Fungal Ecology 57-58 (2022) 101152

Contents lists available at ScienceDirect

Fungal Ecology

journal homepage: www.elsevier.com/locate/funeco

Fungus-insect symbiosis: Diversity and negative ecological role of the hypocrealean fungus *Trichoderma harzianum* in colonies of neotropical termites (Blattodea: Termitidae)

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ARTICLE INFO

Corresponding Editor: Nicolai Vitt Meyling

Keywords:
Antagonism
Host-parasite interactions
Social insects
Entomopathogens
Fungal phylogeny

ABSTRACT

Symbioses between social insects and fungi can drive important processes in both. We show previously unrealised prevalence and diversity of *Trichoderma* species (Ascomycota: Hypocreales) in individuals of two termite species (Blattodea: Termitidae). *Trichoderma* is well known for producing cellulolytic and fungistatic compounds, which can be important to protect colonies against entomopathogenic fungi. We hypothesized that *Trichoderma* species have a positive effect on termite hosts, yet found an unexpected negative effect of *Trichoderma harzianum* on termite survival, the presence of *Trichoderma* species was not determinant for termite nutritional status. Although *T. harzianum* hindered growth of the entomopathogenic fungus *Metarhizium anisopliae* (Ascomycota: Hypocreales) *in vitro,* this was not registered for *T. virens*, suggesting they have different fungistatic roles. Despite the prevalence of *Trichoderma* species at the colony level being low in termites, we propose that *T. harzianum* has no specific ecological role that benefits higher termites and might even be a potential opportunistic parasite for termite colonies.

1. Introduction

Insect-fungus symbioses are widespread and can range from mutualistic to antagonistic (Currie et al., 1999; Vega et al., 2009; Kaltenpoth and Engl, 2014). The nature of these symbioses can be dynamic and vary in ecological time, depending on the outcomes for host and symbiont (Aanen and Boomsma, 2005; Alizon et al., 2009). Different groups of filamentous fungi have diversified strategies of environmental exploitation (Balvanera et al., 2006; Meyling and Eilenberg, 2007; Vega et al., 2009). As a result, these strategies have driven the evolution of both partners in the symbiosis at individual, population, and community levels (Aanen and Boomsma, 2005; Samson et al., 2013; Brune, 2014; Kaltenpoth and Engl, 2014).

Because of the different strategies employed to obtain nutrients, insects and fungi can share diverse habits, resulting in several ecological roles for both symbionts. This is evident for *Metarhizium* species (Ascomycota: Hypocreales), well known insect pathogens, and widely used in biological control (Meyling and Eilenberg, 2007; Samson et al., 2013). Several species of the genus use alternative nutritional modes by interacting with plant tissue and soil, as is the case with *Metarhizium anisopliae* (Meyling and Eilenberg, 2007; Vega et al., 2009; Behie and Bidochka, 2014; Chen and Zhuang, 2017; Dara, 2019). Likewise, the hypocrealean fungal genus *Trichoderma* (Ascomycota: Hypocreales) is a group notable for displaying different strategies depending on their hosts (Harman et al., 2004; Samuels, 2006; Atanasova et al., 2013b). In social insects, many species of *Trichoderma* have been reported as a component of fungal communities present in nests of wasps (Poulsen et al., 2011), ants (Rodrigues et al., 2014) and termites (Zoberi and

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https://doi.org/10.1016/j.funeco.2022.101152
Received 24 September 2021; Received in revised form 2 March 2022; Accepted 6 March 2022
Available online 28 March 2022

Grace, 1990; Sreerama and Veerabhadrappa, 1993; Jayasimha and Henderson 2007; Mevers et al., 2017). A few studies have shown symbioses of *Trichoderma* and other fungal species with termites (Zoberi and Grace, 1990; Sreerama and Veerabhadrappa, 1993; Guswenrivo et al., 2018). For instance, Xiong et al. (2018, 2019) showed avoidance behaviour by the subterranean termite *Odontotermes formosanus* (Blattodea: Termitidae) to up to six different species of *Trichoderma.* This avoidance may reduce the direct contact between termites and fungi, consequently reducing the risk of introducing the fungus in termite nests. A negative effect of *Trichoderma* isolated from different parts of the nests on survival of the subterranean termite *Globitermes sulphureus* (Haviland) (Blattodea: Termitidae) was also shown by Guswenrivo et al. (2018), but fungal identification was done only to genus level. In contrast, there are records of attractive effects and altered entomopathogen-avoiding behaviour by the lower termite *Coptotermes formosanus* (Blattodea: Rhinotermitidae) when exposed to seven species of *Trichoderma* (Wen et al., 2020), or for several days (*e.g.* 14 d in Xiong et al., 2018). However, some of these fungal species may have been completely alien to the termites since these isolates were obtained from isolate banks and commercial formulations. Even though these studies have reported numerous species of fungi interacting with termite guts or nest surfaces, the ecological consequences of the presence of *Trichoderma* species vary within nests of lower and higher termites, and remain unclear (Jayasimha and Henderson 2007; Xiong et al., 2018; Wen et al., 2020). Considering the diverse ecology within the genus *Trichoderma*, it is important to document patterns, such as prevalence and diversity, and ultimately to understand possible processes and ecological roles of *Trichoderma* species in these termite-fungus symbioses.

