



Lack of fungal cultivar fidelity and low virulence of *Escovopsis trichodermoides*

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ABSTRACT

Fungus-growing ants (the attines) are a paramount example of symbiosis, practicing fungiculture for food. Fungi in the genus *Escovopsis* (Ascomycota: Hypocreales) threaten the ant fungal cultivars and show patterns of specificity towards them. *Escovopsis trichodermoides* was described from colonies of the lower attine *Mycocepurus goeldii*, however, its ecological role is still unknown. Here we provide clues of the generalist nature of *E. trichodermoides*, with lack of fidelity to fungal cultivars from different attine ant species and low infection in ant colonies of *M. goeldii*. Inhibitory soluble compounds are produced by *E. trichodermoides* towards different fungal cultivars, as a mechanism of interference competition. Interestingly this generalist lifestyle is not a common trait of *Escovopsis* species, which usually show partner fidelity. Our study indicates that *Escovopsis* has more lifestyles than previously thought, prompting further investigations on its evolution in the attine ant-fungal symbiosis.

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1. Introduction

Symbioses imply a close relationship between organisms and are important models for studying the evolution of beneficial and detrimental interactions. In nature, different organisms live in symbiosis with fungi (Batra, 1963; Weber, 1972; Simard et al., 1997; Helgason et al., 1998; Lutzoni et al., 2001; Dejean et al., 2005; Kämper et al., 2006; Spribille et al., 2016; Martin et al., 2017). The ecological success of these interactions involves many factors, related to the organism's health as well as their environment.

Some social insects maintain mutualistic associations with fungi (Mueller and Gerardo, 2002). Fungus-growing ants (Hymenoptera: Attini: Attina, hereafter named "attine ants") established an ancient obligatory mutualism with basidiomycete fungi (Agaricales: Agaricaceae: *Leucoagaricus gongylophorus* or *Leucocoprinus* spp.) cultivated as the main food source for the colony (Weber, 1972; Schultz and Brady, 2008). In turn, the ants disperse the fungus and provide substrate for its development. Attine ants vertically transmit the fungal cultivars, from parental to offspring colonies by mated

female alates during the establishment of a new colony, securing long-term partner fidelity over evolutionary time (Chapela et al., 1994). In phylogenetically basal lineages of the attines, the so-called lower attines, ants may also recruit new fungi horizontally from free-living stocks (Mueller et al., 1998; Vo et al., 2009; Kellner et al., 2013). These novel ant-cultivar combinations are thought to provide protection towards antagonists, increasing the genetic variability of the fungal partner populations in the symbiosis (Kellner et al., 2018).

In addition to the fungal cultivar, several microbes are found in attine ant fungal gardens. Among them, the fungal genus *Escovopsis* (Ascomycota: Hypocreales) has been reported as a specialized parasite of the ants' fungal cultivars (Currie et al., 1999a; Currie, 2001). Infections with this parasite are supposed to weaken the fungus garden, consequently decreasing the ant workforce (Currie, 2001). *Escovopsis* shows host fidelity (i.e., one strain of the parasite associated with phylogenetically related hosts; Gerardo et al., 2006a; Birnbaum and Gerardo, 2016) and production of inhibitory compounds towards its fungal hosts (Reynolds and Currie, 2004; Varanda-Hafig et al., 2017), the ants and their bacterial symbionts (Dhodary et al., 2018; Heine et al., 2018).

The attine ant fungiculture has been described as a tripartite coevolution, with phylogenetic congruence between the ants, their

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cultivars, and *Escovopsis* (Currie et al., 2003; Gerardo et al., 2006b). Such congruence is maintained by host or parasite adaptations and counter-adaptations under a coevolutionary arms race scenario (Currie et al., 2003). As a result of this coevolution *Escovopsis* shows host fidelity at finer and broader phylogenetic scales (Gerardo et al., 2006a; Birnbaum and Gerardo, 2016; Custodio and Rodrigues, 2019). However, some *Escovopsis* strains overcome the defenses from geographically and phylogenetically distant related fungal hosts, allowing genotype incongruence, local specialization at population level and maintenance of endemic strains (Gerardo and Caldera, 2007; Meirelles et al., 2015). Host-switching events occurred over the evolutionary time (Gerardo et al., 2004, 2006b; Meirelles et al., 2015; Birnbaum and Gerardo, 2016), and could be facilitated by *Escovopsis* horizontal transmission (Currie et al., 1999a) which, however, was only documented for the phylogenetically derived attines (Augustin et al., 2017). Thus, the amplitude of this mechanism in the lower attine ant fungiculture is still elusive.

Escovopsis trichodermoides was described in association with different lower attine ant species (Masiulionis et al., 2015; Montoya et al., 2019). This fungus differs from other *Escovopsis* species by the highly branched conidiophores and absence of conidia-bearing vesicles, in addition to having verrucose conidia (Masiulionis et al., 2015). The patterns of interaction, and the mechanisms involved between *Escovopsis* and the fungal cultivars, are still unknown. To reveal new ecological traits in *Escovopsis*, here we provide clues about the generalist nature of *E. trichodermoides*, with lack of fidelity and low infection towards fungal cultivars of lower attine ants. This lifestyle of *E. trichodermoides* is reported for the first time in the attine ant-fungal symbiosis.

2. Material and methods

2.1. Fungal cultures: preservation and maintenance

In this study, we carried out a series of *in vitro* and *in vivo* experiments to determine the interaction of *E. trichodermoides* and fungi cultivated by attine ants. Fungi examined in this study are kept in the collection of the Laboratory of Fungal Ecology and Systematics (LESF), UNESP - Rio Claro, State of São Paulo, Brazil (Table 1). *Escovopsis* fungi (n = 6 strains) are maintained as conidial suspensions (in glycerol 10%) at −80 °C and in sterile distilled water at 10 °C. Strains were revived on Potato Dextrose Agar medium (PDA, Acumedia) supplemented with 150 µg mL^{−1} of chloramphenicol, and incubated at 25 °C for 7 d in darkness. Working stocks of *Escovopsis* strains were maintained in agar slants with PDA at 10 °C. Since the mutualistic fungi were sensitive to long-term

preservation methods, cultures (n = 4 strains) were maintained by successive transfers on PDA every 20 d and kept at 25 °C in darkness. Cultures were observed periodically to check colony vigor and macroscopic characteristics. Before each assay, fungal strains were previously grown on PDA at 25 °C in darkness up to 7 d for *Escovopsis*; and up to 20 d for fungal cultivars, as well as, for *Trichoderma atroviride* and *Moniliophthora perniciosa*, respectively, used as a comparative group in the experiments.

2.2. Molecular characterization of fungal cultivars

The mutualistic fungi used in this study were characterized by molecular methods (Fig. 1). Mycelium of fungal isolates, previously grown on PDA, was harvested for genomic DNA extraction, following Lacerda et al. (2018). The internal transcribed spacer (ITS) region, a fragment of the ribosomal large subunit gene (LSU), and a segment of the translation elongation factor-1 alpha gene (*tef1*) were amplified (Table S1 for primers and conditions). The amplicons were cleaned up with ExoSAP-IT™ PCR Product Cleanup kit (Thermo Fisher Scientific) and sequenced using BigDye Terminator® v. 3.1 kit (Thermo Fisher Scientific). Forward and reverse sequences were generated on ABI 3500 DNA sequencer (Thermo Fisher Scientific) and assembled in BioEdit v.7.0.5.3 (Hall, 1999). The consensus sequences were compared with homologous ones deposited in GenBank.

