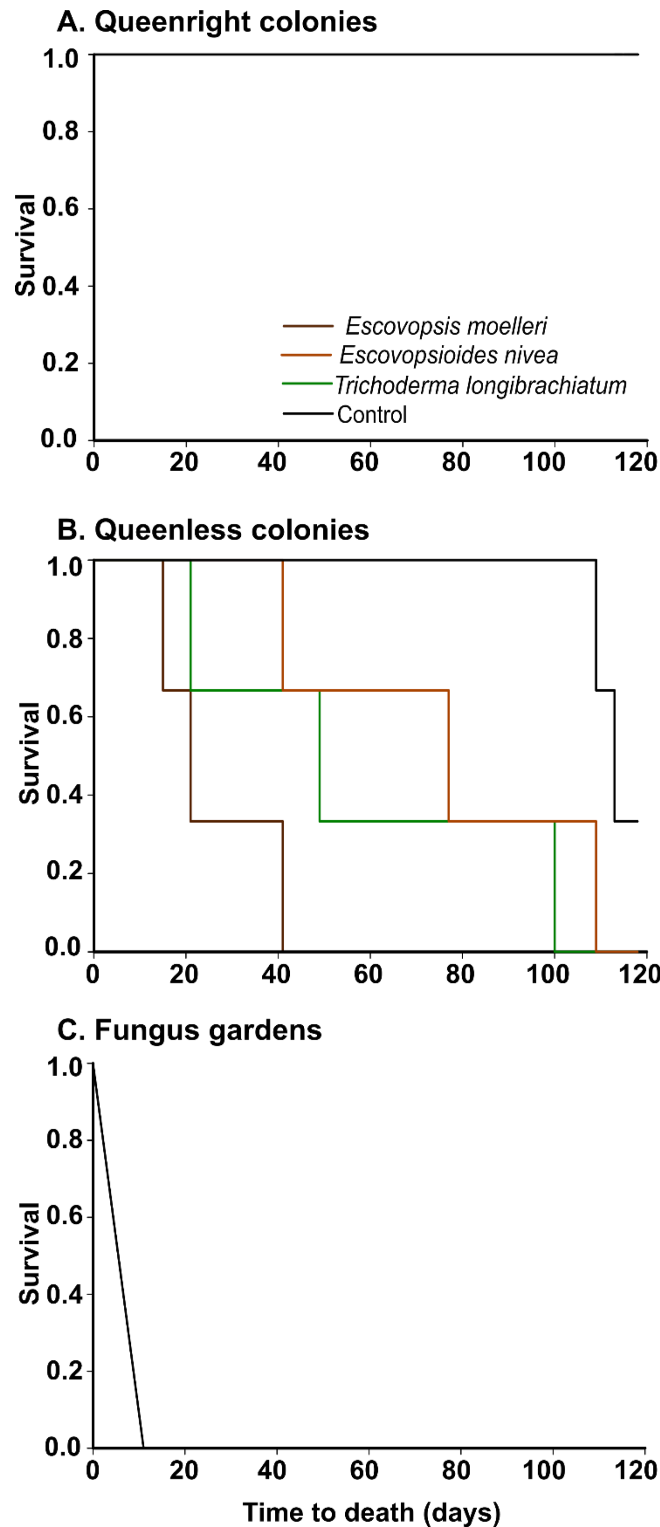


Fig.3 Survival of *Acromyrmex subterraneus subterraneus* leafcutter ant **(A)** queenright colonies, **(B)** queenless colonies and **(C)** fungus gardens exposed to one of three treatments or to control: conidial suspension of the fungi *Escovopsis moelleri*; *Escovopsioides nivea; Trichoderma longibrachiatum*; control (0.01% Tween 80® solution + saline solution - NaCl 0.85%). Survival was checked every day from the day of inoculation with fungal treatments (Day 0) until 118 days post-inoculation. The fungus gardens were considered dead when they were completely covered by other fungi and presented a dry texture. We considered the queenright

colonies and queenless colonies dead when they did not contain any live ants. A survival analyse with a Weibull distribution was conducted and the models compared using "Chi-squared" test (P<0.05).

Weights of queenright colonies, queenless colonies and fungus gardens

We observed that all queenright colonies lost weight the day after the inoculation of conidial suspensions independent of the treatment to which they were exposed (Fig.S1A). Queenright colonies exposed to blank controls and *E. nivea* increased their weight after this period and they did not differ from one other ($F_{(1,8)}$ = 0.279, P = 0.870; Fig.4A, Fig.S1A). On the other hand, queenright colonies exposed to *E. moelleri* presented a decrease in their weight and differed from control and *E. nivea* queenright colonies ($F_{(1,6)}$ = 18.941, P= 0.0001; Fig.4A, Fig.S1A). The queenright colonies exposed to *T. longibrachiatum* maintained similar weight over time and differed from queenright colonies of control and *E. nivea* ($F_{(1,6)}$ = 10.718, P = 0.005; Fig.4A, Fig.S1A), as well as queenright colonies exposed to *E. moelleri* ($F_{(1,8)}$ = 7.007, P = 0.030; Fig.4A, Fig.S1A).

All queenless colonies suffered a substantial decrease in their weight over time (Fig.4B, Fig.S1B). However, this reduction was higher in queenless colonies exposed to *E. moelleri* and this treatment differed from queenless colonies exposed to control, *E. nivea* and *T. longibrachiatum* ($F_{(1,3)}$ = 34.161, P=0.009; Fig.4B, Fig.S1B). The weight of queenless colonies exposed to control, *E. nivea* and *T. longibrachiatum* did not differ from each other over time ($F_{(1,6)}$ = 4.865, p = 0.088; Fig.4B, Fig.S1B). In relation to fungus gardens, all of these decreased their weight and there was no difference between treatments ($F_{(1,4)}$ = 7.536, P = 0.057; Fig.4C, Fig.S1C).

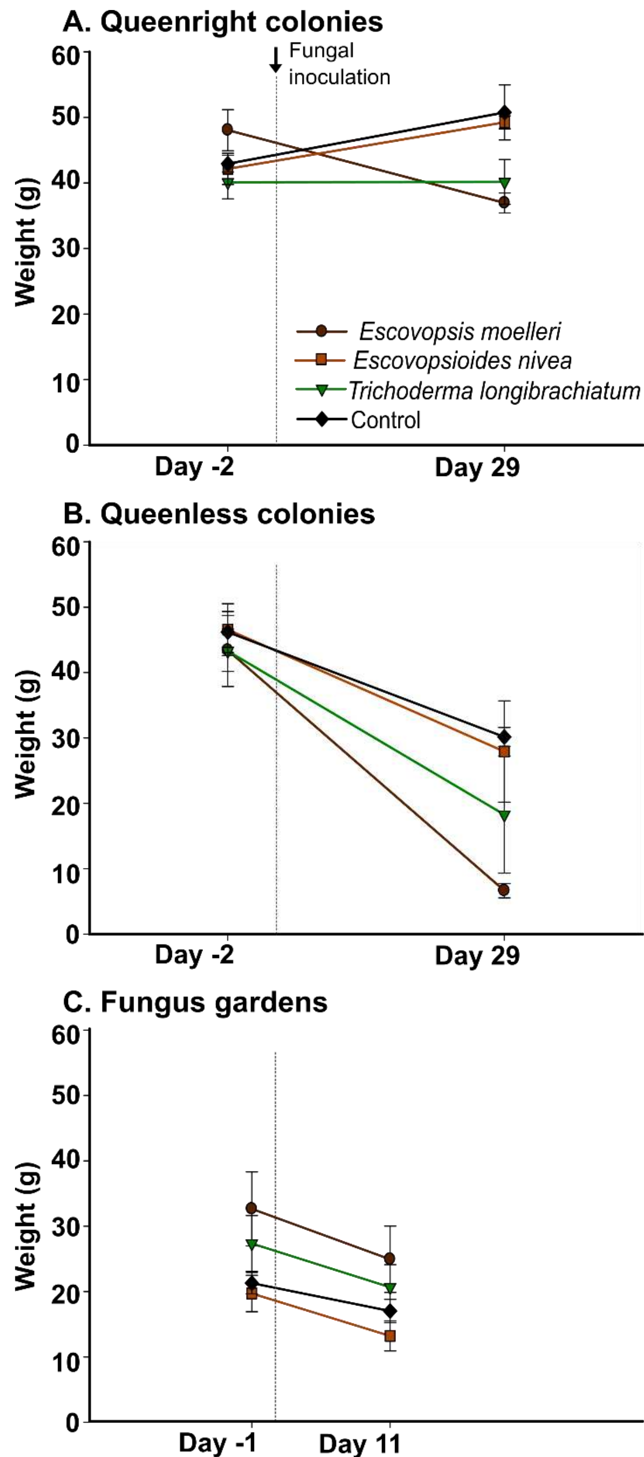


Fig.4 Weights of *Acromyrmex subterraneus subterraneus* leafcutter ant **(A)** queenright colonies, **(B)** queenless colonies and **(C)** fungus gardens exposed to one of three treatments or to control: conidial suspension of the fungi *Escovopsis moelleri*; *Escovopsioides nivea; Trichoderma longibrachiatum*; control (0.01% Tween 80® solution + saline solution - NaCl 0.85%). Conidial suspensions and blank control were inoculated on Day 0 and the queenright colonies and queenless colonies were evaluated until Day 29 after inoculation; the fungus gardens were evaluated until Day 11, when all parcels were considered dead. The dotted line in the graphs indicates the day we inoculated the treatments and the control (Day 0).

The weight of queenright colonies and queenless colonies was measured two days before inoculation (Day -2) while fungus gardens were weighed one day before inoculation (Day -1). Weighing was repeated 48 hours after inoculation of conidial suspensions for all treatments, and then every 72 hours. The differences between treatments were evaluated by comparing: the weight of queenright colonies on days -2 and 29; the weight of queenless colonies on days -2 and 29; the weight of fungus gardens on days -1 and 11. To conduct the analysis we adjusted linear mixed models (LMM) considering each sample as a repeated measure. The significance was evaluated using *F* tests (P<0.05).