Some species of *Trichoderma* described in symbiosis with insects have also been isolated from rhizospheres or living plant tissues, such as *T. atroviride*, *T. spirale, T. harzianum*, and *T. virens* (Druzhinina et al., 2011; Rocha et al., 2017; Vaz et al., 2018; Muthukathan et al., 2020). As an endophyte, the fungus can penetrate the first layers of plant tissues producing glycolytic, chitinolytic or cellulolytic enzymes (Chutrakul et al., 2008; Atanasova and Druzhinina, 2010; Malinich et al., 2019). In some plant hosts, these enzymes can optimize plant resource acquisition and stimulate growth, as is well documented for *T. harzianum* and *T. reesei* (Altomare et al., 1999; Yedidia et al., 2001; Hoyos-Carvajal et al., 2009; Samuels and Hebbar, 2015). In other cases, enzymes produced by *Trichoderma* species can decompose cellulose and this may benefit different organisms that consume cellulose sub-products (Harman, 2006; Xie et al., 2014). Since termites consume cellulose but cannot fully digest it by themselves, the symbiosis with cellulose-decomposing *Trichoderma* species could be a driving force for maintenance of this interaction in the environment of the nest. We might then expect that this fungal symbiosis can favour the acquisition of nutrients by the insect.

Production of toxic and antibiotic metabolites characterizes several species within the genus *Trichoderma* as efficient antagonists of plant pathogens and inducers of plant resistance, such as *T. harzianum* and *T. asperellum* (Howell, 2003; Schuster and Schmoll, 2010; Druzhinina et al., 2011; Malinich et al., 2019). In addition, *T. atroviride* and *T. virens* have been reported parasitizing other fungal species (Atanasova et al., 2013a; Chaverri and Samuels, 2013; Lima et al., 2016; Muthukathan et al., 2020). These mycoparasite species can penetrate the host hyphae and secrete chitinases that degrade the parasitized cells' wall and cytoplasm, provoking their disruption (Howell, 2003; Druzhinina et al., 2011). Considering that some of these species of *Trichoderma* can induce suppression of soil-borne pathogenic fungi (Harman, 2006; Poveda et al., 2019; Muthukathan et al., 2020), interaction with such species could potentially enhance host defence against pathogens. Since *Trichoderma* species are ubiquitous and can be antagonists of entomopathogenic fungi, it is plausible to hypothesize that its presence in termite colonies represents a role as an extended immune component, being favoured in the termite nest environment, and directly affecting termites' survival.

To test these hypotheses, this study focused on describing a termite-*Trichoderma* symbiosis and understanding the role of this group of fungi inside colonies of neotropical termites. First, we aimed to estimate the prevalence of *Trichoderma* and evaluate whether the fungal prevalence differs among two termite species or among individuals within the species. Secondly, we assessed the diversity of *Trichoderma* species interacting with these termites. We also addressed the role of the termite- *Trichoderma* symbiosis. Considering the diversity of strategies that *Trichoderma* species use to exploit their environment, we hypothesized that the presence of the fungus in termite bodies would optimize termite resource acquisition from substrates. If the fungus confers a nutritional benefit to termites, we would then expect that workers carrying the fungus would have higher amounts of body fat. Thus, we tested if the fat content in individuals is directly correlated with the presence of *Trichoderma* species inside its body. We also evaluated the impact of *Trichoderma* species on growth of an entomopathogenic fungus, which can be a threat to termites. As some species of *Trichoderma* are widely described as antagonists of other fungi, we hypothesized that *T. harzianum* and *T. virens* would inhibit the growth of an entomopathogenic fungus, using the species *M. anisopliae* as a model. Lastly, if this symbiosis is beneficial to termites, we also expect positive impacts on termite survival when they are exposed to *T. harzianum*. Therefore, we investigated if the fungus affects the survival of the termite *Cornitermes cumulans* when they are exposed to it.

2. Material and methods

2.1. Study area, species identification and fieldwork

We conducted the fieldwork at *Estação de Pesquisa, Treinamento e Educação Ambiental Mata do Paraíso* of the Universidade Federal de Viçosa (20° 48′ 00″ S, 42° 52′ 00.8 W), at *Sítio Bom Sucesso* (20° 47′ 39.7″ S, 42° 50′ 25″ W) and at *Povoado dos Cristais* (20° 46′ 58.5″ S, 42° 50′ 23″ W), all located within the municipality of Viçosa, Minas Gerais, southeastern Brazil. *Mata do Paraíso* is a fragment of Atlantic Rainforest and the vegetation is predominantly secondary seasonal semideciduous montane forest (Veloso et al., 1991). *Sítio Bom Sucesso* is a private property, and it is predominantly open-grass pasture with small patches of Atlantic Rainforest, whereas *Povoado dos Cristais* is an open-grass pasture.

We performed a preliminary survey of the areas that revealed different species of termites with epigeic nests, that consist of a mound on the ground (Constantino, 2021). The nests are semi-spherical structures, fixed on the soil, consisting of a mixture of soil, saliva and partly digested organic material (Eggleton, 2010). The nest volumes were approximately 0.03–0.6 m^3. From each of the 35 nests found, we collected 10 termite workers and 10 soldiers for morphological identification at the genus level. To perform the prevalence and ecological studies, we selected nests from the two most abundant termite genera: *Cornitermes* and *Diversitermes* (Blattodea: Termitidae). Fieldwork was performed from April 2017 to March 2018. Termite collections were carried out with permanent permission of *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio 23915).

2.2. Prevalence of the fungus Trichoderma

To assess fungi that interact with termite body cavities and nest walls, we sampled four nests of *Cornitermes cumulans* and four nests of *Diversitermes* sp. We broke one side of the nest structure using a pickaxe and a hammer that were washed and sterilized with 70% ethanol between the collection of each nest. Insects and nest fragments were taken to the laboratory for processing.