For the phylogenetic analyses each gene/region were aligned with the dataset available in Mueller et al. (2018). Alignments were carried out in MAFFT v.7. (Katoh and Standley, 2013), followed by deletion of ambiguous sites in GBLOCKS (Castresana, 2000). Accession numbers of sequences used in the phylogenetic analysis along with metadata are available in Table S2. Sequences from the datasets were concatenated in Winclada v. 1.00.08 (Nixon, 2002). The final alignment comprised 165 sequences with a total of 1494 bp (characters sites 1–510, 511–1075 and, 1076–1494 for ITS, LSU and *tef1*, respectively). Phylogenetic trees were reconstructed in MrBayes v.3.2.2 (Ronquist et al., 2012) under the Bayesian inference. Nucleotide substitution models were selected in JModelTest 2 (Darriba et al., 2012), using Akaike Information Criterion (AIC) with 95% of confidence interval. The selected models were: HKY+I+G for ITS, GTR+I+G for LSU, and K80+I+G for *tef1*. Analyses were carried out with 15.000.000 Markov Chain Monte Carlo (MCMC) generations, until the standard deviation of split frequencies was below 0.01. The first 25% of the MCMC generations were discarded, and the final tree was edited in FigTree v.1.4.3 (Rambaut, 2016). *Chlorophyllum agaricoides* (AFTOL 440) was used as outgroup in the analysis, according to Mueller et al. (2018).

Table 1
Fungi examined in the present study.

Fungal ID ^a	Fungi	Isolation source	Ant colony ID	City, State
LESF 003	<i>Escovopsis trichodermoides</i>	<i>Mycocarpus goeldii</i>	VM1	Rio Claro, SP
LESF 310	<i>Escovopsis trichodermoides</i>	<i>Mycetophylax morschi</i>	AR14022604A1	Florianópolis, SC
LESF 311	<i>Escovopsis trichodermoides</i>	<i>Mycetophylax morschi</i>	AR14022604A2	Florianópolis, SC
LESF 312	<i>Escovopsis trichodermoides</i>	<i>Mycetophylax morschi</i>	AR14022604ALA	Florianópolis, SC
LESF 895	<i>Escovopsis trichodermoides</i>	<i>Mycocarpus goeldii</i>	QVM160527-03	Anhembi, SP
LESF 927	<i>Escovopsis trichodermoides</i>	<i>Mycocarpus goeldii</i>	QVM160528-07	Anhembi, SP
LESF 118	<i>Trichoderma atroviride</i>	<i>Atta sexdens</i>	Nest 39	Corumbataí, SP
AR01	<i>Leucocoprinus</i> sp.	<i>Mycetophylax morschi</i>	AR140227-01	Florianópolis, SC
AR02	<i>Leucocoprinus</i> sp.	<i>Mycetophylax morschi</i>	AR140227-02	Florianópolis, SC
QVM2	<i>Leucocoprinus</i> sp.	<i>Mycocarpus goeldii</i>	QVM160527-03	Anhembi, SP
QVM12	<i>Leucocoprinus</i> sp.	<i>Mycocarpus goeldii</i>	QVM160528-01	Anhembi, SP
QVM11	<i>Leucocoprinus</i> sp.	<i>Mycocarpus goeldii</i>	QVM160527-15	Anhembi, SP
RB03	<i>Leucocoprinus</i> sp.	<i>Mycocarpus smithii</i>	RB180518-03	Anhembi, SP
RB02	<i>Leucoagaricus gongylophorus</i>	<i>Acromyrmex coronatus</i>	BLS170701-01	Rio Claro, SP
LESF 1140	<i>Moniliophthora perniciosa</i>	<i>Theobroma cacao</i>	CP44	—

^a LESF: Laboratory of Fungal Ecology and Systematics (UNESP, Rio Claro, SP). Fungal IDs coded as AR, QVM and RB indicate fungi cultivated by the ants.

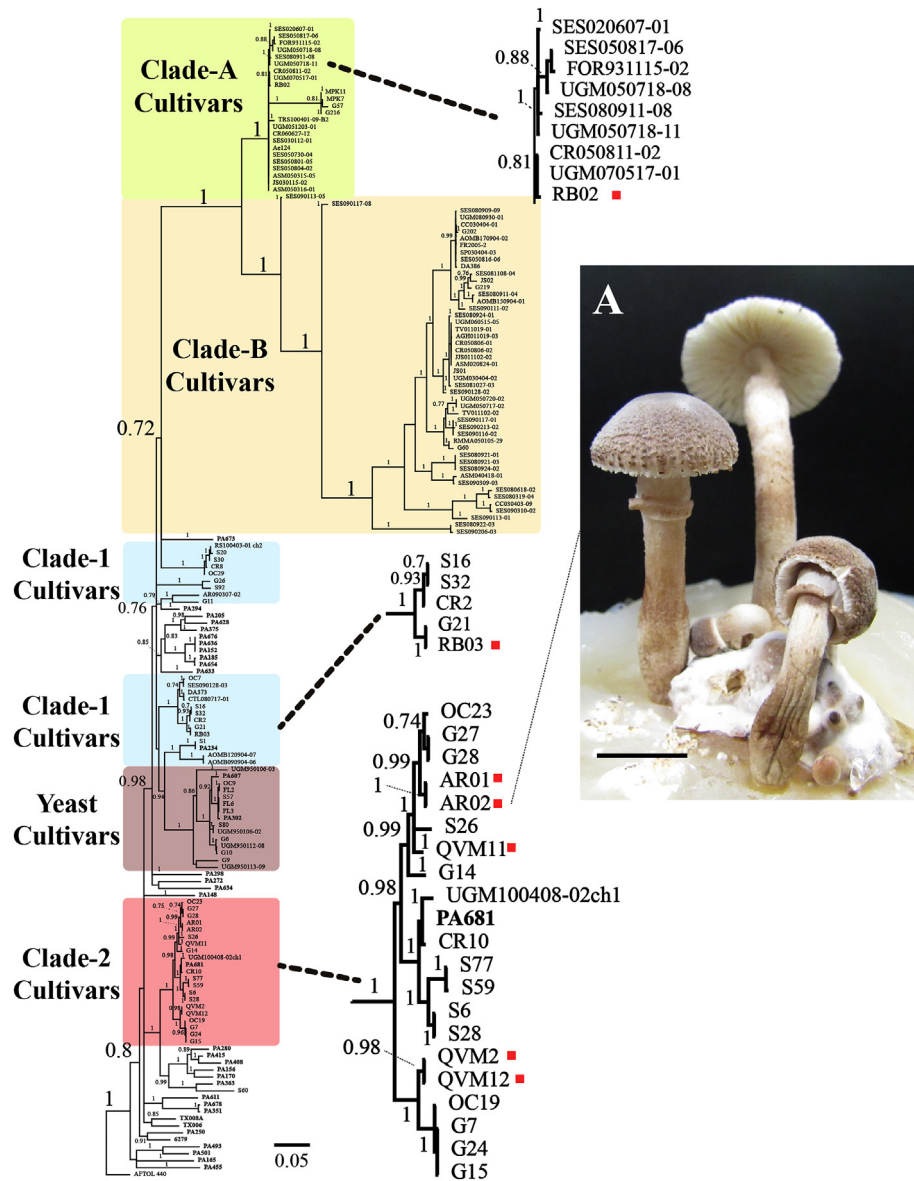


Fig. 1. Phylogeny of fungal cultivars of attine ants based on ITS, LSU and *tef1* markers (with 1494 bp in the final alignment). To characterize the position of strains from this study, the sequences were aligned with sequences from cultivars from Mueller et al. (2018). Free-living fungi (not in association with ant colonies) are shown in bold. *Chlorophyllum agaricoides* (AFTOL 440) were used as outgroup. Red squares on each clade indicate the position of strains from this study. The analysis was performed using the Bayesian inference algorithm and the numbers on branches indicate posterior probabilities greater than or equal to 0.7. Information on the strains, including the ant host, is available in Table S2. Each strain is indicated by the Sample ID code. (A) Basidiome produced in culture by *Leucocoprinus* sp. (AR02) associated with *Mycetophylax morschi* (see supplementary material for details). Photo by Rodolfo Bizarria Jr.