Midden weights

The weight of midden produced by ants in queenright colonies exposed to control and *E. nivea* was similar over time and did not differ from each other ($F_{(1,8)}$ = 0.277, P = 0.870; Fig.5A, Fig.S2A). In contrast, midden production was high one day after the inoculation of *E. moelleri* on queenright colonies, and this treatment differed from control and *E. nivea* queenright colonies ($F_{(1,6)}$ = 15.275, P = 0.004; Fig.5A, Fig.S2A). Despite queenright colonies exposed to *T. longibrachiatum* producing a larger amount of midden in relation to controls and *E. nivea* queenright colonies one day after fungus inoculation , these treatments did not differ from each other over time ($F_{(1,6)}$ = 7.294, P = 0.121; Fig.5A, Fig.S2A).

Similarly to queenright colonies, the queenless colonies exposed to control and to *E. nivea* maintained similar productions of midden over time, and these treatments did not differ from each other ($F_{(1,8)}$ = 1.377, P = 0.502; Fig.5B, Fig.S2B). On the other hand, the weight of midden produced in queenless colonies submitted to *E. moelleri* was high on the day after inoculation of the fungus, and differed from control and from *E. nivea* queenless colonies ($F_{(1,8)}$= 26.593, P <.0001; Fig.5B, Fig.S2B). Likewise, queenless colonies treated with *T. longibrachiatum* also produced a considerable amount of midden one day after the fungus inoculation and differed from queenless colonies of control and *E. nivea*

($F_{(1,8)}$ = 7.250, P = 0.026; Fig.5B, Fig.S2B). Although the amount of midden produced in queenless colonies exposed to *E. moelleri* and *T. longibrachiatum* was high after fungal inoculation, these treatments differed from each other over time ($F_{(1,8)}$ = 14.189, P = 0.0008; Fig.5B, Fig.S2B).

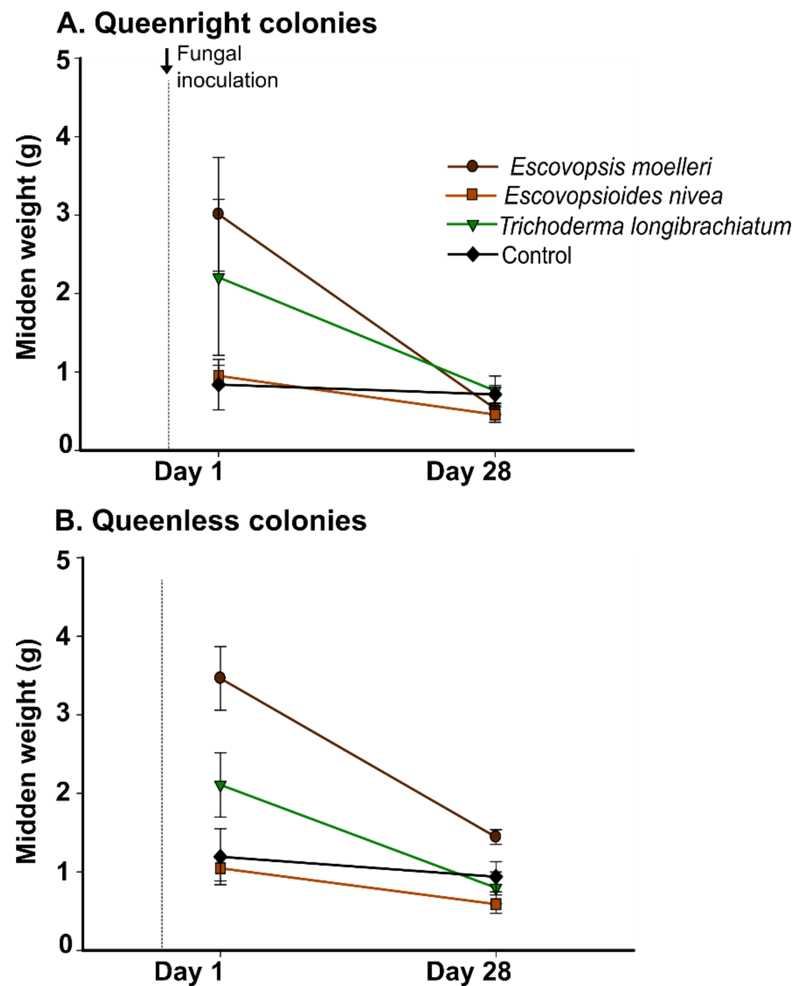


Fig.5 Weights of midden produced by *Acromyrmex subterraneus subterraneus* leafcutter ant **(A)** queenright colonies and **(B)** queenless colonies exposed to one of three treatments or to control: conidial suspension of the fungi *Escovopsis moelleri*; *Escovopsioides nivea; Trichoderma longibrachiatum*; control (0.01% Tween 80® solution + saline solution - NaCl 0.85%). Conidial suspensions and blank control were inoculated on Day 0 and the weight of midden produced by the queenright colonies and queenless colonies was evaluated until Day 28 after inoculation. The dotted line in the graphs indicates the day we inoculated the treatments and the control (Day 0). The weight of midden was measured 24 hours after inoculation of conidial suspensions for all treatments (Day 1) and then, this procedure was repeated every 72 hours. The differences between treatments were evaluated by comparing: the weight of midden produced by queenright colonies on days 1 and 28; the weight of midden produced by queenless colonies on days 1 and 28. To

conduct the analysis we adjusted linear mixed models (LMM) considering each sample as a repeated measure. The significance was evaluated using *F* test ($P<0.05$).

Weights of leaves cut by ants

The weight of leaves cut by the ants from control and *E. nivea* queenright colonies was variable over time (Fig.S3A), nevertheless, the treatments did not differ from each other ($F_{(1,8)}$ = 3.189, *P*= 0.203; Fig.6A). Queenright colonies exposed to *E. moelleri* increased the amount of cut leaves and differed from control and *E. nivea* queenright colonies ($F_{(1,6)}$ = 16.632, *P* = 0.0002; Fig.6A, Fig.S3A). This increase was also observed in queenright colonies exposed to *T. longibrachiatum*, and this treatment differed from queenright colonies submitted to control and *E. nivea* ($F_{(1,6)}$ = 8.720, *P* = 0.013; Fig.6A, Fig.S3A). There was no difference between the amount of cut leaves from queenright colonies exposed to *T. longibrachiatum* and *E. moelleri* ($F_{(1,8)}$ = 2.273, *P* = 0.321; Fig.6A, Fig.S3A).

In queenless colonies, the amount of cut leaves was higher in those exposed to control, and differed from those submitted to *E. nivea* treatment ($F_{(1,5)}$ = 6.620, *P* = 0.010; Fig.6B, Fig.S3B), as well from *E. moelleri* ($F_{(1,5)}$ = 10.716, *P* = 0.001; Fig.6B, Fig.S3B) and *T. longibrachiatum* treatments ($F_{(1,8)}$ = 6.584, *P* = 0.037; Fig.6B, Fig.S3B). Queenless colonies exposed to *E. nivea*, *E. moelleri* and *T. longibrachiatum* treatments cut variable amounts of leaves over time (Fig.S3B), however, these treatments did not differ from each other ($F_{(1,4)}$ = 1.676, *P* = 0.433; Fig.6B).

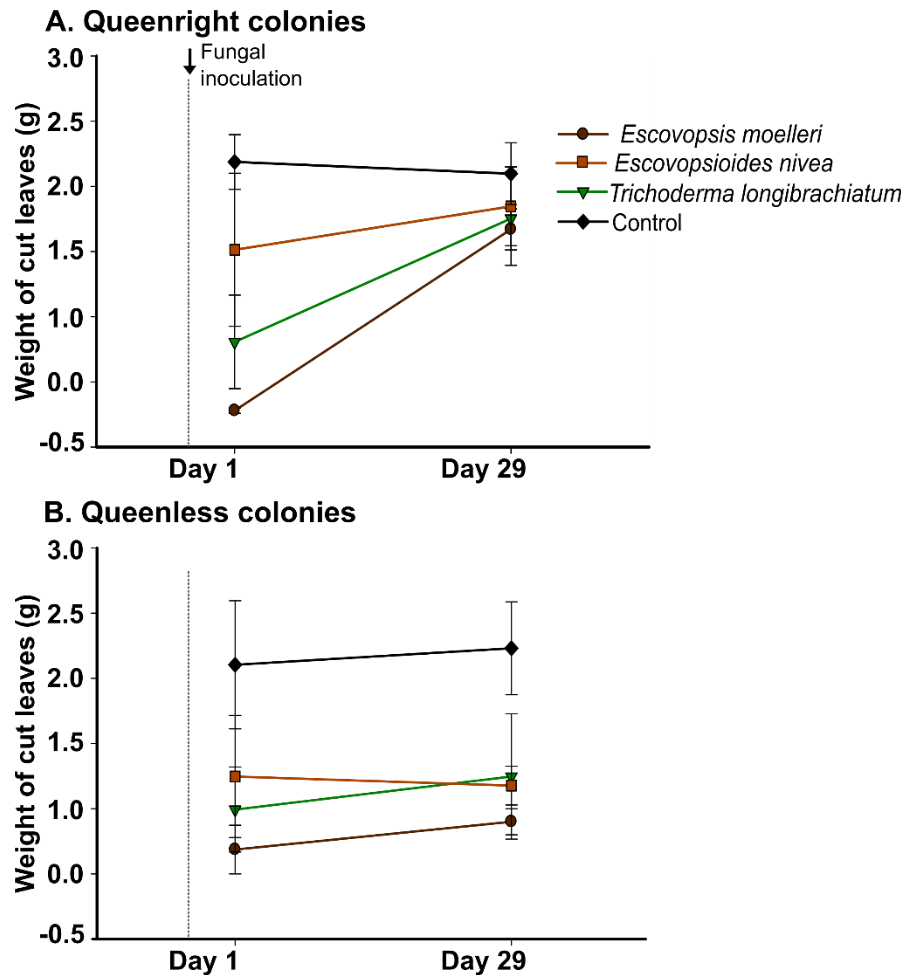


Fig.6 Weights of leaves cut by *Acromyrmex subterraneus subterraneus* leafcutter ant **(A)** queenright colonies and **(B)** queenless colonies exposed to one of three treatments or to control: conidial suspension of the fungi *Escovopsis moelleri*; *Escovopsioides nivea; Trichoderma longibrachiatum*; control (0.01% Tween 80® solution + saline solution - NaCl 0.85%). Conidial suspensions and blank control were inoculated on Day 0 and the weight of cut leaves by queenright colonies and queenless colonies was evaluated until Day 29 after inoculation. The dotted line in the graphs indicates the day we inoculated the treatments and the control (Day 0). The weight of cut leaves was measured 24 hours after inoculation of conidial suspensions for all treatments (Day 1) and then, this procedure was repeated daily. The differences between treatments were evaluated by comparing: the weight of cut leaves produced by queenright colonies on days 1 and 29; the weight of cut leaves produced by queenless colonies on days 1 and 29. To conduct the analysis we adjusted linear mixed models (LMM) considering each sample as a repeated measure. The significance was evaluated using F test (P<0.05).