2.2.1. Fungal isolation and identification

For each of the eight nests, we collected 30 immature termites, 30 soldiers, and 30 workers. As the genus *Diversitermes* have more than one type of soldier, that are distinguishable from one another by body size

and head capsule shape (Oliveira and Constantino, 2016), we collected 30 of each of the two types, totalling 840 individuals. We separated these individuals from nest material using flexible forceps and transferred them immediately to a −20 °C freezer. Forceps were surface sterilized using 70% ethanol after processing each nest sample to avoid cross-contamination. After 24 h, we removed the termite cadavers from the freezer, surface-sterilized them using 70% ethanol for 30 s and 5% sodium hypochlorite for 1 min, then rinsed them in sterile distilled water for 1 min (Lacey, 2012). After this procedure, each individual was dried on sterile filter paper and placed in a Petri dish (49 mm × 12 mm) containing Potato Dextrose Agar medium 20% (PDA; 7.8 g/l, Sigma Aldrich®) with 0.03 g/l of chloramphenicol and incubated at 25 °C. All the insects were inspected daily for 10 d to evaluate the emergence of fungal hyphae from body cavities.

In addition to the individual insects, we also collected internal wall fragments. We collected 30 arbitrarily selected pieces (1 cm^3) of each nest, totalling 240 fragments. To avoid external contamination, we separated these fragments using sterile forceps. We then placed these fragments in Petri dishes (90 mm × 15 mm) containing modified PDA medium (20% PDA + 0.02 g of rose bengal, 0.03 g of chloramphenicol, 0.02 g of streptomycin sulphate per litre of medium). This medium has previously been used for the selective isolation of *Trichoderma* from soil (Vargas-Gil et al., 2009). The plates were incubated at 25 °C and inspected daily for 10 d to evaluate the emergence of fungal hyphae from the nest fragments.

Once fungi emerged from termites or nest fragments, we transferred all strains to new Petri dishes (49 mm × 12 mm, PDA 20%) and incubated these at 25 °C for 7 d. After fungal growth and conidiation, we classified the strains as morphotypes according to their morphological features. From each morphotype with similar features to the genus *Trichoderma*, we prepared slides for microscopic inspection. All strains morphologically identified as *Trichoderma* were preserved in 10% glycerol at −80 °C (Lacey, 2012) and held at the Laboratory of Insect-Microbe Interactions, Viçosa, Brazil. Fungal collections were carried out with permission of ICMBio (23920).

2.2.2. Prevalence of Trichoderma

To quantify the prevalence of *Trichoderma* in termites, we calculated which proportion of samples from each group [immatures (n=30), soldiers (n=30), workers (n=30), and fragments (n=30)] had *Trichoderma*. Thus, we estimated the prevalence of *Trichoderma* in immatures, soldiers, workers, and fragments of nest wall separately. Each nest was considered as a biological replicate. To evaluate whether the prevalence of *Trichoderma* differs within and between species, we adjusted generalized linear mixed models (GLMM) with binomial distributions and random intercepts. In two separate analyses, the origin of the fungus in insects (immatures, soldiers, or workers) or in fragments were considered as the explanatory factors. Termite species was considered as a fixed factor and nest was considered as a random factor in both analyses. Prevalence of *Trichoderma* was the response factor. Significance was evaluated using χ^2 tests. Analyses were performed in R (R Core Team, 2021).

2.3. Diversity of Trichoderma symbionts of termites

2.3.1. DNA extraction, amplification, and sequencing

To evaluate the diversity of *Trichoderma* symbionts of termites, we prepared monosporic cultures from all isolates. Isolates were grown in 20% PDA medium for 7 d in the dark. Mycelium was collected from the agar surface and macerated in liquid nitrogen. Genomic DNA from each sample was extracted using the Wizard® Genomic DNA Purification Kit (Promega Corporation, WI, USA), following the manufacturer's protocol. Although the Internal Transcribed Spacer (ITS) region is used as a barcode for most fungal species (Atanasova et al., 2013b), there is low intraspecific ITS resolution for the genus *Trichoderma* (Druzhinina and Kubicek, 2005; Atanasova et al., 2013b; Chaverri et al., 2015; Montoya et al., 2016). Thus, we opted to amplify two other molecular markers used in the identification of species from this genus (Chaverri et al., 2015; Chen and Zhuang, 2017): the partial sequences of the gene coding for the elongation factor 1 *alpha* (*tef1*) (ca. 600 bp) and the gene coding for the largest subunit of RNA polymerase II (*rpb2*) (ca. 1000 bp). For *tef1*, we used the primer pair EF1-728F (5′ CATCGAGAAGTTCGAGAAGG 3′) and TEF1R (5′ GCCATCCTTGGAGATACCAGC 3′) (Carbone and Kohn, 1999; Samuels et al., 2002). For *rpb2*, we used the primer pair fRPB2-5F (5′ GAYGAYMGWGATCAYTTYGG 3′) and fRPB2-7Cr (5′ CCCATRGCTTGYTTRCCCAT 3′) (Liu et al., 1999). We performed amplifications for the two markers in a final volume of 25 μl (12.5 μl of Dream Taq PCR Master Mix Thermo Scientific®; 8.5 μl of water free nuclease; 1 μl of each primer [10 μmol]; 2 μl diluted genomic DNA [25 μl]. The PCR conditions for *tef1* and *rpb2* were 94 °C/2 min followed by 15 cycles at 94 °C/30 s, 65 °C/30 s and 72 °C/1 min; followed by 35 cycles at 94 °C/30 s, 48 °C/30 s, 72 °C/1 min and final extension at 72 °C/10 min. PCR products were purified and sequenced by Macrogen®, South Korea (http://www.macrogen.com).