2.3. Pairwise culture assays

To determine the antagonism of *E. trichodermoides* towards different strains of *Leucocoprinus* sp. (i.e. the ant fungal cultivars), dual-culture assays were performed following the method by Silva et al., 2006, and herein referred to as Dual-culture type 1. Mycelial fragments (0.5 cm²) of the mutualistic fungus were used in all assays. These fragments were obtained by cutting the colonies with a sterile micropipette tip. Fragments were placed 1.5 cm from the edge of a Petri dish containing PDA. This plate was incubated for 14 d at 25 °C in darkness. Then, a mycelial fragment (0.5 cm²) from each of the six *E. trichodermoides* strains was placed 3 cm from the mycelium of the mutualistic fungus (Fig. S1). Two controls were prepared: (i) *Leucocoprinus* sp. strains growing alone, and (ii) *E. trichodermoides* strains growing alone.

The dual-cultures as well as the controls were incubated for 10 d at 25 °C in darkness, and plates were scanned after 1, 2, 3, 5, 7 and 10 d. Growth areas of both fungi were measured (in cm²) in ImageJ v1.8.0_112 (Schneider et al., 2012). Each of the six *E. trichodermoides* strains was considered a biological replicate in these experiments (using the mean of eight plates per strain). Each control was prepared containing six plates.

Mycelial growth areas of the mutualistic fungi were compared to the control after 3, 5 and 10 d of experiment by: (i) two-sample *t*-test with an alpha threshold of 0.05, or Welch two-sample *t*-test for treatments that violated the parametric assumptions. We selected these days since they represented the first contact between fungi (3 d); the complete overgrowth of mutualistic fungi by *E. trichodermoides* (5 d); and the last day of experiment (10 d); (ii) one-way ANOVA followed by Tukey *posthoc* test with an alpha

threshold of 0.05 for multiple comparisons, using the model available in *Agricolae* package (De Mendiburu, 2014). In this analysis we compared the relative growth of each mutualistic fungus (ratio of treatment by the respective control); (iii) inhibition percentage (I %) of each mutualistic fungi with the formula: $I = [(C - Et)/C] * 100$, where C indicates mean growth of control group, and Et the growth in the presence of *E. trichoderma* on the tenth day of incubation. Shapiro-Wilk and Bartlett tests were applied to check the normality and homoscedasticity assumptions of the data, respectively. Analyses were conducted in R v. 3.3.3 (R Core Team, 2017).

The growth of *E. trichoderma* towards the different mutualistic fungi was compared to the control using: (i) mixed-ANOVA using treatments (between-subjects) and the days of culture (within-subjects) as factors. Multiple comparisons were conducted with two-sample *t*-test with an alpha threshold of 0.05 with Bonferroni correction. Data were transformed to log (x) for validation of parametric assumptions; (ii) the mycelial growth over time was also analysed with non-parametric test for longitudinal data for repeated measures (*npard* with an alpha threshold of 0.05), using the same factors. The *npard* analysis was conducted using the F1-LD-F1 model. Wald-type and ANOVA-type analyses were used, followed by paired comparisons between curves with model available in package *npard* (Noguchi et al., 2012). Both analyses were conducted during five days of growth, since the mycelium of *E. trichoderma* completely covered the mycelium of *Leucocoprinus* at this time. Analyses were conducted in R v. 3.3.3 (R Core Team, 2017).

2.4. Bioassays with multiple ant fungal cultivar possibilities

To determine the fidelity of *E. trichoderma* towards different fungal cultivar possibilities, we performed bioassays with choice-bioassays (herein referred as Dual-culture type 2). These bioassays were conducted according to Gerardo et al. (2006a) and an experimental design was performed with Petri dishes (150 × 15 mm) containing 60 mL of PDA (Fig. S1). The culture medium was cut with a sterile scalpel to create six equidistant tracks. Two sets were carried out to provide multiple fungal possibilities: in the first set (i) mycelium fragments of the four *Leucocoprinus* strains (AR01, AR02, QVM2 and QVM12) were placed at the end of the four tracks, a mycelium fragment of *M. perniciosus* (LESF 1140) was placed on the fifth end, and the sixth end was left blank (control). In the second set (ii) mycelium fragments of *Leucocoprinus* strains (AR01, QVM2, QVM11 and RB03) were placed at the end of the four tracks, a mycelium fragment of *Leucoagaricus gongylophorus* (RB02) was placed on the fifth end, and the sixth end was left blank (control). The plates were incubated for 7 d at 25 °C in darkness. The selection of *M. perniciosus* as a distant group was based on its phylogenetic distance, its distinct ecological role (i.e. plant pathogen, Mondego et al., 2008), and also the non-related lifestyle with attine ant gardens. In addition, this fungus was also selected as a comparative group in another study (Augustin et al., 2017).

Afterwards, a mycelium fragment (0.5 cm²) of each *E. trichoderma* strain or *T. atroviride* (LESF 118) was placed at the center of the plate. This system was incubated at 25 °C in the dark for 28 and 14 d for the first and second bioassays, respectively. Growth distances (in cm) towards each end of the tracks were measured as described (section 2.3). *T. atroviride* (LESF 118) was used as a comparative group for *E. trichoderma*, due to its ecological role (i.e. mycoparasite) and because it belongs to Hypocreaceae (Druzhinina et al., 2011; Kubicek et al., 2011) but did not coevolve with the ants. Each assay was conducted with ten plates, and each *E. trichoderma* strain was considered a biological

replicate (using the mean of ten plates per strain).

Fidelity patterns were evaluated in radar charts disposing length values (in cm) over time, with each track of the Petri dish as the axis of the chart. Growth data were compared daily with Friedman test with an alpha threshold of 0.05, followed by Wilcoxon signed-rank test with an alpha threshold of 0.05 for multiple comparisons. Analyses were conducted separately for each day of growth in R v. 3.3.3.

2.5. Production of soluble antifungal metabolites

To evaluate if the antagonism of *E. trichoderma* could be mediated by interference competition, the production of metabolites was assessed following the method by Varanda-Haifig et al. (2017) with modifications. Two types of *E. trichoderma* filtrates were obtained: (i) in the absence of the mutualistic fungi (Et1) and (ii) in the presence of the mutualistic fungi (Et2). For the production of both filtrates, *E. trichoderma* strains were previously grown on PDA at 25 °C for 10 d. Conidial suspensions were prepared according to Newmeyer (1990) in 0.05% Tween 80 solution and adjusted to 10⁶ conidia mL⁻¹ in a Neubauer chamber.

Two Erlenmeyer flasks (125 mL) with 90 mL of Potato Dextrose Broth medium (PDB; Acumedia, final pH: 5.1 ± 0.2) were used for production of filtrates. To prepare the Et1 filtrates, 1 mL of the conidia suspension was inoculated in flasks and then incubated at 25 °C at 120 rpm for 14 d. To prepare the Et2 filtrates, five fragments (0.5 cm²) of each mutualistic fungus were inoculated and the flasks incubated at 25 °C at 120 rpm for 3 d. Then, 1 mL of conidia suspension of *E. trichoderma* was inoculated, and the flasks incubated under the same conditions for 14 d. After incubation the medium was filtrated in a 0.45 µm membrane (MF-Millipore, MCE membrane) and mixed with double-strengthened PDA medium in a 1:1 ratio (v/v). For the control, PDB was added in a 1:1 ratio (v/v) with double-strengthened PDA, simulating absence of metabolites.

Then, a mycelium fragment (0.5 cm²) of each mutualistic fungus was placed at the center of a Petri plate with the respective prepared media. Plates were incubated at 25 °C in darkness, and growth areas (in cm²) were recorded at the 3, 7, 10, 14, 21, 28 and 35 d of incubation (item 2.3). Each of the six *E. trichoderma* strain was considered a biological replicate (using the mean of eight plates per strain). The control consisted of six plates.