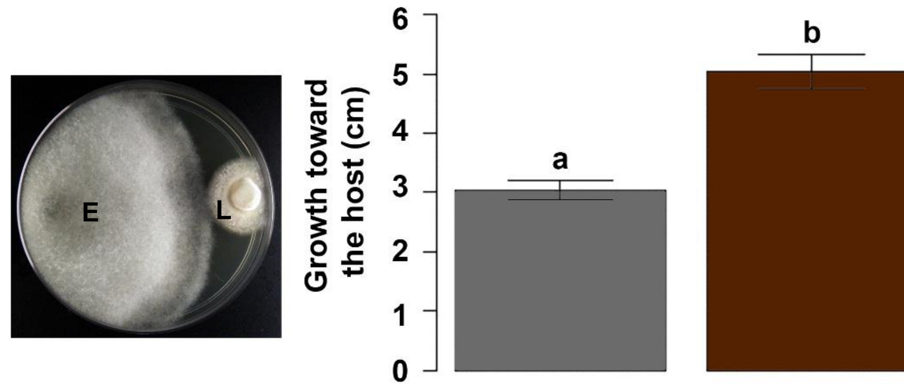
Experiment II (*In vitro*)

Interaction of *Escovopsis* and *Escovopsioides* with the fungal cultivar of leafcutter ants

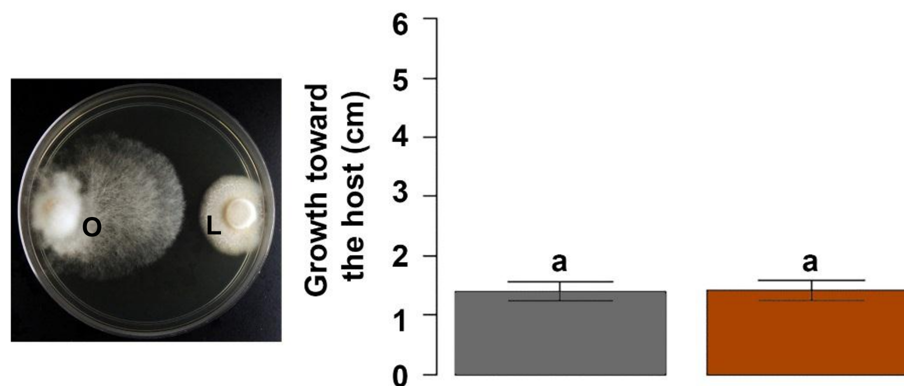
Paired culture bioassay

The growth of *E. moelleri* was greater in the presence of *L. gongylophorus* and differed from the control ($F_{(1,15)} = 24.753$, $P= 0.0002$; Fig.7A). On the other hand, there was no difference in the growth of *E. nivea* in the presence or absence of the fungal cultivar ($F_{(1,16)} = 0.01$, $P= 0.921$; Fig.7B). The mycelial growth of *T. longibrachiatum* was reduced in the presence of *L. gongylophorus*, and this treatment differed from the control ($F_{(1,19)} = 18.068$, $P= 0.0005$; Fig.7C).

A. ***Escovopsis moelleri***



B. ***Escovopsioides nivea***



C. ***Trichoderma longibrachiatum***

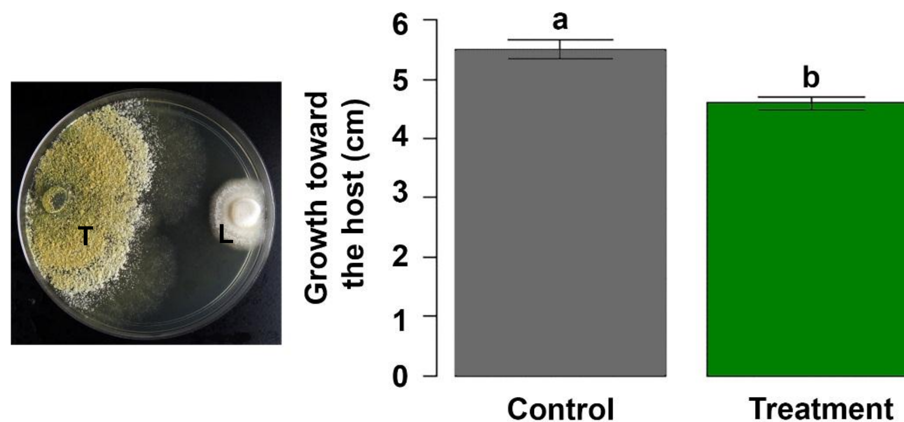


Fig.7 Growth of the fungi **(A)** *Escovopsis moelleri,* **(B)** *Escovopsioides nivea* and **(C)** *Trichoderma longibrachiatum* toward the fungal cultivar of leafcutter ants, *Leucocoprinus gongylophorus*. Mycelium disks of *E. moelleri*, *E. nivea* and *T. longibrachiatum* were individually placed in Petri dishes (90x15mm) containing a disk of *L. gongylophorus*. These fungi were inoculated on the opposite side of the fungal cultivar. The control plates consisted disk of MEA (malt extract agar

medium) in place of the fungal cultivar disk. From this, we obtained the following combinations: **(A)** MEA disk × *E. moelleri* (control), *L. gongylophorus* × *E. moelleri* (treatment); **(B)** MEA disk × *E. nivea* (control), *L. gongylophorus* × *E. nivea* (treatment); **(C)** MEA disk × *T. longibrachiatum* (control), *L. gongylophorus* × *T. longibrachiatum* (treatment). To evaluate the growth of the fungi we photographed and scanned the plates every 12 hours for 10 days. Posteriorly, from the obtained images we measured the growth of fungi toward *L. gongylophorus* using ImageJ 1.49v software. The analysis was conducted using the data from the fifth day, when the first fungi isolate reached *L. gongylophorus* on the opposite side of the plate. We adjusted generalized linear models (GLM) with the significances evaluated using *F* test ($P<0.05$). Different letters indicate statistical difference. In photos of the paired culture of each fungi with *L. gongylophorus*, the letters indicate: L= *L. gongylophorus;* E= *E. moelleri*; O= *E. nivea*; T= *T. longibrachiatum*.

DISCUSSION

Our objective was to investigate whether the parasitic fungus *Escovopsis* and the antagonist *Escovopsioides* represent threats to the health of leafcutter ants colonies. We also tested whether the virulence level of these fungi varied according to levels of colony complexity. The *in vivo* experiment showed that although the queenright colonies exposed to *E. moelleri* suffered a reduction in their weight, this was not enough to compromise their survival. This indicates that *Escovopsis* may not always present a high virulence to colonies as has been previously suggested (Currie et al., 1999a; Currie, 2001). We hypothesized that workers controlled the growth of this fungus inside the colonies through their defence mechanisms. Some of these mechanisms are already well-known, such as the production of antimicrobial compounds by the metapleural glands (Poulsen et al., 2002; Bot et al., 2002; Fernández-Marín et al., 2006; Fernández-Marín et al., 2009; Yek et al., 2012), hygienic behaviours such as grooming and weeding (Currie & Stuart, 2001) and a symbiotic association with a filamentous bacterium (actinobacteria) that secretes antimicrobial substances (Currie et al,1999b). This control of alien fungi growth by the workers may also have occurred in relation to *E. nivea* and *T. longibrachiatum*, since neither fungus affected negatively the weight of the queenright colonies and nor were they lethal to them. Moreover, according to Frank (1996) it is predicted that some parasites can obtain nutritional resources from their hosts in a sustainable manner, not causing great harm to it, i.e. with low virulence. We propose that this is the case for *E. moelleri*.

In relation to the queenless colonies, most suffered a decrease in their weight leading to their subsequent death. Some studies have shown that the queen is not only important for reproduction but can also influence colony cohesion (Vienne et

al., 1998; Della Lucia et al., 2003). According to Sousa-Souto & Souza (2006), the absence of the queen in a colony of leafcutter ants *Atta sexdens rubropilosa* increased the workers' mortality and decreased the refuse disposal that could indicate a disruption in colony´s internal tasks. Considering this and based on our results, it seems that the absence of the queen naturally causes a negative impact on queenless colonies. As demonstrated here, *E. moelleri* promoted a great decrease in the weight of queenless colonies. In this manner, since the queenless colonies seem to be debilitated, probably turning it a limited resource, it is possible that a better strategy of *E. moelleri* would be increase the exploitation of its host resource, which reflects in an increase of its virulence. This strategy may allow an early parasite reproduction and transmission to new hosts in a natural setting. Although *E. nivea* and *T. longibrachiatum* also led to a decrease in the weight of the queenless colonies, this reduction was not different from that observed in control queenless colonies. Thus, it seems that these fungi are less aggressive than *E. moelleri*, even when its host is in a debilitating condition.

Similar to queenless colonies, the fungus gardens without ants also reduced their weight. However, they died more rapidly, with only eleven days after the inoculation of fungi treatments. It is important to note that we did not observe any fungal sporulation in queenright colonies and queenless colonies. In contrast, *Escovopsis*, *Escovopsioides*, *Trichoderma* and other fungi were observed growing in fungus gardens without ants. These results indicate that the ants are very important to the protection of a colony against parasites as the fungal cultivar seems not to defend itself alone. Thus, the absence of ants probably rendered the fungus gardens more susceptible to infection. Our results clearly demonstrated that ant-free fungus gardens represent a completely unreal condition. In addition, these results

are in line with other studies that showed the growth of alien fungi and signals of infection in fungus gardens not tended by workers (Currie et al, 1999a; Augustin, 2011; Wallace et al., 2014; Kellner et al., 2017).