2.3.2. Phylogenetic analyses

We edited and assembled *Trichoderma* sequences with CodonCode Aligner (Codon Code Corporation, 2020). The *tef1* and *rpb2* contigs were compared to homologous sequences using NCBI nucleotide database BLASTn to ensure that all the sequences were *Trichoderma* species (Altschul et al., 1990). We also included in our phylogenetic analyses representative sequences from other studies (Table S1). Sequences from previous studies were selected from the NCBI-GenBank database by analysing initial phylogenetic trees for each *Trichoderma* clade, avoiding sequences that were too divergent and could mislead the global analyses. After sequence selection, the final dataset consisted of 138 partial *tef1* sequences and 117 partial *rpb2* sequences.

The alignments of the two regions were improved manually, annotated, and concatenated into a single combined dataset using MEGA v. 10 (Kumar et al., 2018). Gaps were treated as missing data and ambiguously aligned regions were excluded from phylogenetic analyses. The final alignment length was 1857 bp: 789 bp for *tef1* and 1068 bp for *rpb2*. Maximum Likelihood and Bayesian Inference analyses were performed to reconstruct our phylogenetic trees. Maximum Likelihood was performed with RAxML HPC-BlackBox (Stamatakis, 2014; Kozlov et al., 2019) on the independent regions *tef1* and *rpb2* using the CIPRES Science Gateway v. 3.3 webserver (Miller et al., 2010). We then performed this again on a concatenated dataset containing the two loci. The dataset consisted of six data partitions, including one for each of the three codon positions of the protein coding genes, *tef1* and *rpb2*. The GTR-GAMMA nucleotide substitution model was employed during the generation of 1000 bootstrap replicates. Bayesian Inference was performed with MrBayes v. 3.2 (Ronquist et al., 2012), using the GTR+I+G nucleotide substitution model for *tef1* and SYM+I+G for *rpb2*, selected according to the Akaike Information Criterion (AIC) in MrModeltest 2.4 (Nylander, 2004). For Bayesian Inference, two parallel runs, consisting of four chains, were subjected to Markov Chain Monte Carlo (MCMC) analysis until the runs converged with a split frequency <0.01. The MCMC analysis started with a heating parameter 0.1 from a random tree topology and lasted 40 millions of generations. Trees were saved every 1000 generations. Finally, 25% of trees were discarded in the burn-in phase. Phylogenetic trees were visualized using FigTree v. 3.5.9 (Rambaut, 2017) and edited using the packages *ape* v. 5.5 (Paradis and Schliep, 2019) and *ggtree* v. 3.0.2 (Yu, 2020) in R (R Core Team, 2021), and using Inkscape (www.inkscape.org). The species *Hypomyces aurantius* and *Sphaerostilbella aureonitens* were used as outgroups to root the trees (Chaverri and Samuels, 2013).

2.4. Trichoderma and termite fat content

To assess if the fat content in individuals is positively correlated with the presence of *Trichoderma* in body cavities, we compared the fat

contents of workers in which the fungus was present *versus* absent. For this, we collected an additional 226 workers from 5 colonies of *C. cumulans*. This species had a higher prevalence of *Trichoderma* in our prevalence study (see section 2.2), so it was chosen for this analysis. Termites were frozen at −20 °C for 24 h, and then weighed on an analytical scale (Shimadzu ATX224, 0.0001 g precision). To evaluate the emergence of fungus from workers' internal body cavities, we processed the individuals as described in section 2.2. After 48 h of incubation, we removed all termites from Petri dishes. To evaluate whether the time spent in Petri dishes was relevant for termites' body mass loss, we then weighed the termites again and compared these values with fresh body mass. Plates were incubated at 25 °C to verify the fungal strains which emerged from the insects over 7 d. We further grouped the samples as: no fungi, *Trichoderma*, and other fungi.

We assessed the amount of fat content in the bodies of 226 workers. For this, we dried the workers – immediately after we removed them from Petri dishes – in an oven at 50 °C (±2 °C) for 3 h. We weighed them again and placed each termite into a 1.5 ml microtube containing 1 ml of chloroform for 72 h for lipid extraction (adapted from Plaistow and Siva-Jothy, 1999; Peixoto and Benson, 2012; Junior and Peixoto, 2013). After extraction, we dried the individuals in an oven at 50 °C (±2 °C) for an additional 3 h and weighed them again. We used the difference of body mass before and after immersing the workers in chloroform to estimate the individual fat body mass content (Peixoto and Benson, 2012; Junior and Peixoto, 2013) post-fungal emergency.

To estimate values of fat content accounting for the individual size (residual fat), we used standardized residuals obtained from a linear regression between the fat content (response variable) and body mass of the individual after chloroform extraction (explanatory variable). We used body mass in this case because it was correlated to body length ($F=7.75$; d.f.=55; $p<0.001$). To test whether fat content in individuals was directly correlated with the presence of *Trichoderma* in termite bodies, we used a logistic regression between the probability of presence of *Trichoderma* (response variable) and the residual fat of individuals (explanatory variable). Analyses were performed in R (R Core Team, 2021).

2.5. Growth of an entomopathogenic fungus in the presence of Trichoderma

To test whether *Trichoderma* can inhibit the growth of an entomopathogenic fungus, we evaluated *in vitro* growth of *M. anisopliae* in the presence of *T. harzianum* (Fig. S1A) and *T. virens* (Fig. S1B). Besides these two different species of *Trichoderma*, we added a species of the opportunistic fungus *Fusarium* sp. (Fig. S1C). This allowed us to verify if the potential inhibitory effects of *Trichoderma* were only a consequence of the presence of other fungi competing for resources. We used two isolates of *M. anisopliae* (GenBank accession no. MZ712199 and MZ712200) that we isolated from soil adjacent to two nests of the termite *C. cumulans* from *Mata do Paraíso* research station (in this study). We molecularly identified these isolates using the genomic region *tef1*, under the same conditions that we previously used to identify the isolates of *Trichoderma*. *Trichoderma virens* (GenBank accession no. MZ816947) and *T. harzianum* (GenBank accession no. MZ816948) were obtained from fragments of nest wall from the same two colonies from which *M. anisopliae* isolates were collected. The isolate of *Fusarium* sp. was provided by the Laboratory of Insect-Microbe Interaction fungal collection, having been isolated from soil from *Mata do Paraíso* research station (discarded after experiment).