The final growth of the mutualistic fungus in the presence of metabolites of *E. trichoderma* was evaluated by: (i) one-way ANOVA followed by Tukey *posthoc* test with an alpha threshold of 0.05 for multiple comparisons. For treatments that violated the parametric assumptions, we applied Kruskal-Wallis, followed by Mann-Whitney *U* tests both with an alpha threshold of 0.05; (ii) Relative growth of each mutualistic fungus after 35 d of culture with one-way ANOVA, followed by Tukey *posthoc* test with an alpha threshold of 0.05 for multiple comparisons; (iii) Inhibition percentage (I %) using the growth values of control and treatments in the presence of metabolites (Et1 or Et2) after 35 d of incubation. Analyses were conducted in R v. 3.3.3.

2.6. Assays in colonies of *Mycocepurus goeldii*

We performed assays in colonies of *Mycocepurus goeldii* to characterize the effects of *E. trichoderma* infections. A total of twenty queen-less colonies were collected in Anhembi (State of São Paulo, Brazil), from March 18th to 20th, 2018. After excavation, fungus gardens along with tending workers and brood were collected in plastic containers with a fine layer of plaster at the base, previously subjected to UV exposure for 30 min. Fungal isolation from colonies was conducted following Rodrigues et al. (2008a)

transferring seven gardens fragments to PDA plates supplemented with 150 $\mu\text{g mL}^{-1}$ of chloramphenicol (see details in the Supplementary Material). Colonies were transferred to new containers of 250 mL or 500 mL depending on the size of the fungus gardens. These containers had one or two holes (1.0 cm in diameter) for ant mobility. Finally, the containers with fungus gardens were placed in a larger container (1000 mL) with a hole to add or remove cornmeal flour as substrate for ant foraging (Fig. S1). Such system was kept for acclimation in darkness for three days.

The experimental design comprised 20 colonies distributed in groups of five, considering the size and age for homogeneity between treatments. Conidial suspensions of *E. trichoderma* LESF 003, LESF 895 and LESF 927 (selected for these experiments because they were isolated from *M. goeldii* colonies) were prepared in 0.05% Tween 80 solution and adjusted to 10^6 , 10^7 and 10^8 conidia mL^{-1} in a Neubauer chamber. Using a hand spray (previously exposed to UV light for 30 min) 2 mL of each suspension were sprayed on the fungus garden starting from 10^6 conidia mL^{-1} and increasing the concentrations in intervals of seven days, for a total of 21 d. The control-treated group consisted of 0.05% of sterile Tween 80 solution only. Each conidial suspension was also spread on PDA plates to check for conidia viability. Every second day, 0.2 g of cornmeal flakes were offered to the ants and the plaster humidified with 1 mL of sterile deionized water.

The colonies were evaluated daily regarding: the (i) survival-ship, (ii) food incorporation on fungus gardens, (iii) presence of fungal infection indicated by fungal mycelium overgrowing the fungus gardens, (iv) final aspect of the fungus garden after consecutive exposures, and (v) accumulative waste weight (considering the sum of the ratio between waste and garden weight for each exposure).

For the *in vivo* assays, the colony survival percentage was expressed in a survival chart over time (Kaplan-Meier curve) with survival package (Lumley and Therneau, 2004) in R. The effect of successive exposures was evaluated by non-metric multidimensional scaling (NMDS) using Bray-Curtis as the dissimilarity index and by principal coordinates analysis (PCoA) using Gower distance. Binary values were used to indicate survival and presence of fungal infection; absolute values for the amount of waste produced and number of times that food was incorporated; as well as ordinal data for final aspect of the fungus gardens (Fig. S2). Charts were computed in PAST v.3.22 (Hammer et al., 2001), in two-dimensions with the first two coordinates.

3. Results

3.1. Inhibition of different ant fungal cultivars by *Escovopsis trichoderma*

The four *Leucocoprinus* strains used in this assay clustered in two distinct clades within clade-2 of the lower attine ant fungiculture (Fig. 1). Despite these genetic differences, *E. trichoderma* inhibited all strains of *Leucocoprinus* (Table S3; two-sample *t*-test and Welch two-sample *t*-test, $P < 0.05$) with the lowest values of relative growth for strain QVM12 (Table 2; Tukey *posthoc* test, $P < 0.05$). Mycelial area was reduced at least 1.78 times for all cultivar strains towards *E. trichoderma* compared to the control. In addition, we observed a darkening pattern in the mutualistic fungal mycelia at the contact zones with the antagonist (Fig. 2), followed by fungal cultivar mycelial degeneration (Table S3), characterized by reduction in colony size and loss of colony opacity. Inhibition was observed on the fifth day when cultivars were overgrown by *E. trichoderma* mycelium, and on the third day for *Leucocoprinus* sp. QVM12 (Fig. 2, Table 2 and Table S3; two-sample *t*-test and Welch two-sample *t*-test, $P < 0.05$). High inhibition

Table 2

Leucocoprinus growth in the dual-culture assays. Figures indicate the mean of relative mycelial area ($\pm$ SD) between the growth towards *Escovopsis trichoderma* and the control group. Different letters indicate significant statistical differences between groups on each day (Tukey test at 5%).

Days	AR01 ^a	AR02 ^a	QVM2 ^b	QVM12 ^b
3	0.94 $\pm$ 0.07a	0.98 $\pm$ 0.06a	0.96 $\pm$ 0.03a	0.84 $\pm$ 0.03b
5	0.75 $\pm$ 0.05 ab	0.82 $\pm$ 0.06a	0.81 $\pm$ 0.04a	0.69 $\pm$ 0.02b
10	0.52 $\pm$ 0.05a	0.56 $\pm$ 0.04a	0.54 $\pm$ 0.03a	0.43 $\pm$ 0.02b

^a Mutualistic fungi of *Mycetophylax morshi*.

^b Mutualistic fungi of *Mycocarpus goeldii*.

percentage was observed in the tenth day (48.0%, 43.8%, 46.2%, 57.5% for AR01, AR02, QVM2 and QVM12, respectively).

In contrast to expectations, we did not observe growth maximization of *E. trichoderma* towards the different fungal cultivars (Fig. 2). No statistical differences were observed between treatments and the control group (Fig. 2; mixed-ANOVA, $P > 0.05$).

3.2. Lack of fungal cultivar fidelity by *Escovopsis trichoderma*

Absence of preference was observed for *E. trichoderma* towards the different mutualistic fungi (Figs. 1 and 3). Growth until the end of the track was observed towards all mutualistic fungi (Tables S4 and S5), including *L. gongylophorus* (RB02), the fungus cultivated by some leafcutter ant species and distantly related to the lower attine cultivars (Tables S4 and S5). This growth pattern of *E. trichoderma* was similar to *T. atroviride*, a fungus that did not coevolve in the attine ant-fungus symbiosis (Fig. S3). On the other hand, *E. trichoderma* was inhibited only by *M. perniciosus* (Fig. S4). Thus, the absence of efficient defensive barriers towards *E. trichoderma* was observed for all fungal cultivars but not for *M. perniciosus*, a fungus that did not coevolve in association with the attines (Figs. 1 and 3; Wilcoxon signed-rank test, $P < 0.05$).

3.3. Interference competition by *Escovopsis trichoderma*

Soluble metabolites produced by *E. trichoderma* inhibited all *Leucocoprinus* strains in culture (Fig. 4). Chemical compounds produced in both Et1 and Et2 filtrates reduced the mycelial growth area of the mutualistic fungi (Table 3; Tukey *posthoc* test, $P < 0.05$; Mann-Whitney *U* test, $P < 0.05$).