The protection of the fungal cultivar by leafcutter ants can be performed through main two hygienic behaviours, fungus grooming and weeding (Currie & Stuart, 2001). Fungus grooming is characterized by the removal of parasite spores from the fungus gardens while weeding is the removal of infected garden pieces or of vegetal material contaminated (Currie & Stuart, 2001). The infected material is discarded in the midden, located in specific underground chambers or outside the colony (Hölldobler & Wilson 2009; Lacerda et al., 2011). The removal of contaminants from the fungus gardens is very important to control infections and consequently the maintenance of colony health. In our study, we found that queenright colonies and queenless colonies responded similarly to the presence of alien fungi on their fungus gardens in terms of midden production. In both of these colonies complexity levels, the amount of midden produced by workers was high in the day post-inoculation of *E. moelleri*. We hypothesized that workers tried to remove *E. moelleri* from their fungus gardens. This high production of midden was also observed in queenright colonies and queenless colonies exposed to *T. longibrachiatum*, although only the queenless colonies differed from the control. We suspect that workers from queenless colonies also tried to remove *T. longibrachiatum* probably to control its growth inside of their fungus gardens. On the other hand, both queenright colonies and queenless colonies exposed to control and *E. nivea* maintained the production of midden similar in the first and last day of evaluations. This may indicate that workers do not put much effort into removing

E. nivea from their fungus gardens, probably because this fungus does not pose a great threat to them.

In the day after the inoculation of *E. moelleri* and *T. longibrachiatum* in the queenright colonies, workers cut small amounts of leaves. We suspect that the workers, at first, used efforts to remove the parasites from their queenright colonies rather than cutting leaves to be incorporated into fungus gardens. This result seems to be supported by the results of midden production, because in this same day the amount of midden produced was high, as discussed above. After this period, the queenright colonies exposed to both fungi increased the amount of cut leaves. It is possible that workers increase the cutting of vegetal material to incorporate them in the fungus gardens, promoting the fungal cultivar grow. Similar results were found by Augustin (2011) when colonies of *Atta sexdens rubropilosa* were exposed to *Escovopsis microspora* and it was observed that workers increased the incorporation of leaf fragments in the fungus gardens 50 hours post fungal inoculation. According to this author, this could act as a mechanism that circumvents the possible negative effects caused by this fungus on the colonies and we believe this same hypothesis can be applied to our results. In contrast, the amount of cut leaves, in queenright colonies treated with *E. nivea,* did not differ from the control. This can indicate once again that fungus does not pose a risk to the health of queenright colonies and the ants do not alter the foraging activity to overcome its presence in their fungus gardens. In relation to the queenless colonies, we observed those exposed to control cut a higher amount of vegetal material compared to queenless colonies belonging to the fungi treatments. It is possible that queenless colonies exposed to fungi invested in other tasks such as fungus garden

maintenance instead of foraging activity, as an alternative mechanism to control the fungi in their fungus gardens.

The results of the *in vitro* bioassay have shown that the parasite fungus *E. moelleri* presented a greater growth in the presence of its host. We believe that this parasite may easily overgrow the fungal cultivar because there are no workers to control it in the culture medium. Furthermore, the absence of other microorganisms in this interaction, which could compete for the same nutritional resources, can have facilitated its growth as well. In fact, this was observed in our assay after approximately seven days post-inoculation of *E. moelleri* in the culture medium with the fungal cultivar, in contrast to what we observed in queenright colonies. The overgrowth of the fungal cultivar by *Escovopsis in vitro* has also been shown in previous studies (Folgarait et al., 2011a; Varanda-Haifig et al., 2017). On the other hand, the antagonist fungus *E. nivea* did not present a differential growth in the presence of the fungal cultivar. We also observed that *E. nivea* only overgrew the fungal cultivar after approximately 10 days post-inoculation in the culture medium with *L. gongylophorus*. This result, associated with the data obtained from the *in vivo* experiment, may suggest that this fungus has a low virulence strategy toward the fungal cultivar of leafcutter ants. According to Varanda-Haifig et al. (2017), *E. nivea* isolates were capable of inhibiting the growth of *L. gongylophorus* in the culture medium, however, their isolates seem to be less aggressive compared to *Escovopsis*, similar to what we observed here. Interestingly, *T. longibrachiatum* had its growth reduced in the presence of the fungal cultivar. Rocha et al. (2017) also observed a reduction in the growth of some isolates of *Trichoderma* in paired culture assays with *L. gongylophorus.* This may indicate that the fungal cultivar presents own defences mechanisms. In addition, considering the long

coevolutionary history between *Escovopsis* and leafcutter ants (Currie et al. 2003a) it is possible that this parasite is able to evade some defences of *L. gongylophorus*, which we did not observe in relation to *Trichoderma*.

Our study aimed to investigate the virulence of fungi commonly found in colonies of leafcutter ants. From this study, we showed how the virulence of these microorganisms can be influenced by the complexity of interactions that composed a colony. Our results showed that, in general, the fungi *E. moelleri*, *E. nivea* and *T. longibrachiatum* were not capable to cause a great negative impact on queenright colonies of leafcutter ants. However, most of the queenless colonies and fungus gardens died, which suggests that the queen and workers are very important to the maintenance of colony health and stability. These results highlight the importance of considering the whole superorganism in studies that investigate the virulence of parasites in colonies of eusocial insects. It is important to point out that *Escovopsis* has been suggesting as a potential agent of biological control of leafcutter ants (Folgarait et al., 2011a, b; Wallace et al., 2014). In this manner, we emphasize the importance of conducting experiments to test this possibility using queenright colonies, since *in vitro* experiments may not represent a realistic approach. In fact, a colony of leafcutter ants is not only composed of the fungal cultivar or the queen and workers but is a complex of interactions involving all these organisms and this must be considered. In addition, we observed that *E. nivea* and *T. longibrachiatum* seems to be less aggressive than *E. moelleri*, and this was observed both in the *in vivo* and *in vitro* experiments. Our study is one of the first to investigate the impact of *Escovopsioides* on colonies of leafcutter ants. This may open new avenues for future research that seek to understand the role of this fungus in the leafcutter ants-fungus symbiosis. It would be interesting to develop new studies that investigate

other factors related to the virulence of each these fungi species. This could help us to understand the diversity of strategies and virulence these fungi can present, especially because some studies showed that different strains can vary in their virulence level (Silva et al., 2006; Wallace et al., 2014; Marfetán et al., 2015).

To conclude, we propose that *Escovopsis* is far from being the highly virulent parasite it has been cast as in the literature and that this is also true of its sister-genus *Escovopsioides*. This would make evolutionary sense if one considers the comparatively long-lived nature of its (superorganism) hosts. Where *Escovopsis* may be virulent, we propose that this is a change in strategy when its host is weakened, which would seem not be true for *Escovopsioides*.

REFERENCES

Agnew, P., & Koella, J. C. (1997). Virulence, parasite mode of transmission, and host fluctuating asymmetry. *Proceedings of the Royal Society B,* 264, 9–15. https:// doi.org/10.1098/rspb.1997.0002

Ali, S., Huang, Z., Zou, S., Bashir, M. H., Wang, Z., & Ren, S. (2012). The effect of insecticides on growth, germination and cuticle-degrading enzyme production by *Isaria fumosorosea*. *Biocontrol Science and Technology*, 22, 1047–1058. https://doi.org/10.1080/09583157.2012.708394

Alizon, S., Hurford, A., Mideo, N., & Van Baalen, M. (2009). Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. *Journal of Evolutionary Biology*, 22, 245–259. https://doi.org/10.1111/j.1420-9101.2008.01658

Antunes, E. C., & Della Lucia, T. M. C. (1999). Consumo foliar em *Eucalyptus urophylla* por *Acromyrmex laticeps nigrosetosus* Forel (Hymenoptera-Formicidae). *Ciência e Agrotecnologia*, 23, 208-211.

Augustin, J. O. (2011). Novas espécies de *Escovopsis*, sua dispersão e virulência na simbiose formigas Attini - *Leucoagaricus gongylophorus*. D.Sc. Thesis. Viçosa, MG, Brazil: Universidade Federal de Viçosa, p.131. http:// locus.ufv.br/handle/123456789/897

Augustin, J. O., Groenewald, J. Z., Nascimento, R. J., Mizubuti, E. S. G., Barreto, R.W., Elliot, S. L., & Evans, H. C. (2013). Yet more "weeds" in the garden: fungal novelties from nests of leaf-cutting ants. *PLoS ONE*, 8, e82265. https://doi.org/10.1371/journal.pone.0082265

Augustin, J. O., Simões, T. G., Dijksterhuis, J., Elliot, S. L., & Evans, H. C. (2017). Putting the waste out: a proposed mechanism for transmission of the mycoparasite *Escovopsis* between leafcutter ant colonies. *Royal Society Open Science,* 4, 161013. http://dx.doi.org/10.1098/rsos.161013

Bot, A. N. M., Ortius-Lechner, D., Finster, K., Maile R., & Boomsma, J. J. (2002). Variable sensitivity of fungi and bacteria to compounds produced by the metapleural glands of leaf-cutting ants. *Insectes Sociaux,* 49, 363–370. https://doi.org/10.1007/PL00012660

Brown, M. J. F., Loosli, R., & Schmid-Hempel, P. (2000). Condition-dependent expression of virulence in a trypanosome infecting bumblebees. *Oikos*, 91, 421–427. https://doi.org/10.1034/j.1600-0706.2000.910302.x

Brown, M. J. F., Schmid-Hempel, R., & Schmid-Hempel, P. (2003). Strong context-dependent virulence in a host – parasite system: reconciling genetic evidence with theory. *Journal of Animal Ecology*, 72, 994–1002. https://doi.org/10.1046/j.1365-2656.2003.00770.x

Bull, J. J., Molineux, I. J., & Rice, W. R. (1991). Selection of benevolence in a host-parasite system. *Evolution*, 45, 875-882. https://doi.org/10.2307/2409695