We inoculated all fungi individually in Petri dishes with PDA medium and incubated them at 25 °C in the dark for 15 d. After this time, these Petri dishes were used as sources of inoculum for the *in vitro* experiment. We then inserted a 0.5 cm^3 plug with fungus in each side of fresh Petri dishes (90 mm × 15 mm, PDA medium), according to treatments described in Table 1. For each treatment, we inoculated 20 Petri dishes with one division at the centre, comprising two compartments, totalling 160 plates. Nevertheless, replicates that presented contamination during the experiment were excluded (Table 1).

Table 1
Treatment inoculated in each side of the Petri dishes with one division at the centre on the first and seventh days of an experiment to evaluate *Metarhizium anisopliae* growth in presence of fungi. We inoculated *M. anisopliae* on one side of the two compartments of the Petri dishes with PDA medium on the first day of the experiment and inoculated other fungi after seven days at the opposite side of the plate. We initially inoculated 20 samples but considered as replicates only the plates that did not show contaminants at the end of the experiment, after 12 d.

Fungi inoculated on 1st day	Treatment inoculated on 7th day	Total replicates
Metarhizium anisopliae Isolate 1	*Trichoderma virens*	16
M.etarhizium anisopliae Isolate 1	*T. harzianum*	16
M. anisopliae Isolate 1	Control (agar)	16
M. anisopliae Isolate 1	*Fusarium* sp.	12
M. anisopliae Isolate 2	*T. virens*	16
M. anisopliae Isolate 2	*T. harzianum*	18
M. anisopliae Isolate 2	Control (agar)	20
M. anisopliae Isolate 2	*Fusarium* sp.	15

Since *M. anisopliae* grows more slowly than *T. harzianum* and *T. virens*, we inoculated *M. anisopliae* on the Petri dishes first and incubated these for 7 d at 25 °C in the dark. At day 7, there was no difference in colony growth among samples of *M. anisopliae* Isolate 1 ($z=0.41$; $p=0.34$) or among samples of *M. anisopliae* Isolate 2 ($z=1.36$; $p=0.08$). After this time, on the opposite side of each plate with *M. anisopliae*, we inoculated plugs of *T. harzianum*, *T. virens*, *Fusarium*, or plain agar (control) (Fig. S1). We then evaluated the entomopathogenic fungus daily for further 5 d, the time taken by both species of *Trichoderma* to grow across the Petri dish and reach the *M. anisopliae* inoculum at the opposite side. To assess *M. anisopliae* growth, we photographed each plate daily for 12 d with a NIKON D5100 camera with an AF-S DX NIKKOR 18–55 mm f/3.5–5.6G VR lens and measured the colony radius (mm) of *M. anisopliae* isolates towards the fungus at the opposite side of the plate in each image using ImageJ (Schneider et al., 2012).

To compare the growth of each *M. anisopliae* isolate in the presence of fungi, we adjusted generalized linear mixed models (GLMM) for each response variable/*M. anisopliae* isolate with normal distributions and random intercepts. For this, we considered *T. harzianum*, *T. virens*, *Fusarium* sp., control, and time as explanatory factors, and colony radius (mm) of *M. anisopliae* as a response factor. The identity of each plate was considered as a random factor in both analyses. We then tested the interaction between each fungus and time. Also, we compared *M. anisopliae* growth, performing multiple comparisons with *multcomp* package v. 1.4–13 (Hothorn et al., 2008), to verify the potential differences of *M. anisopliae* growth in the presence of *T. harzianum* and *T. virens* compared to *Fusarium* sp. and plain agar before inoculation of fungi and control (after 7 d) and at the end of the experiment (after 12 d). We performed all analyses using R (R Core Team, 2021).

2.6. Termite survival when exposed to Trichoderma

2.6.1. Arena set-up

We created 24 arenas, each comprised of one 90 mm × 15 mm Petri dish connected to a 60 mm × 15 mm Petri dish by a 2 cm-length cylindrical tube (Fig. 4A and B), summing approximately 95 cm^2. Prior to the experiment, arenas were washed with neutral detergent, immersed in 5% sodium hypochlorite solution for 24 h and rinsed 2 times with sterile water.

In a new field collection event, we collected eight colonies of *C. cumulans*. The individuals were removed from the nest and the fragments of nest walls were macerated and sieved to homogenize the size of nest particles. Nest wall was used in natural conditions (not sterilized)

because a previous assay using sterile nest walls resulted in lower survival of termites exposed to sterile rather than non-sterile walls (p=0.005, Methods S1). We then filled three arenas with nest soil of each colony. In each arena, we inserted 12 g of processed material in the larger plate and 5 g in the smaller plate and added 5 ml of sterile water to each arena (Fig. 4B). Arenas were kept closed, being opened only three times a day, when we evaluated termite survival. During the experiment, we injected 1.89±0.09 ml of distilled and sterile water daily in each arena to maintain humid conditions.

From each of the eight colonies, we selected three groups of 24 termites, comprising 20 workers and 4 soldiers, totalling 576 individuals. The numbers of individuals and caste ratio workers: soldiers of *C. cumulans* used in the bioassay were chosen according to natural caste proportions (5:1) to maximize termite survival (adapted from Cristaldo et al., 2016). The groups were introduced into the arenas with soil of their respective nest and kept in the dark for 24 h at room temperature to acclimatize. Lastly, we separated the arenas of each colony in three treatments described below.