Overall, the results indicated no significant differences in inhibition between Et1 and Et2 filtrates by the end of the assays. However, in some cases, inhibition above 50% was observed in relation to the control group (Table 3). The fungal cultivar QVM2 was the least inhibited (Tukey *posthoc* test, $P < 0.05$). Curiously, the mutualistic fungus AR02 presented initial basidiome formation in the presence of metabolites of *E. trichoderma* (Fig. 4 and Fig. S5).

3.4. Low virulence of *Escovopsis trichoderma* in ant colonies

The experiments using queen-less colonies showed that *E. trichoderma* was not destructive *in vivo* (Fig. 5). The effects observed on infected *M. goeldii* colonies indicated that the arsenal of *E. trichoderma* is insufficient to overcome the colony defenses (i.e., mutualistic fungus, the ants and associated fungus garden microbes). Colony deaths were observed in five out of twenty colonies, usually associated with the total removal of the fungus garden by the ants (Figs. 5 and S2). In two out of five colonies, we also observed mycelia of *E. trichoderma* and from a pink-spored *Escovopsis* on the fungus garden. The first colony died after 12 d of experiment, despite the large amount of conidia inoculated in

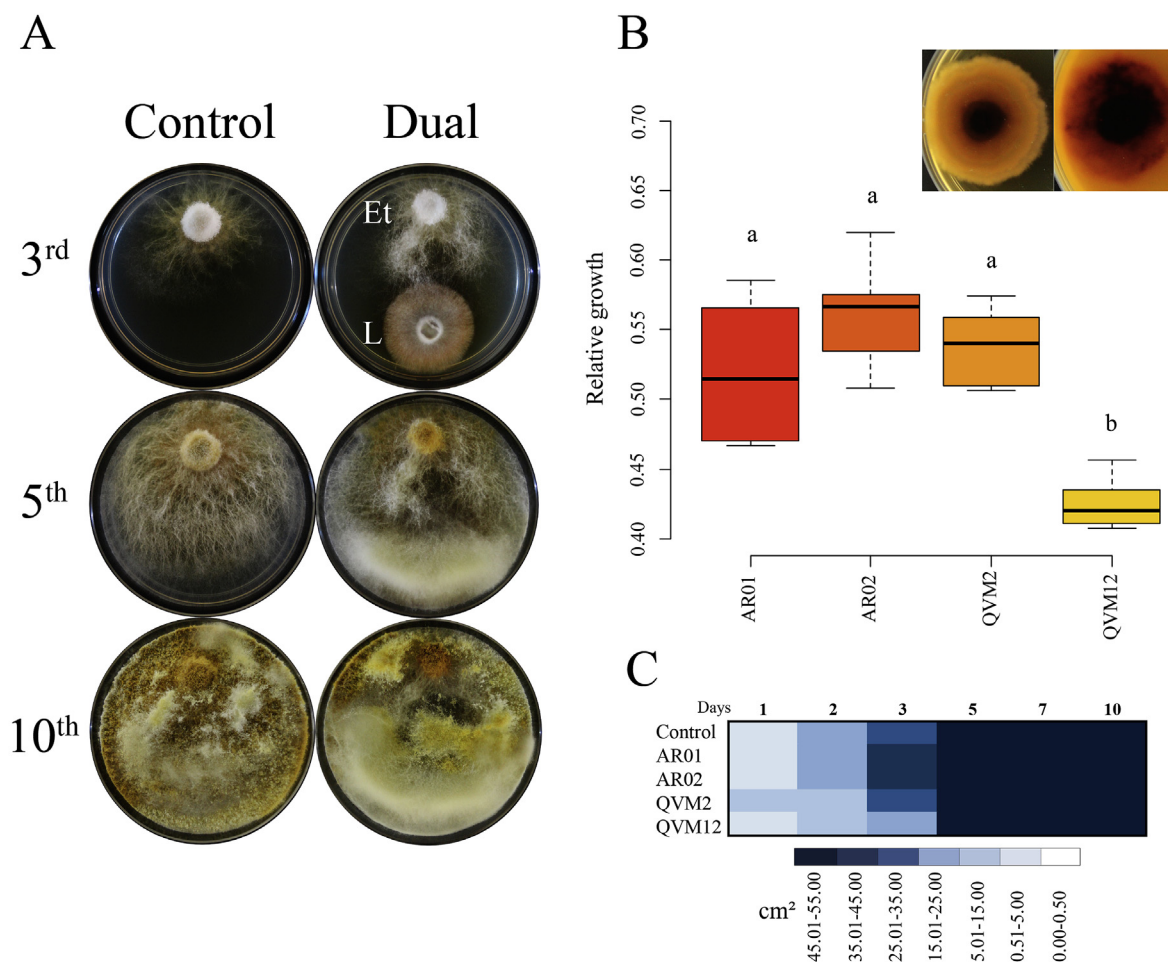


Fig. 2. *Escovopsis trichodermoides* shows a generalist pattern of inhibition. **(A)** Mycelial growth pattern in the absence (Control) and in interaction (dual-culture) with *Leucocoprinus* fungi. Photos indicate the 3rd, 5th and 10th d of interaction between *E. trichodermoides* (Et) LESF 927 and *Leucocoprinus* (L) QVM2. **(B)** Boxplot of relative growth of mutualistic fungi after 10 d of culture, different letters indicate significant differences (Tukey test, $P < 0.05$). Dual-culture plate after 10 d of assay showing darkening of the mutualistic fungus. Right and left indicate the mutualistic fungus in the absence and in the presence of *E. trichodermoides*, respectively. **(C)** Heat maps of *E. trichodermoides* growth in dual-culture (values in cm^2). No significant differences with the control group (Mixed-ANOVA, $P > 0.05$ and npairLD, $P > 0.05$).

the second exposure (Fig. 5). Thus, colony viability was stable until the second exposure, when the first death was recorded (Fig. 5).

Phylogenetic analyses indicated that the fungi cultivated by the majority of *M. goeldii* colonies clustered with the same fungal strains used in the dual-culture bioassays (Fig. S6). Thus, essentially the fungal cultivars from these colonies were similar to the ones used in the *in vitro* experiments. Therefore, the generalist trait of *E. trichodermoides* is unable to overcome the colony defenses.

Colonies exposed to *E. trichodermoides* showed stability towards the large number of viable conidia sprayed. Food incorporation was only observed in two colonies exposed to *E. trichodermoides* LESF 927 (Fig. S2), and may have contributed to colony stability. On the other hand, the five colonies exposed to strain LESF 895 presented different spatial dispersion of the data for the evaluated parameters (Fig. 6), such as survival, food incorporation, infection and final aspect of fungus gardens. After the third and final exposure, healthy colonies had viable conidia of *E. trichodermoides* isolated from the garden surface (Fig. S7), indicating that the fungus remained in the system.

4. Discussion

Symbiotic interactions are mediated by chemical metabolites for recognition, interference, and nutrition of the partners involved (Akiyama et al., 2005; Gerardo et al., 2006a; Heine et al., 2018).

Escovopsis fungi show host fidelity mediated in part by chemical interaction between the parasite and its host (Gerardo et al., 2006a; Birnbaum and Gerardo, 2016). However, occasional host-switching events occurred over the evolution of this interaction (Gerardo et al., 2004, 2006b; Taerum et al., 2007; Meirelles et al., 2015). Here, we showed that *E. trichodermoides* has a generalist pattern with an absence of fidelity and chemotaxis to different strains of *Leucocoprinus*. Such pattern is reported for the first time for the *Escovopsis*-ant cultivar association.