Carreiro, S. C., Pagnocca, F. C., Bueno, O. C., Bacci Jr., M., Hebling, M. J. A., & Silva, O. A. (1997). Yeasts associated with nests of the leaf-cutting ant *Atta sexdens rubropilosa*. *Antonie van Leeuwenhoek*, 71, 243–248. https://doi.org/10.1023/A:1000182108648

Clayton, D. H., & Tompkins, D. M. (1994). Ectoparasite virulence is linked to mode of transmission. *Proceedings of the Royal Society B: Biological Sciences,* 256, 211–217. https://doi.org/10.1098/rspb.1994.0072

Cowley, B. G. T., & Lichtenstein, E. P. (1970). Growth inhibition of soil fungi by insecticides and annulment of inhibition by yeast extract or nitrogenous nutrients. *Journal of General Microbiology*, 62, 27-34. https://doi.org/10.1099/00221287-62-1-27

Cremer, S., Pull, C. D., & Fürst, M. A. (2017). Social immunity: emergence and evolution of colony-level disease protection. *Annual Review of Entomology*, 63, 105–23. https://doi.org/10.1146/annurev-ento-020117-043110

Cremer, S., & Sixt, M. (2009). Analogies in the evolution of individual and social immunity. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364, 129–142. https://doi.org/10.1098/rstb.2008.0166

Cressler, C. E., Mcleod, D.V., Rozins, R., Hoogen, J. V. D., & Day, T. (2015). The adaptive evolution of virulence: a review of theoretical predictions and empirical tests. *Parasitology*, 143, 915–930. https://doi.org/10.1017/S003118201500092X

Currie, C. R., Mueller, U. G., & Malloch, D. (1999a). The agricultural pathology of ant fungus gardens. *Proceedings of the National Academy of Sciences of the United States of America*, 96, 7998–8002. https://doi.org/10.1073/pnas.96.14.7998

Currie, C. R., Scott, J. A., Summerbell, R. C., & Malloch, D. (1999b). Fungus-growing ants use antibiotic producing bacteria to control garden parasites. *Nature,* 398, 701–704. https://doi.org/10.1038/nature01563

Currie, C. R., & Stuart, A. E. (2001). Weeding and grooming of pathogens in agriculture by ants. *Proceedings of the Royal Society B: Biological Sciences,* 268, 1033–1039. https://doi.org/10.1098/rspb.2001.1605

Currie, C. R. (2001). Prevalence and impact of a virulent parasite on a tripartite mutualism. *Oecologia*, 128, 99–106. https://doi.org/10.1007/s004420100630

Currie, C. R., Wong, B., Stuart, A. E., Schultz, T. R., Rehner, S. A., Mueller, U.G., Sung, G. H., Spatafora, J.W., & Straus, N. A. (2003a). Ancient tripartite coevolution in the attine ant-microbe symbiosis. *Science*, 299, 386–388. https://doi.org/10.1126/science.1078155

Currie, C. R., Bot, A. N. M., & Boomsma, J. J. (2003b). Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites. *Oikos* 101, 91-102. https://doi.org/10.1034/j.1600-0706.2003.12036.x

Della Lucia, T. M. C., Peternelli, E. F. O., Lacerda, F. G., & Peternelli, L. A. (2003). Colony behavior of *Atta sexdens rubropilosa* (Hymenoptera: Formicidae) in the absence of the queen under laboratory conditions. *Behavioural Processes*, 64, 49–55. https://doi.org/10.1016/S0376-6357(03)00078-0

Detrain, C., & Deneubourg, J. L. (2006). Self-organized structures in a superorganism: do ants "behave" like molecules? *Physics of Life Reviews,* 3, 162–187. https://doi.org/10.1016/j.plrev.2006.07.001

Ebert, D. (2013). The epidemiology and evolution of symbionts with mixed-mode transmission. *Annual Review of Ecology, Evolution, and Systematics*, 44, 623–643. https://doi.org/10.1146/annurev-ecolsys-032513-100555

Ewald, P. W. (1987). Transmission modes and evolution of the parasitism-mutualism continuum. *Annals of the New York Academy of Science*, 503, 295-306. https://doi.org/10.1111/j.1749-6632.1987.tb40616.x

Farji-brener, A. G., Elizalde, L., Fernández-Marín, H., & Amador-Vargas, S. (2016). Social life and sanitary risks: evolutionary and current ecological conditions determine waste management in leaf-cutting ants. *Proceedings of the Royal Society B: Biological Sciences*, 283, 20160625. http://dx.doi.org/10.1098/rspb.2016.0625

Fernández-Marín, H., Zimmerman, J. K., Rehner, S. A., & Wcislo, W. T. (2006). Active use of the metapleural glands by ants in controlling fungal infection. *Proceedings of the Royal Society B: Biological Sciences*, 273, 1689–1695. http://dx.doi.org/10.1098/rspb.2006.3492

Fernández-Marín, H., Zimmerman, J. K., Nash, D. R., Boomsma, J. J., & Wcislo, W. T. (2009). Reduced biological control and enhanced chemical pest management in the evolution of fungus farming in ants. *Proceedings of the Royal Society B: Biological Sciences*, rspb-2009. http://dx.doi.org/10.1098/rspb.2009.0184

Folgarait, P., Gorosito, N., Thomas-Poulsen, M., & Currie, C.R. (2011a). A preliminary *in vitro* insights into the use of natural fungal pathogens of leaf-cutting ants as biocontrol agents. *Current Microbiology*, 63, 250–258. http://dx.doi.org/10.1007/s00284-011-9944-y

Folgarait, P. J., Marfetán, J. A., & Cafaro, M. J. (2011b). Growth and conidiation response of *Escovopsis weberi* (Ascomycota: Hypocreales) against the fungal cultivar of *Acromyrmex lundii* (Hymenoptera: Formicidae). *Environmental Entomology*, 4040, 342-349. http://dx.doi.org/10.1603/EN10111

Frank, S. A. (1996). Models of parasite virulence. *The Quarterly Review of Biology*, 71, 37-78. https://doi.org/10.1086/419267

Gandra, L. C. (2014). Mecanismo de supressão de colônias da formiga cortadeira *Acromyrmex subterraneus subterraneus* por fipronil. M.Sc. Thesis. Viçosa, MG, Brazil: Universidade Federal de Viçosa, p.37. http://locus.ufv.br/handle/123456789/3988

Hölldobler, B., & Wilson, E. O. (2009). *The Superorganism: The beauty, elegance and strangeness of insect societies*, WW Norton & Company.

Hughes, W. O. H., & Boomsma, J. J. (2004). Genetic diversity and disease resistance in leaf-cutting ant societies. *Evolution*, 58, 1251–1260. http://dx.doi.org/10.1554/03-546

Hughes, D. P., Pierce, N. E., & Boomsma, J. J. (2008). Social insect symbionts: evolution in homeostatic fortresses. *Trends in Ecology and Evolution*, 23, 672–677. http://dx.doi.org/10.1016/j.tree.2008.07.011

Jokela, J., Taskinen, J., Mutikainen, P., & Kopp, K. (2005). Virulence of parasites in hosts under environmental stress: experiments with anoxia and starvation. *Oikos*, 108, 156–164. https://doi.org/10.1111/j.0030-1299.2005.13185.x

Kellner, K., Kardish, M. R., Sea, J. N., Linksvayer, T. A., &. Mueller, U. G. (2017). Symbiont-mediated host-parasite dynamics in a fungus-gardening ant. *Microbial Ecology*, 1–14. https://doi.org/10.1007/s00248-017-1124-6

Lacerda, F. G., Della Lucia, T. M. C., & Souza, D. J. (2011). Biologia comportamental das operárias do lixo das colônias de formigas-cortadeiras. In: Della Lucia, T. M. C. (Ed.), *Formigas-cortadeiras: da bioecologia ao manejo*. (pp. 226-235). Editora UFV

Manley, R., Boots, M., & Wilfert, L. (2017). Condition-dependent virulence of slow bee paralysis virus in *Bombus terrestris*: are the impacts of honeybee viruses in wild pollinators underestimated? *Oecologia*, 184, 305–315. https://doi.org/10.1007/s00442-017-3851-2

Marfetán, J. A., Romero, A. I., & Folgarait, P. J. (2015). Pathogenic interaction between *Escovopsis weberi* and *Leucoagaricus* sp.: mechanisms involved and virulence levels. *Fungal Ecology*, 17, 52–61. http://dx.doi.org/10.1016/j.funeco.2015.04.002

Montoya, Q. V., Meirelles, L. A., Chaverri, P., & Rodrigues, A. (2016). Unraveling *Trichoderma* species in the attine ant environment: description of three new taxa. *Antonie van Leeuwenhoek,* 109, 633–651. http://dx.doi.org/10.1007/s10482-016-0666-9

Moreira, S. M., Rodrigues, A., Forti, L. C., & Nagamoto, N. S. (2015). Absence of the parasite *Escovopsis* in fungus garden pellets carried by gynes of *Atta sexdens*. *Sociobiology*, 62, 34–38. http://dx.doi.org/10.13102/sociobiology.v62i1.34-38

Mueller, U. G., Ishak, H. D., Bruschi, S. M., Smith, C. C., Herman, J. J., Solomon, S. E., Mikheyev, A. S., Rabeling, C., Scott, J. J., Cooper, M., Rodrigues, A., Ortiz, A., Brandão, C. R. F., Lattke, J. E., Pagnocca, F. C., Rehner, S. A., Schultz, T. R., Vasconcelos, H. L, Adams, R. M. M., Bollazzi, M., Clark, R. M., Himler, A. G., LaPolla, J. S., Leal, I. R., Johnson, R. A., Roces, F., Sosa-Calvo, J., Wirth, R., & Bacci Jr, M. (2017). Biogeography of mutualistic fungi

cultivated by leafcutter ants. *Molecular Ecology,* 26, 6921-6937. http://dx.doi.org/10.1111/mec.14431

Nidelet, T., Koella, J. C., & Kaltz, O. (2009). Effects of shortened host life span on the evolution of parasite life history and virulence in a microbial host-parasite system. *BMC Evolutionary Biology*, 9, 1–10. http://dx.doi.org/10.1186/1471-2148-9-65