2.6.2. Fungal selection and inoculation

The impact of one strain of *T. harzianum* on termite survival was tested (VIMI 17.0133 isolated from a termite in section 2.2 – GenBank accession no. MZ675966 and MZ675875). We also selected the most prevalent fungus from the insects we isolated in section 2.4, *Lichtheimia brasiliensis* (Mucoromycota: Mucorales) (VIMI 18.0001– GenBank accession no. MZ734290), to use as a control for fungal presence. It was found in 36.9% of our samples and there are no reports of its pathogenicity to termites (Schwartze et al., 2014). Both strains are preserved in 10% glycerol at −80 °C, at the Laboratory of Insect-Microbe Interactions, Viçosa, Brazil.

To introduce the treatment inside the arena, sugar cane fragments of approximately 1 cm^3 were used as baits, as they can serve as a food source for termites as well as substrate for fungus growth. We sterilized the sugar cane baits and kept them without fungus (control) or directly inoculated them with a few spores of the fungi *T. harzianum* or *L. brasiliensis.* Baits kept without fungus were manipulated for the same time as those that had been inoculated. All fragments were incubated at 25 °C. Once the fungi had grown for 5 d, we estimated fungal growth by calculating the proportional area covered by fungi (all six sides of the cubes) in each bait for each treatment. The surface area covered by *T. harzianum* varied from 11.40% to 19.45%, whereas the area covered by *L. brasiliensis* varied from 15.65% to 33.30% (Fig. S2). The baits of each treatment (inoculated with *T. harzianum*, *L. brasiliensis*, or without fungi – control) were placed in the smaller plates of the arenas. Thus, for each of the eight nests, one group was exposed to *T. harzianum,* a second group was exposed to *L. brasiliensis*, whereas a last group was exposed to sugar cane with no fungi. The first baits were placed 24 h after termites were inserted in the arenas and were replaced daily with a fresh fragment of the same treatment.

2.6.3. Termite survival

The 24 arenas were maintained in the dark in an incubator at 25 °C and 70% relative humidity during the experiment. Termite mortality counts were performed by opening the arenas every 8 h over 25 d, totalling 75 monitoring events. Death was determined by a lack of movement in response to tactile stimulus. In each monitoring event, dead termites were removed, and surface sterilized as described in section 2.2. To verify the presence of the fungi in the termites, we placed the sterile cadavers in Petri dishes containing modified PDA medium and incubated these at 25 °C to determine if the fungi from the designated treatments emerged from within the insect bodies. Incubated cadavers were inspected daily for emergence of fungus. To compare mortalities among treatments, we performed a survival analysis with Weibull distributions and censored data (Therneau and Grambsch, 2000). The colony of origin was included as a frailty factor. The frailty model is used when survival is correlated or clustered. In this case, data were correlated to colony of origin. So, we used the shared frailty gamma model (Rondeau et al., 2003), adding a random effect (colony) which acts in a multiplicative fashion on the survival function (Rondeau et al., 2012). Analysis was performed in R (R Core Team, 2021) with *survival* package v. 2.38 (Therneau, 2015).

3. Results

3.1. Prevalence of Trichoderma

We compared the prevalence of *Trichoderma* species among termite groups and nest fragments separately. Among *C. cumulans* groups, fungus prevalence varied from 0 to 0.27, with a global mean of all nests being 0.058±0.022 (mean±S.E.) (n=4). Among *Diversitermes*, *Trichoderma* prevalence varied from 0 to 0.1, with a global mean of 0.015 ±0.009 (n=4). Thus, *Trichoderma* prevalence in *C. cumulans* was higher than in *Diversitermes* sp. (χ^2_5=5.08, n=8, p=0.02, Fig. 1A). Meanwhile, *Trichoderma* prevalence was higher among workers of *C. cumulans* (0.133±0.045) and *Diversitermes* (0.042±0.021) than among their soldiers (0.042±0.021 and 0, respectively) (χ^2_5=10.04, n=8, p=0.002, Fig. 1A). Additionally, no fungi were isolated from immatures of either termite species. In line with the prevalence of *Trichoderma* in termites, its prevalence in nest fragments was higher in *C. cumulans* (0.57±0.06) than in *Diversitermes* (0.14±0.05) (χ^2_4=7.10; n=8; p<0.01; Fig. 1B).

3.2. Diversity of Trichoderma symbionts of termites

Eighty one isolates comprising 16 distinct currently accepted species of *Trichoderma* were obtained from insects and fragments of 8 nests (Bissett et al., 2015) (Table S2). The distribution of the species according to nest, substrate of origin and termite species is described in Table 2. Four species, *T. afroharzianum*, *T. hamatum*, *T. harzianum*, and *T. koningiopsis*, were isolated for the first time from insects, representing a novel niche for these species. *Trichoderma harzianum,* the most prevalent species (n=23), was found in nest walls, soldiers, and workers (Table 2). *Trichoderma koningiopsis*, *T. camerunense,* and *T. hamatum* were present in nest walls and workers, while *T. afroharzianum* and *T. lentiforme* were isolated from nest walls and soldiers. Eight other *Trichoderma* species were present only in nest walls: *T. ghanense, T. longibrachiatum*, *T. ovalisporum, T. simplex*, *T. spirale*, *T. strigosellum, T. subviride,* and *T. virens.* Meanwhile, *T. koningii* was isolated only from soldiers, and *T. inhamatum* was found only in workers.