Escovopsis trichodermoides caused high growth inhibition towards the different cultivars tested *in vitro*. The defensive barriers of ant cultivars were insufficient to prevent inhibition by the antagonist. On the other hand, the mechanisms of infection of *E. trichodermoides* were insufficient towards queen-less colonies of *M. goeldii*, and damage was only observed after three successive exposures, with increased conidia dosages. Such patterns differ from other *Escovopsis* species, which were described as having high virulence (Currie et al., 1999a; Currie, 2001) and high host fidelity (Gerardo et al., 2006a; Birnbaum and Gerardo, 2016; Custodio and Rodrigues, 2019). *Escovopsis* virulence may be an outcome of the interaction between ant-cultivar genotypes (Kellner et al., 2018), microbial associated symbionts (Currie et al., 1999b) and chemical interactions that mediate *Escovopsis* fidelity (Birnbaum and Gerardo, 2016). With *L. gongylophorus*, the fungus cultivated by

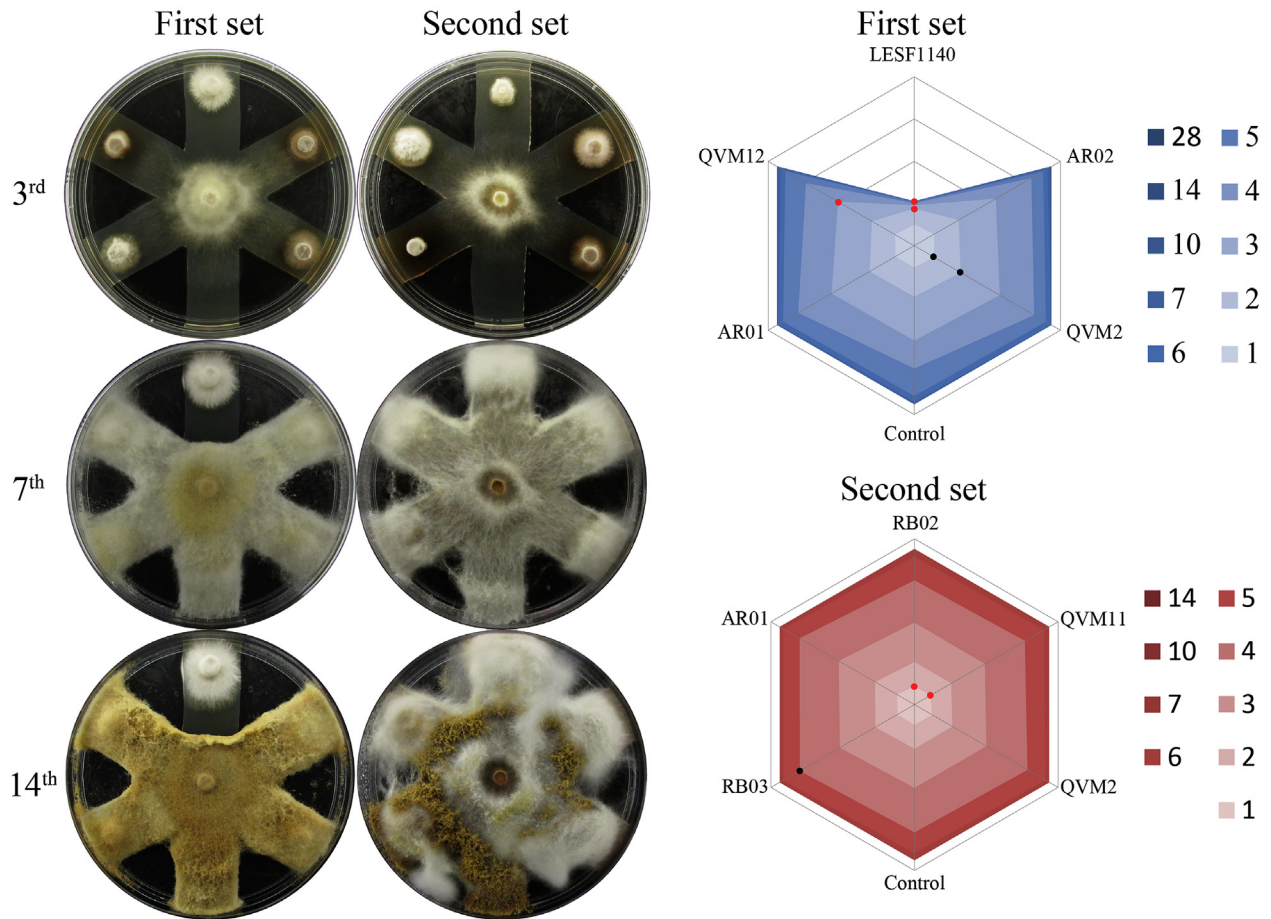


Fig. 3. Lack of fungal cultivar fidelity by *Escovopsis trichodermoides*. Growth pattern of *E. trichodermoides* on PDA towards multiple fungal cultivars in two sets, after 3, 7 and 14 d of incubation. Information regarding the sets was described in section 2.4 of Material and Methods. Pictures show the growth of *E. trichodermoides* LESF 311 (First set) and LESF 895 (Second set). Radar charts on the right show the growth of *E. trichodermoides* over time (squares indicate each day of growth). The fungal cultivar strains IDs are indicated on the vertices of each chart. LESF 1140 and RB02 stand for *Moniliophthora perniciosa* and *Leucoagaricus gongylophorus*, respectively. The others IDs stand for *Leucocoprinus* sp. strains. Red dots on axis indicate lower values in relation to control group, while black dots indicate higher values (Wilcoxon signed-rank test, $P < 0.05$).

some leafcutter ant species (Mueller et al., 2018), we also observed no defensive barriers that prevented *E. trichodermoides* from overgrowing this cultivar (even considering the large phylogenetic distance from the lower attine ant cultivars). This is not the case for other *Escovopsis* (Gerardo et al., 2006a; Birnbaum and Gerardo, 2016; Custodio and Rodrigues, 2019), and other symbiotic systems (Gilbert and Webb, 2007). Growth inhibition of *E. trichodermoides* was only observed against *M. perniciosa*, a fungus distantly related to the attine ant cultivars and not associated with the ants. These observations support the generalist pattern of *E. trichodermoides* towards the ant fungal cultivars.

Additional defensive barriers may prevent *E. trichodermoides* infections from establishing in nature. Ant-associated bacterial symbionts (Currie et al., 1999b; Li et al., 2018), hygienic behavior along with antimicrobial secretions produced by the ants (Currie and Stuart, 2001; Fernández-Marín et al., 2006; Rodrigues et al., 2008b) and complexity of colonies (Birnbaum and Gerardo, 2016) may play a role in preventing successful infections in lower attine colonies and in other fungicultures of the attine ants. Collectively, such barriers may explain the low frequency of *E. trichodermoides* in lower attine ant colonies compared to other *Escovopsis* species (A. Rodrigues, personal observation), since not only the fungal cultivar defenses account for the host-parasite interaction (Currie et al., 1999b; Currie and Stuart, 2001; Fernández-Marín et al., 2006;

Rodrigues et al., 2008b).

Chemical mechanisms are associated with inhibition by *E. trichodermoides*. We observed a darkening pattern and mycelium degeneration of the mutualistic fungi at the contact zones with the antagonist, a pattern also observed in other studies that performed similar experiments (Silva et al., 2006; Folgarait et al., 2011; Varanda-Haifig et al., 2017). Darkening of the colony might be associated with cell degeneration or antibiosis as a response by the host (Savoie et al., 1998; Silva et al., 2006; Folgarait et al., 2011; Varanda-Haifig et al., 2017). The production of soluble compounds as a mechanism of interference competition was observed for *E. trichodermoides*, and also for other *Escovopsis* strains (Reynolds and Currie, 2004; Varanda-Haifig et al., 2017). Secondary metabolites produced by *Escovopsis* were observed to inhibit fungus gardens, bacterial symbionts and affect the behavior of ants (Dhodary et al., 2018; Heine et al., 2018) indicating the chemical complexity of the symbiosis. The metabolites from *E. trichodermoides* could be essential for accessing the fungal cultivar, but it seems to be insufficient to cause colony death in the *in vivo* experiments. Other mechanisms could be associated with *E. trichodermoides* antagonism, such as competition for resources, since *E. trichodermoides* covers the entire plate and overgrows *Leucocoprinus* fungi. The mechanisms involved in this interaction should be further investigated to understand *E. trichodermoides* ecology.