Olmert, I., & Kenneth, R. G. (1972). Sensitivity of the entomopathogenic fungi, *Beauveria bassiana, Verticillium lecanii,* and *Verticillium* sp. to fungicides and insecticides. *Environmental Entomology,* 3, 33–38. https://doi.org/10.1093/ee/3.1.33

Pagán, I., Montes, N., Milgroom, M. G., & García-Arenal, F. (2014). Vertical transmission selects for reduced virulence in a plant virus and for increased resistance in the host. *PLoS Pathogens*, 10, 23–25. https://doi.org/10.1371/journal.ppat.1004293

Pagnocca, F. C., Masiulionis, V. E., & Rodrigues, A. (2012). Specialized fungal parasites and opportunistic fungi in gardens of attine ants. *Psyche*, 2012. https://doi.org/10.1155/2012/905109

Poulsen, M., Bot, A. N., Nielsen, M. G., & Boomsma, J. J. (2002). Experimental evidence for the costs and hygienic significance of the antibiotic metapleural gland secretion in leaf-cutting ants. *Behavioral Ecology and Sociobiology*, 52, 151–157. https://doi.org/10.1007/s00265-002-0489-8

R Core Team (2017). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

Reis, B. M. S., Silva, A., Alvarez, M. R., Oliveira, T. B., & Rodrigues, A. (2015). Fungal communities in gardens of the leafcutter ant *Atta cephalotes* in forest and cabruca agrosystems of southern Bahia State (Brazil). *Fungal Biology*, 119, 1170–1178. http://dx.doi.org/10.1016/j.funbio.2015.09.001

Rocha, S. L., Jorge, V. L., Della Lucia, T. M. C., Barreto, R.W., Evans, H. C., & Elliot, S. L. (2014). Quality control by leaf-cutting ants: evidence from communities of endophytic fungi in foraged and rejected vegetation. *Arthropod-Plant Interactions*, 8, 485–493. http://dx.doi.org/10.1007/s11829-014-9329-9

Rocha, S. L., Evans, H. C., Jorge, V. L., Cardoso, L. A. O., Pereira, F. S. T., Rocha, F. B., Barreto, R. W., Hart, A. G., & Elliot, S. L. (2017). Recognition of endophytic *Trichoderma* species by leaf-cutting ants and their potential in a Trojan-horse management strategy. *Royal Society Open Science*, 4, 160628. http://dx.doi.org/10.1098/rsos.160628

Rodrigues, A., Pagnocca, F. C., Bueno, O. C., Pfenning, L. H., & Bacci Jr., M. (2005). Assessment of microfungi in fungus gardens free of the leaf-cutting ant *Atta sexdens rubropilosa* (Hymenoptera: Formicidae). *Sociobiology*, 46, 329–334.

Rodrigues, A., Bacci Jr., M. , Mueller, U. G., Ortiz, A., & Pagnocca, F. C. (2008). Microfungal “weeds” in the leafcutter ant symbiosis. *Microbial Ecology*, 56, 604–614. http://dx.doi.org/10.1007/s00248-008-9380-0

Schmid-Hempel, P. (1998). *Parasites in social insects*. Princeton University Press.

Seeley, T. D., & Tarpy, D. R. (2007). Queen promiscuity lowers disease within honeybee colonies. *Proceedings of the Royal Society B: Biological Sciences,* 274, 67–72. http://dx.doi.org/10.1098/rspb.2006.3702

Silva, A., Rodrigues, A., Bacci Jr., M., Pagnocca, F. C., & Bueno, O. C. (2006). Susceptibility of the ant-cultivated fungus *Leucoagaricus gongylophorus* (Agaricales: Basidiomycota) towards microfungi. *Mycopathologia*, 162, 115–119. http://dx.doi.org/10.1007/s11046-006-0037-6

Sousa-Souto, L., & Souza, D. J. (2006). Queen influence on workers behavior of the leaf-cutting ant *Atta sexdens rubropilosa* Forel, 1908. *Brazilian Journal of Biology*, 66, 503–508. http://dx.doi.org/10.1590/S1519-69842006000300016

Stearns, S. C., & Hoekstra, R. F. (2005). *Evolution: an introduction*, 2nd ed. Oxford University Press.

Stewart, A. D., Logsdon Jr., J. M., & Kelley, S. E. (2005). An empirical study of the evolution of virulence under both horizontal and vertical transmission. *Evolution*, 59, 730–739. https://doi.org/10.1554/03-330

Suen, G., Scott, J. J., Aylward F. O., Adams, S. M., Tringe, S. G., Pinto-Tomás, A. A., Foster, C. E., Pauly, M., Weimer, P. J., Barry, K. W., Goodwin, L. A., Bouffard, P., Lewyn, L., Osterberger, J., Harkins, T. T., Slater, S. C., Donohue, T. J., & Currie, C. R. (2010). An insect herbivore microbiome with high plant biomass-degrading capacity. *PLoS Genetics*, 6, e1001129. https://doi:10.1371/journal.pgen.1001129

Tarpy, D. R., & Seeley, T. D. (2006). Lower disease infections in honeybee (*Apis mellifera*) colonies headed by polyandrous vs monandrous queens. *Naturwissenschaften*, 93, 195–199. https://doi.org/10.1007/s00114-006-0091-4

Tompkins, D. M., Jones, T., & Clayton, D. H. (1996). Effect of vertically transmitted ectoparasites on the reproductive success of Swifts (*Apus apus*). *Functional Ecology*, 10, 733–740. https://doi.org/10.2307/2390508.

Varanda-Haifig, S. S., Albarici, T. R., Nunes, P. H., Haifig, I., Vieira, P. C., & Rodrigues, A. (2017). Nature of the interactions between hypocrealean fungi and the mutualistic fungus of leaf-cutter ants. *Antonie van Leeuwenhoek*. 110, 593-605. https://doi.org/10.1007/s10482-016-0826-y

Verza, S. S., Diniz, E. A., Chiarelli, M. F., Mussury, R. M., & Bueno, O. C. (2017). Waste of leaf-cutting ants: disposal, nest structure, and abiotic soil factors around internal waste chambers. *acta ethologica*, 20, 119-126. https://doi.org/10.1007/s10211-017-0255-6

Vienne, C., Errard, C., & Lenoir, A. (1998). Influence of the queen on worker behaviour and queen recognition behaviour in ants. *Ethology*, 104, 431–446. https://doi.org/10.1111/j.1439-0310.1998.tb00081.x

Wallace, D. E. E., Asensio, J. G. V., & Tomás, A. A. P. (2014). Correlation between virulence and genetic structure of *Escovopsis* strains from leaf-cutting ant colonies in Costa Rica. *Microbiology*, 160, 1727–1736. https://doi.org/10.1099/mic.0.073593-0

Watson, M. J. (2013). What drives population-level effects of parasites? Meta-analysis meets life-history. *International Journal for Parasitology: Parasites and Wildlife*, 2, 190–196. http://dx.doi.org/10.1016/j.ijppaw.2013.05.001

Yek, S. H., Nash, D. R., Jensen, A. B., & Boomsma, J. J. (2012). Regulation and specificity of antifungal metapleural gland secretion in leaf-cutting ants. *Proceedings of the Royal Society B: Biological Sciences*, 279, 4215–4222. http:// doi:10.1098/rspb.2012.1458

APPENDIX

APPENDIX S1 – Sampling of colonies fungus gardens before the experiments

We conducted a sampling of the fungus gardens from the 12 colonies collected to check whether they naturally presented *Escovopsis* and *Escovopsioides*. For each colony, we collected ten fragments of fungus garden from three regions: top, middle and base, totalizing 30 fragments. For this, we first sampled the top and the base region of the fungus gardens. Subsequently, each fungus garden was carefully split in half with the aid of a spatula to allow access to the middle region and then we conducted our sampling of this region. The fragments of each region were plated onto petri dishes containing PDA 20% following incubation at 25ºC for 15 days. The plates were checked daily to verify the presence of *Escovopsis* and *Escovopsioides* on fragments.

None of the colonies presented *Escovopsis* and *Escovopsioides* growing on the top region of their fungus gardens. We found *Escovopsis* growing in the middle region from only one colony, while *Escovopsioides* was not observed in this region in any of colonies. The presence of *Escovopsis* and *Escovopsioides* in the base region was detected in four colonies and one colony, respectively. In total, *Escovopsis* was found in five of the 12 colonies and *Escovopsioides* was found in one.

APPENDIX S2 - Weights of queenright colonies, queenless colonies and fungus gardens