Regarding the host species, five species of *Trichoderma* were found in both termite species (Table 2): *T. camerunense, T. harzianum, T. koningiopsis, T. lentiforme,* and *T. subviride.* Ten species of the genus were unique to *C. cumulans*: *T. afroharzianum*, *T. ghanense*, *T. hamatum*, *T. inhamatum*, *T. koningii*, *T. longibrachiatum*, *T. simplex*, *T. spirale*, *T. strigosellum*, and *T. virens.* One species of *Trichoderma* was unique to *Diversitermes* sp.: *T. ovalisporum.* One isolate from nest wall was identified only to genus level (VIMI 17.0084), described as *Trichoderma* sp.

3.2.1. Phylogenetic analyses

Sequences of the 81 isolates of *Trichoderma* were used for the phylogenetic analyses (Fig. 2), and the only isolate (VIMI 17.0084) not identified to species level did not group within the proposed clades. Regarding the molecular markers used, VIMI 17.0084 showed 100% (*tef1*) similarity with *T. lentiforme* (**FJ463309**) and 99.88% (*rpb2*) similarity with *T. hamatum* (**KJ665275**). The 16 phylogenetic species found belong to 4 different clades (Atanasova et al., 2013b; Chaverri et al., 2015): **Clade Harzianum** (Fig. 2, green square): *T. afroharzianum* (n=2), *T. camerunense* (n=10), *T. harzianum* (n=23), *T. inhamatum* (n=1), *T. lentiforme* (n=4), and *T. simplex* (n=1); **Clade Longibrachiatum** (Fig. 2, yellow square): *T. longibrachiatum* (n=1); **Clade Virens** (Fig. 2, orange square), also known by their characteristic green

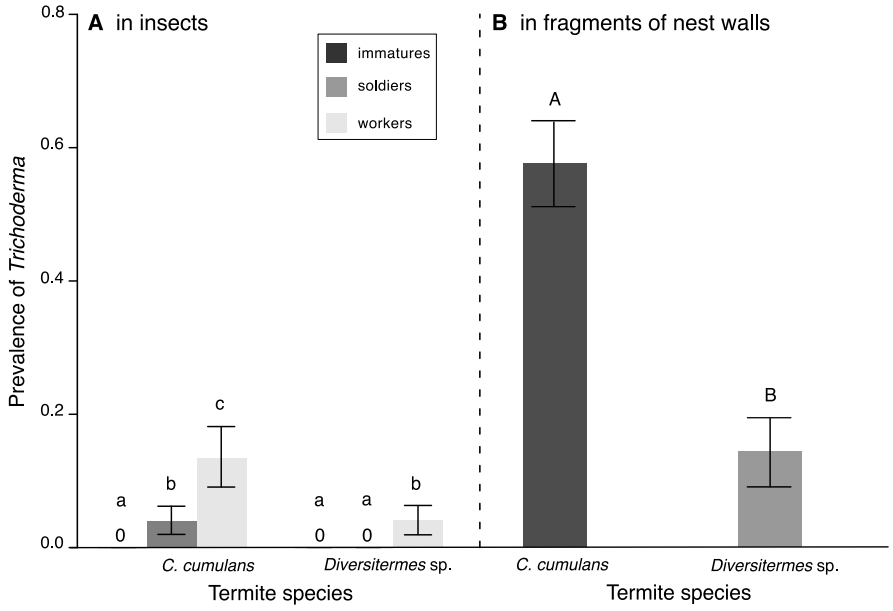


Fig. 1. Prevalence of *Trichoderma* species in termites (mean±S.E.). The prevalence of *Trichoderma* at the genus level was estimated (A) according to termite species and insect of origin, and (B) according to termite species and nest wall. Eight nests of termites (*Cornitermes cumulans*, n=4; *Diversitermes* sp., n=4) were collected at *Mata do Paraíso* Research Station, Viçosa, Minas Gerais, Brazil. Insects (immatures, soldiers and workers, n=720) and fragments of nest wall (n=240) were placed in Petri dishes to stimulate emergence of *Trichoderma*. (A) Prevalence of *Trichoderma* species in *C. cumulans* was higher than in *Diversitermes* sp. and *Trichoderma* prevalence in workers of *C. cumulans* and *Diversitermes* sp. was higher than in soldiers (lower case letters show difference among insect groups and termite species. (B) Prevalence of *Trichoderma* species in nest walls was again higher in *C. cumulans* than *Diversitermes* sp. (upper case letters show difference between species).

Table 2
Trichoderma species and numbers of isolates from each substrate (workers, soldiers, immatures, or nest walls) and termite nest collected (host species). Shown are the number of termite nests in which we found each species of the fungus *Trichoderma* and total number of isolates from each fungal species. Species in bold were isolated for the first time from insects, characterizing a new niche for the fungal species.

Species	Worker	Soldier	Immature	Nest wall	Hosts	# nests	Total of isolates
T. afroharzianum	0	1	0	1	*Cornitermes cumulans*	2	2
T. camerunense	1	0	0	9	*C. cumulans, Diversitermes* sp.	5	10
T. ghanense	0	0	0	2	*C. cumulans*	1	2
T. hamatum	1	0	0	4	*C. cumulans*	2	5
T. harzianum	7	1	0	14	*C. cumulans, Diversitermes* sp.	8	23
T. inhamatum	1	0	0	0	*C. cumulans*	1	1
T. koningii	0	1	0	0	*C. cumulans*	1	1
T. koningiopsis	3	0	0	15	*C. cumulans, Diversitermes* sp.	5	18
T. lentiforme	1	1	0	2	*C. cumulans, Diversitermes* sp.	3	4
T. longibrachiatum	0	0	0	1	*C. cumulans*	1	1
T. ovalisporum	0	0	0	1	*Diversitermes* sp.	1	1
T. simplex	0	0	0	1	*C. cumulans*	1	1
T. spirale	0	0	0	1	*C. cumulans*	1	1
T. strigosellum	0	0	0	1	*C. cumulans*	1	1
T. subviride	0	0	0	2	*C. cumulans, Diversitermes* sp.	2	2
T. virens	0	0	0	7	*C. cumulans*	3	7
Trichoderma sp.	0	0	0	1	*C. cumulans*	1	1

spores: *T. spirale* (n=1), *T. virens* (n=7); **Clade Viride** (Fig. 2, purple square): *T. ghanense* (n=2), *T. hamatum* (n=5), *T. koningii* (n=1), *T. koningiopsis* (n=18), *T. strigosellum* (n=1), *T. subviride* (n=2), *T. ovalisporum* (n=1).