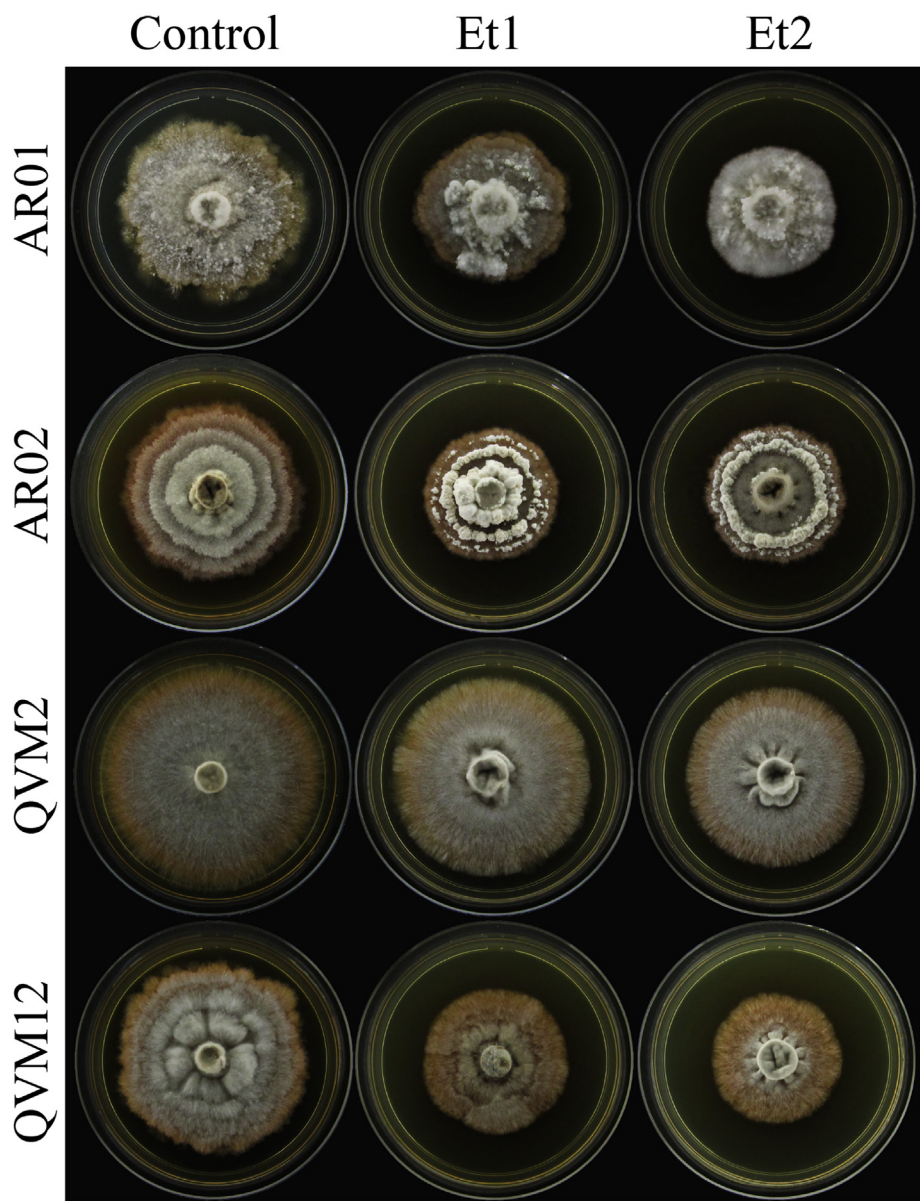


Fig. 4. Interference competition by *Escovopsis trichodermoides* via metabolites production. Mycelial growth pattern of fungal cultivar *Leucocoprinus* spp. (AR01, AR02, QVM2 and QVM12) in the presence of metabolites of *E. trichodermoides* obtained in isolated culture (Et1), in dual-culture (Et2), and in the absence of metabolites (Control). Plates represent 35 days-old cultures. Note the presence of early stages of basidiome formation for AR02 (in Et1 and Et2) in the presence of metabolites.

Table 3
Leucocoprinus spp. growth in the presence and absence of *Escovopsis trichodermoides* metabolites. Values indicate mycelial area in cm² (±SD) of the control group (C), in the presence of metabolites of *E. trichodermoides* grown alone (Et1) and in dual culture (Et2). Bold values are statistically different from respective control group in final day (Tukey test, *P* < 0.05; Mann-Whitney *U* test, *P* < 0.05 for QVM12). Different letters indicate significant differences between groups of relative growth (RG) after 35 days of culture (Tukey test, *P* < 0.05).

Days	AR01 ^a			AR02 ^a			QVM2 ^b			QVM12 ^b		
	C	Et1	Et2	C	Et1	Et2	C	Et1	Et2	C	Et1	Et2
0	0.50 ± 0.0	0.50 ± 0.0	0.50 ± 0.0	0.50 ± 0.0	0.50 ± 0.0	0.50 ± 0.0	0.50 ± 0.0	0.50 ± 0.0	0.50 ± 0.0	0.50 ± 0.0	0.50 ± 0.0	0.50 ± 0.0
3	0.89 ± 0.1	0.87 ± 0.0	0.92 ± 0.1	0.81 ± 0.1	0.79 ± 0.0	0.85 ± 0.0	0.87 ± 0.1	0.79 ± 0.1	0.83 ± 0.1	0.75 ± 0.1	0.71 ± 0.0	0.75 ± 0.1
7	1.79 ± 0.2	1.82 ± 0.3	1.44 ± 0.2	2.02 ± 0.2	1.59 ± 0.2	1.40 ± 0.1	2.08 ± 0.3	1.62 ± 0.3	1.53 ± 0.1	1.40 ± 0.3	1.21 ± 0.1	1.09 ± 0.2
10	4.27 ± 0.3	2.78 ± 0.5	2.24 ± 0.6	3.92 ± 0.7	2.46 ± 0.3	2.56 ± 0.5	4.23 ± 0.5	2.82 ± 0.5	3.22 ± 0.5	4.30 ± 0.4	1.80 ± 0.1	1.98 ± 0.8
14	7.43 ± 0.8	4.63 ± 0.8	3.58 ± 1.2	6.53 ± 0.2	4.27 ± 0.5	4.12 ± 0.4	8.22 ± 0.8	6.03 ± 0.8	5.81 ± 0.6	6.44 ± 0.5	3.24 ± 0.4	3.69 ± 1.7
21	14.71 ± 2.0	8.44 ± 0.8	6.41 ± 2.0	12.56 ± 0.9	7.79 ± 0.6	6.98 ± 0.9	17.70 ± 1.8	14.48 ± 2.1	12.75 ± 2.0	13.56 ± 1.6	6.84 ± 1.0	7.81 ± 3.5
28	25.87 ± 2.5	13.48 ± 1.3	11.37 ± 2.8	22.11 ± 1.7	12.51 ± 1.3	12.04 ± 1.6	32.63 ± 2.3	29.03 ± 3.4	26.04 ± 2.9	24.43 ± 1.4	12.81 ± 1.7	14.74 ± 5.5
35	34.31 ± 2.9	18.94 ± 1.8	15.86 ± 3.8	30.21 ± 2.5	17.72 ± 1.6	16.47 ± 2.5	45.98 ± 3.9	41.73 ± 3.2	38.20 ± 4.3	33.79 ± 2.4	19.84 ± 2.3	21.23 ± 6.4
RG ^c		0.55b	0.46b		0.59b	0.55b		0.91a	0.83a		0.59b	0.63b
I% ^d		44.8	53.8		41.3	45.5		9.2	16.9		41.3	37.2

^a Mutualistic fungi of *Mycetophylax morshi*.