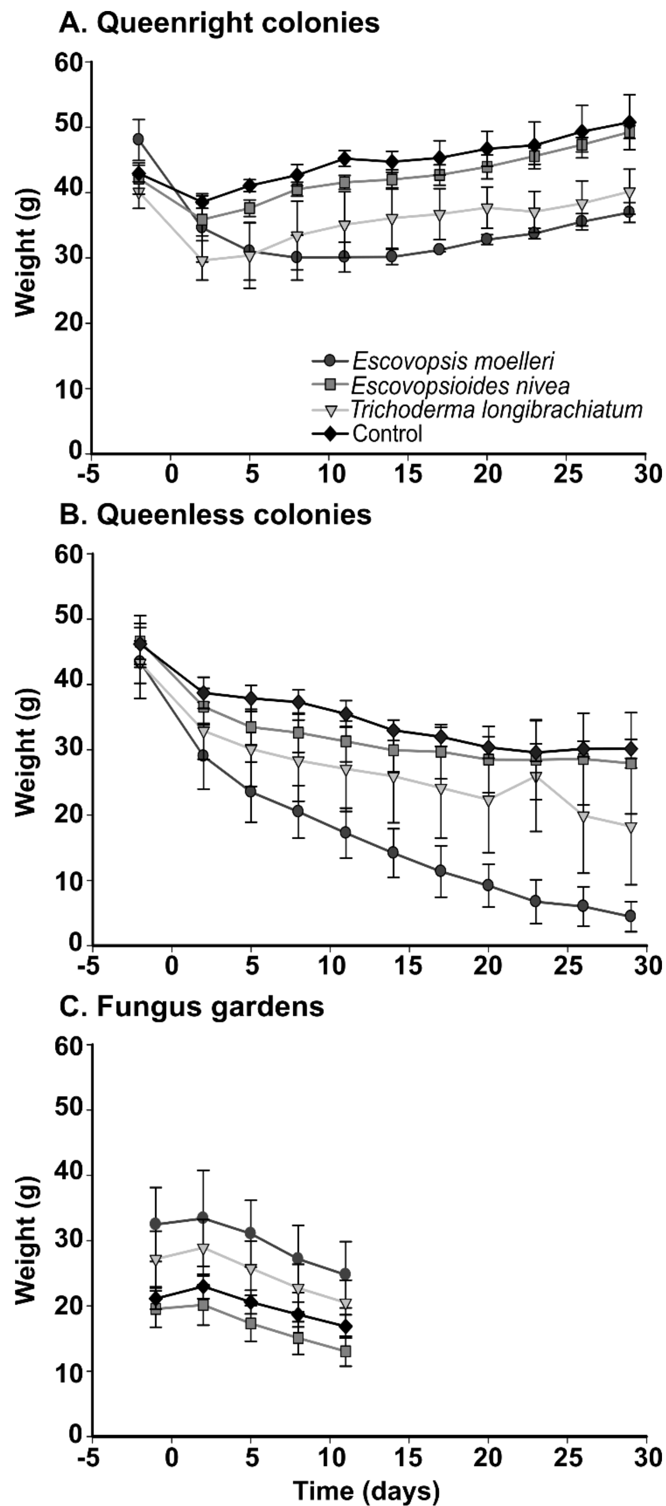


Fig. S1 Weights of *Acromyrmex subterraneus subterraneus* leafcutter ant **(A)** queenright colonies, **(B)** queenless colonies and **(C)** fungus gardens exposed to one of three treatments or to control: conidial suspension of the fungi *Escovopsis moelleri*; *Escovopsioides nivea; Trichoderma longibrachiatum*; control (0.01% Tween 80® solution + saline solution - NaCl 0.85%). Conidial suspensions and blank control were inoculated on Day 0 and the queenright colonies and queenless

colonies were evaluated until Day 29 after inoculation; the fungus gardens were evaluated until Day 11, when all parcels were considered dead. The weight of queenright colonies and queenless colonies was measured two days before inoculation (Day -2) while fungus gardens were weighed one day before inoculation (Day -1). Weighing was repeated 48 hours after inoculation of conidial suspensions for all treatments, and then every 72 hours. The graphs show the weight of each colony complexity level over time. The bars present on each evaluated day represent the standard error.

APPENDIX S3 - Midden weights

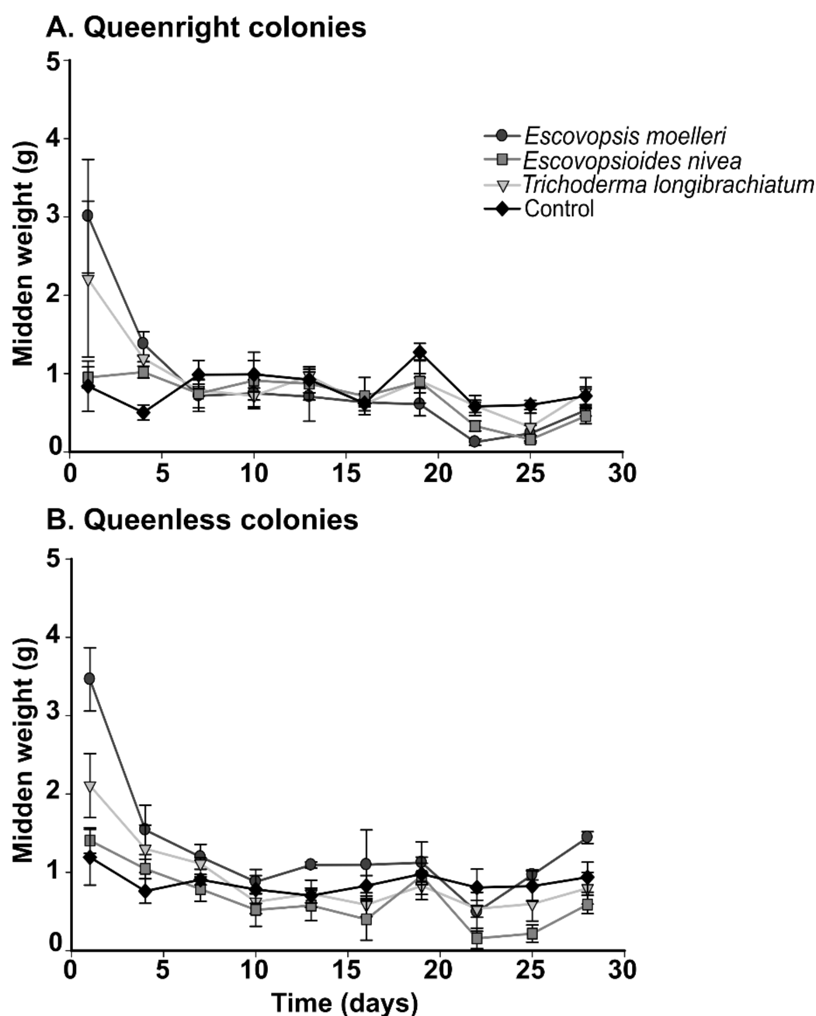


Fig. S2 Weights of middens produced by *Acromyrmex subterraneus subterraneus* leafcutter **(A)** queenright colonies and **(B)** queenless colonies exposed to one of three treatments or to control: conidial suspension of the fungi *Escovopsis moelleri*; *Escovopsioides nivea; Trichoderma longibrachiatum*; control (0.01% Tween 80® solution + saline solution - NaCl 0.85%). Conidial suspensions and blank control were inoculated on Day 0 and the weight of midden produced by the queenright colonies and queenless colonies was evaluated until Day 28 after inoculation. The weight of midden was measured 24 hours after inoculation of conidial suspensions

for all treatments (Day 1) and then, this procedure was repeated every 72 hours. Graphs show the weight of midden produced by the queenright colonies and queenless colonies over time. The bars present on each evaluated day represent the standard error.

APPENDIX S4 - Weights of leaves cut by ants

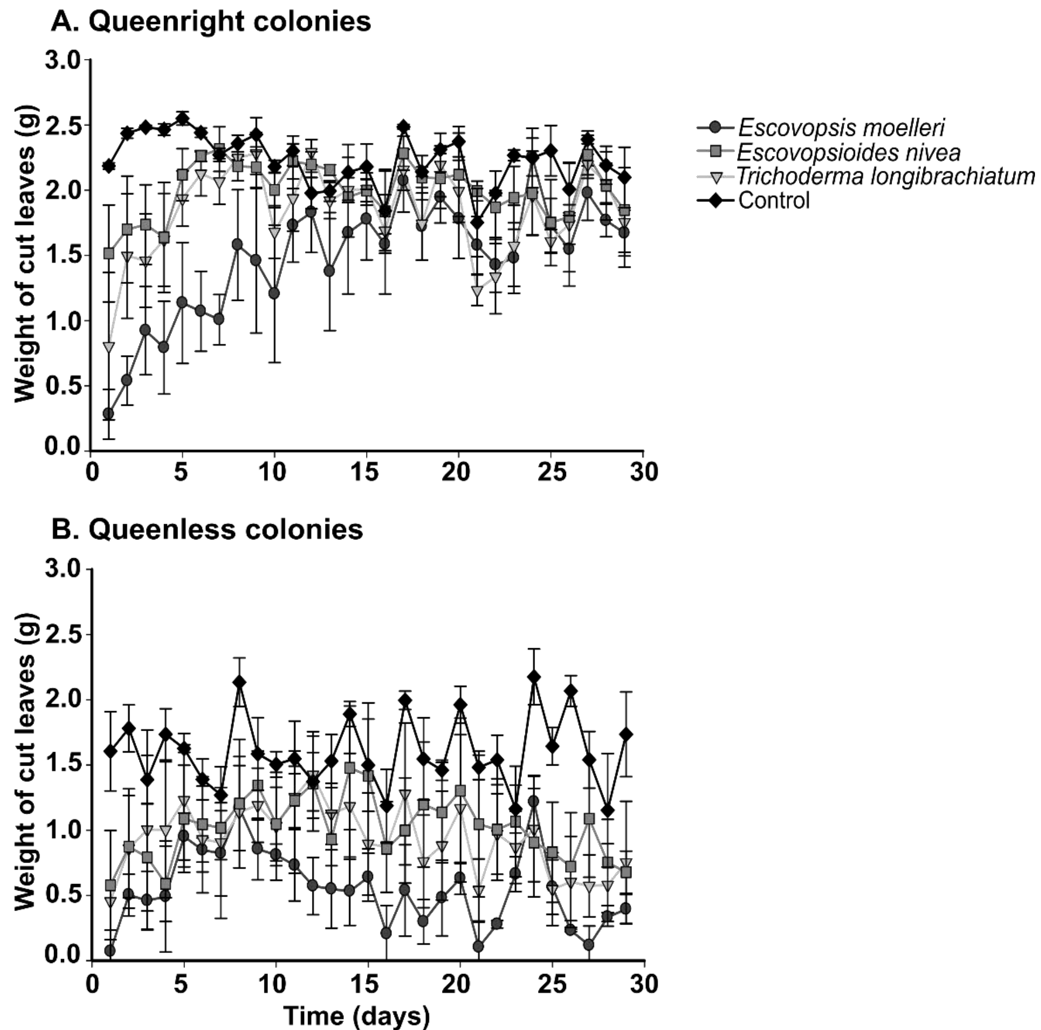


Fig. S3 Weights of leaves cut by *Acromyrmex subterraneus subterraneus* leafcutter ant **(A)** queenright colonies and **(B)** queenless colonies exposed to one of three treatments or to control: conidial suspension of the fungi *Escovopsis moelleri*; *Escovopsioides nivea; Trichoderma longibrachiatum*; control (0.01% Tween 80® solution + saline solution - NaCl 0.85%). Conidial suspensions and blank control were inoculated on Day 0 and the weight of cut leaves by queenright colonies and queenless colonies was evaluated until Day 29 after inoculation. The weight of cut leaves was measured 24 hours after inoculation of conidial suspensions for all treatments (Day 1) and then, this procedure was repeated daily. Graphs show the weight of leaves cut by the queenright colonies and queenless colonies over time. The bars present on each evaluated day represent the standard error.

APPENDIX S5 - Sampling from fungus gardens and middens

Fungus gardens

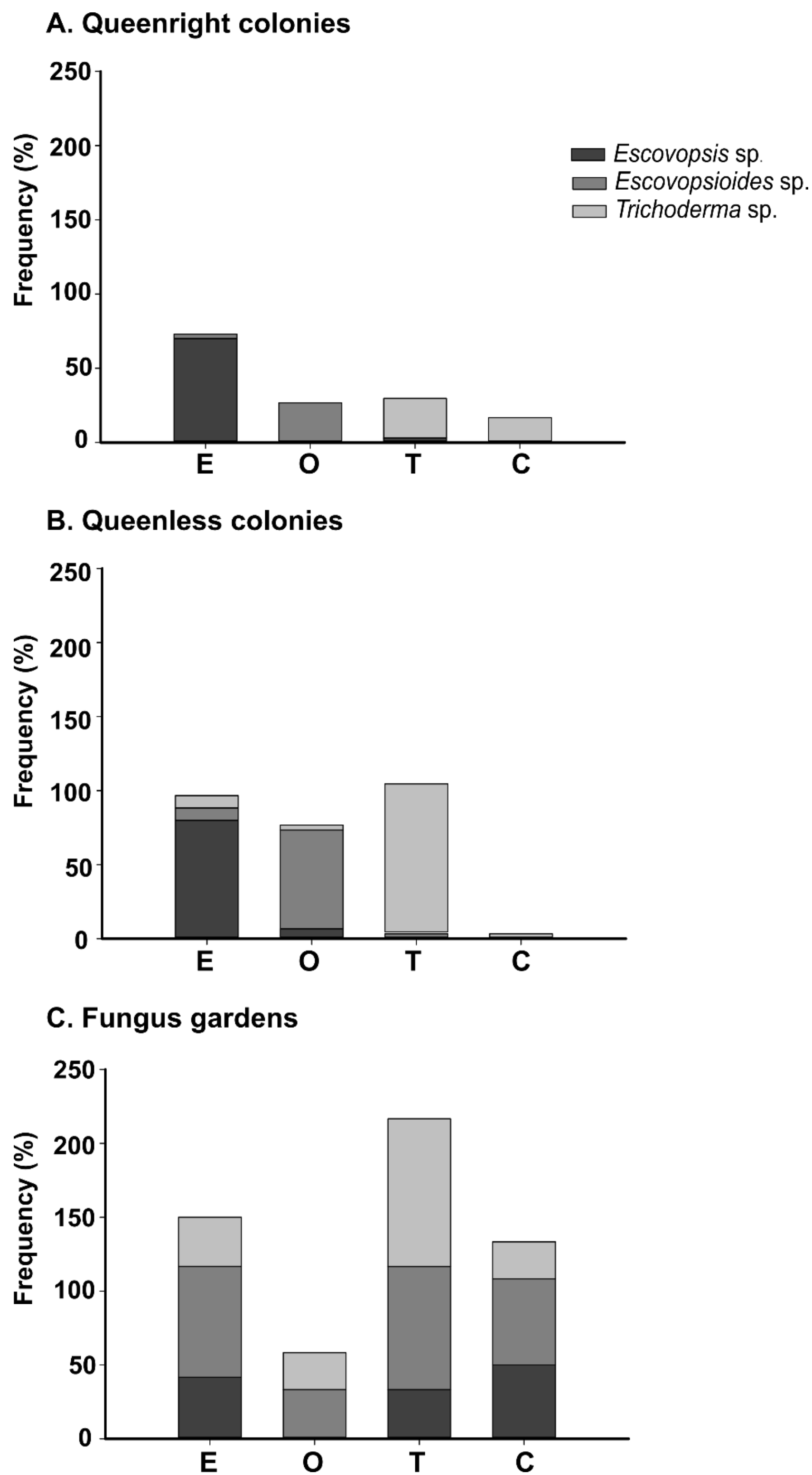


Fig. S4 Frequency of the fungi genera *Escovopsis*, *Escovopsioides* and *Trichoderma* found in the fungus gardens of **(A)** queenright colonies, **(B)** queenless colonies and **(C)** fungus gardens of *Acromyrmex subterraneus subterraneus*

leafcutter ants exposed to one of three treatments or to control: conidial suspension of the fungi *Escovopsis moelleri* (E); *Escovopsioides nivea* (O); *Trichoderma longibrachiatum* (T); control (0.01% Tween 80® solution + saline solution - NaCl 0.85%; C). The fungus gardens from each queenright colony, queenless colony and fungus garden were sampled 48 hours after inoculation of conidial suspensions for all treatments (Day 2) and then, every 72 hours, totalling 10 days of sampling. For this, we transferred aseptically 10 pieces of fungus gardens to plates containing PDA 20% and incubated at 25ºC. We checked the plates daily to verify the occurrence of *Escovopsis, Escovopsioides* and *Trichoderma*. The bars represent the medium frequency of these fungi presence in the 10 days of sampling. We recognized that the ideal would identify these fungi genera at a species level, however, this was not possible due to practical reasons. The presence of *Escovopsis*, *Escovopsioides* and *Trichoderma* was detected in the queenright colonies, queenless colonies and fungus gardens. However, the frequency of these fungi was variable depending on the treatment these colonies complexity levels were exposed.

Midden

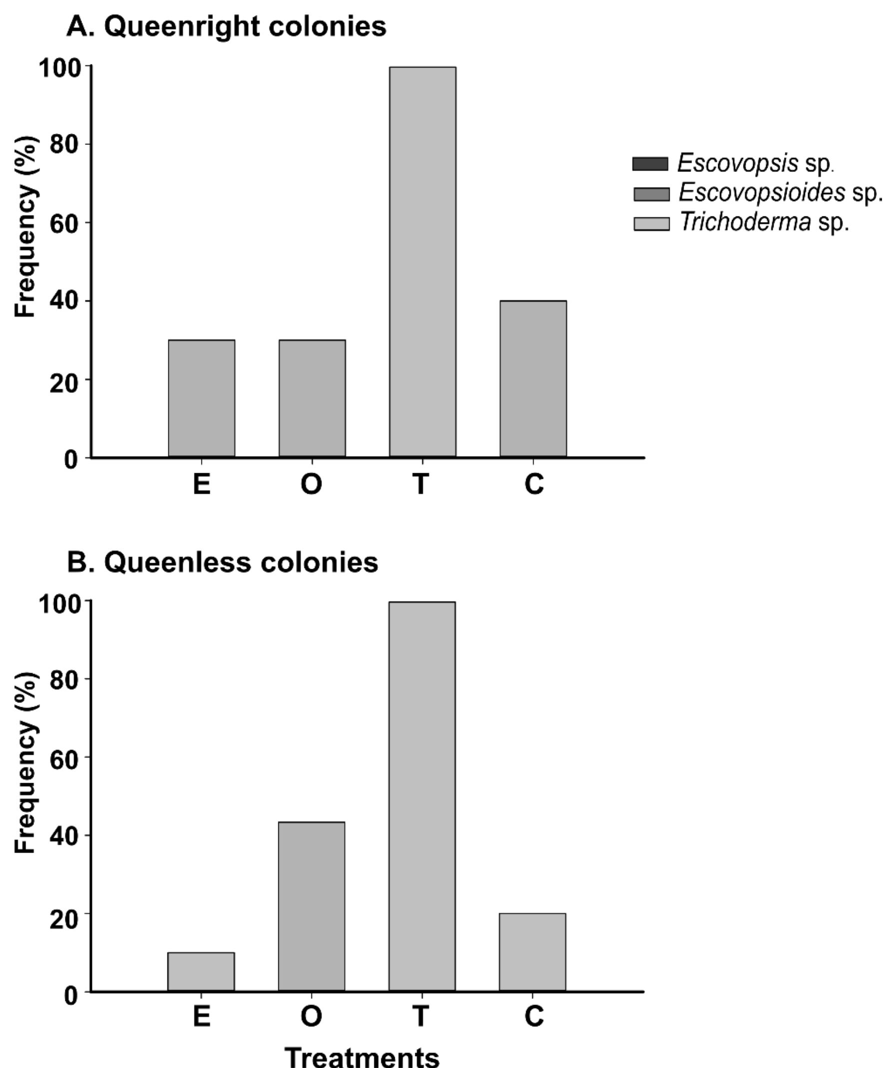


Fig. S5 Frequency of the fungi genera *Escovopsis*, *Escovopsioides* and *Trichoderma* found in the midden of **(A)** queenright colonies and **(B)** queenless colonies of *Acromyrmex subterraneus subterraneus* leafcutter ants exposed to one of three treatments or to control: conidial suspension of the fungi *Escovopsis moelleri* (E); *Escovopsioides nivea* (O); *Trichoderma longibrachiatum* (T); control (0.01% Tween 80® solution + saline solution - NaCl 0.85%; C). We plated five fragments of midden from each queenright colony and queenless colony on Petri dishes containing PDA 20% and then incubated at 25°C for 15 days. This procedure was conducted 24 hours after inoculation of conidial suspensions for all treatments (Day 1) and then every 72 hours, totalling 10 days of sampling. We checked the plates daily to verify the occurrence of *Escovopsis, Escovopsioides* and *Trichoderma*. The bars represent the medium frequency of these fungi presence in the 10 days of sampling. In the same manner as the previous parameter (sampling of fungus gardens), we recognized that the ideal would identify these fungi genera at a species level, however, this was not possible due to practical reasons. No growth of *Escovopsis* and *Escovopsioides* was observed in the midden of queenright colonies and queenless colonies. We suspect that midden could present these fungi, nevertheless, other microorganisms that are also present may have inhibited their growth in the culture medium. On the other hand, *Trichoderma* was found growing the midden from the queenright colonies and queenless colonies, independent of the treatment they were exposed.