^b Mutualistic fungi of *Mycocyclus goeldii*.

^c Relative growth.

^d Inhibition percentage.

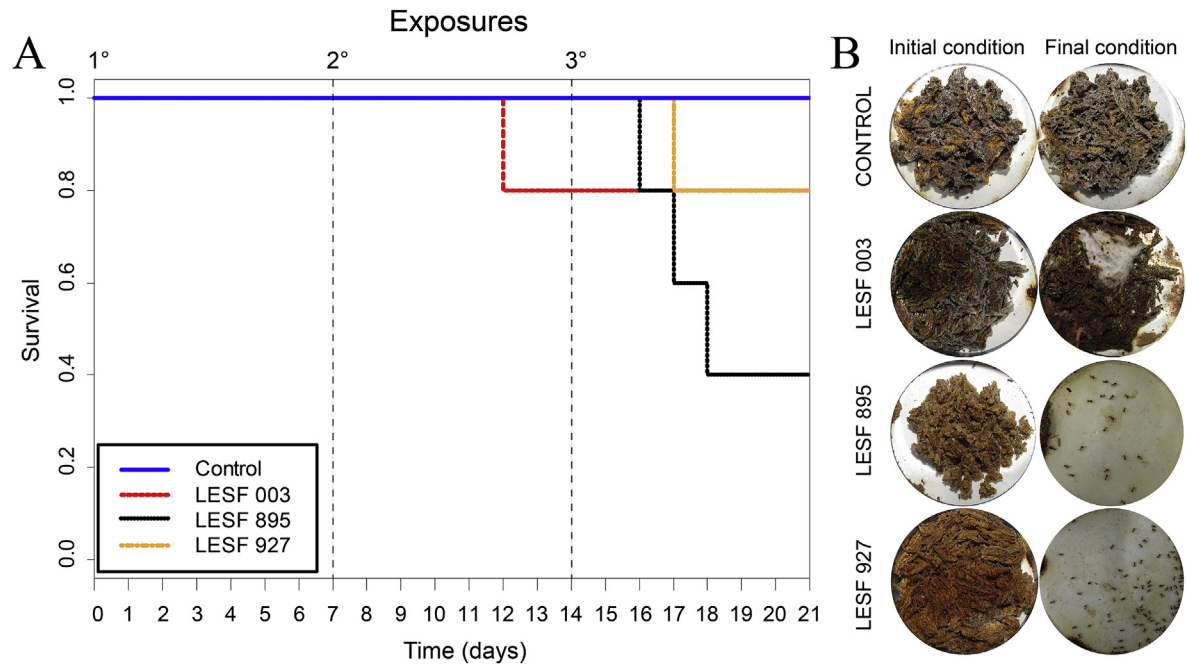


Fig. 5. Low virulence of *Escovopsis trichodermoides* in colonies of the lower attine ant *Mycocepurus goeldii*. **(A)** Colony survival after three successive exposures with increased concentrations of conidia over time (days). Vertical dashed lines indicate the second and third exposures. Concentrations of 10^6 , 10^7 and 10^8 conidia mL^{-1} were used for the first, second and third exposures, respectively. **(B)** Initial and final condition (after the third exposure) of some dead colonies on trials with each *E. trichodermoides* strain. No control colonies died during this experiment.

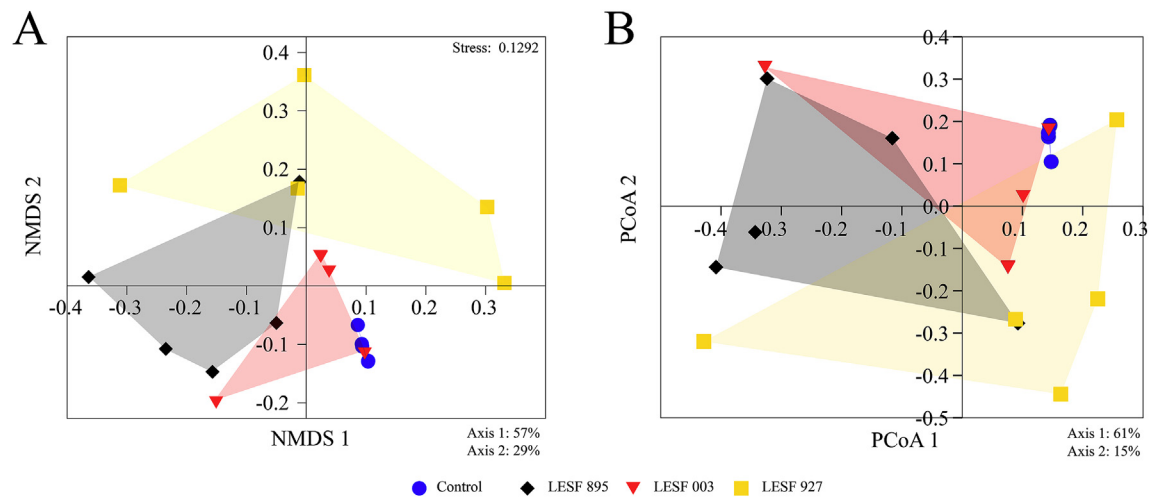


Fig. 6. Effects of *Escovopsis trichodermoides* conidia exposure on *Mycocepurus goeldii* colonies. **(A)** Non-metric multidimensional scaling (NMDS) using Bray-Curtis dissimilarity index. NMDS analyses clearly discriminates control colonies and colonies exposed with conidia of *E. trichodermoides* LESF 895. **(B)** Principal Coordinates analysis (PCoA) using Gower distance. PCoA analyses also indicate a distinction between control colonies and colonies exposed with conidia of *E. trichodermoides* LESF 895.

Interestingly, the strain AR02 of *Leucocoprinus* initiated the formation of a basidiome only in the presence of metabolites of *E. trichodermoides*. The event of basidiome formation was previously reported in laboratory conditions for *Leucocoprinus* fungi associated with lower attines (reviewed by Mueller, 2002). This response might be associated with disruption of the protein:carbohydrate ratio (Shik et al., 2016), stress conditions or activation of metabolic pathways for basidiome formation by the soluble compounds of *E. trichodermoides*, or a combination of these factors.

Low infection was observed on queen-less colonies even when experimentally infected with high amounts of conidia. In our

assays, the colonies were not highly affected by *E. trichodermoides*, and the majority of them resisted to three successive exposures to conidia. Although *in vitro* assays with isolated cultivars showed high inhibition of the mutualistic fungus, *in vivo* assays indicated the role of the ants in maintaining the stability of the system. Colony susceptibility can be understood as the outcome of the interaction between ants, *Escovopsis* and fungal cultivar, as pointed out by Kellner et al. (2018). Here, the colonies showed high survival percentage when treated with *E. trichodermoides* conidia, even considering the fact of not having a queen. In queen-less colonies there is a potential chance of production of worker offspring (Keller and Nonacs, 1993; Villesen and Boomsma, 2003), which may divert

energy costs to reproduction instead of social behaviors, such as hygienic traits. Therefore, even facing this challenge, colonies of *M. goeldii* can deal with *E. trichodermoides* infections.

In the lower attine fungiculture, acquisition of free-living fungal cultivars by ants promotes the genetic diversity of the association (Mueller et al., 1998; Vo et al., 2009; Kellner et al., 2013). Such diversity can provide a better defense for the colonies against specialized pathogens (Kellner et al., 2018). On the other hand, generalist antagonists may increase their own fitness by host-switching events. Our study revealed new ecological traits in the *Escovopsis*-fungal cultivar interaction, with low infection and lack of fungal cultivar fidelity, an antagonistic lifestyle that may have allowed host-switching events over the evolutionary time in the lower attine-ant fungiculture.

Declaration of competing interest

The authors have declared no conflict of interests.

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Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.funeco.2020.100944>.

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