3.3. *Trichoderma* and termite fat content

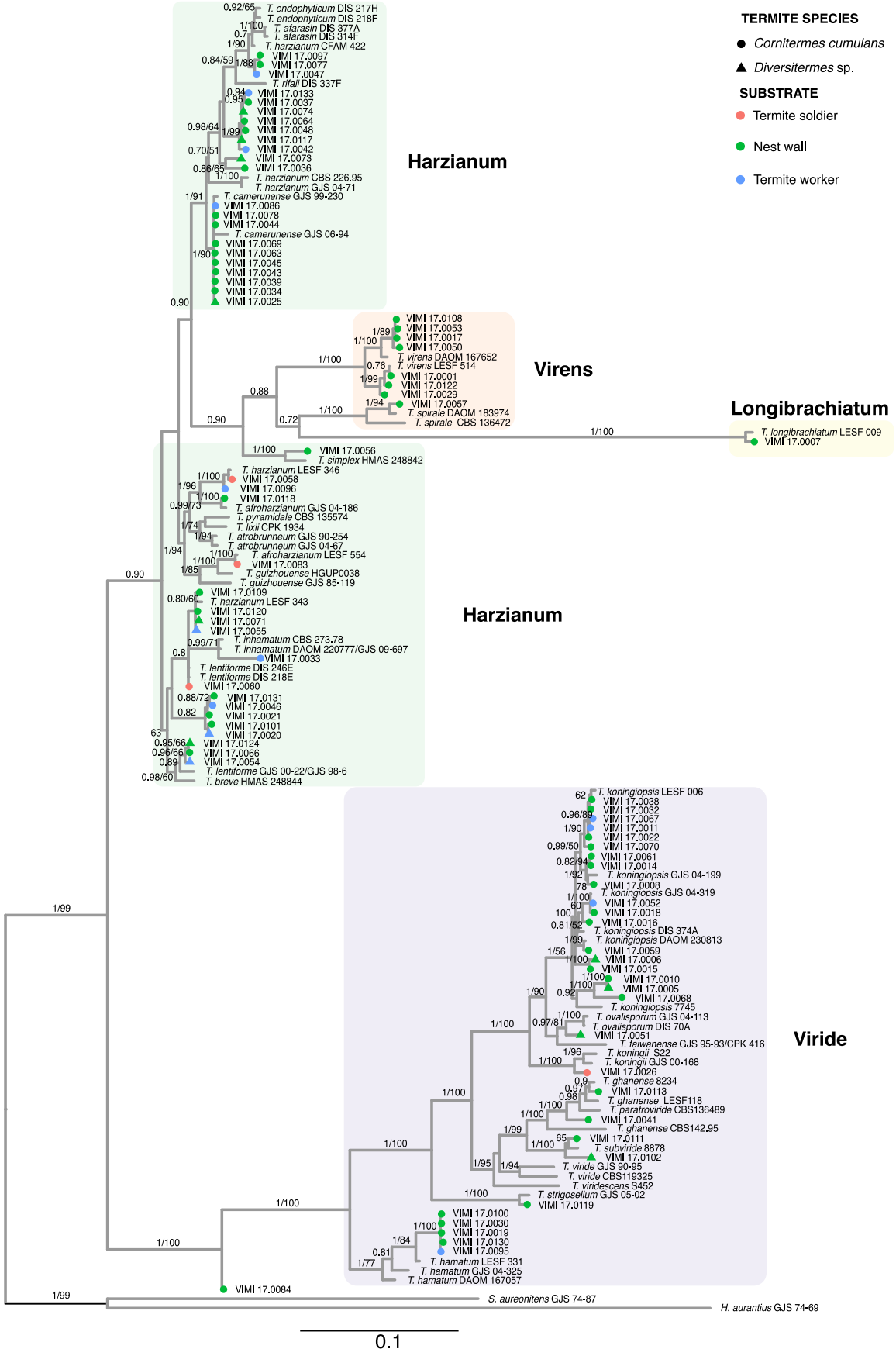
Of the 226 workers for which we measured fat content, 26 presented the fungus *Trichoderma* inside their bodies, 165 presented other fungi, and 35 had no fungi. Fat content varied from 0 to 0.5 mg and the presence of *Trichoderma* was not related to residual fat content (χ^2_1=0.001, p=0.96; Fig. S3).

3.4. Growth of an entomopathogenic fungus in the presence of *Trichoderma*

The growth of the entomopathogenic fungus *M. anisopliae* was slightly reduced in the presence of the two species of *Trichoderma* for *M. anisopliae* Isolate 1 (χ^2_3=29.66, p<0.001, Fig. 3A) and *M. anisopliae* Isolate 2 (χ^2_3=61.73, p<0.001, Fig. 3B) throughout the *in vitro* experiment. For *M. anisopliae* Isolate 1, after 12 d, colony radius in the presence of *T. harzianum* (19.40±0.38 mm) (mean±S.E.) was smaller than colony radius observed in the presence of *T. virens* (21.95±0.75 mm), *Fusarium* sp. (22.44±0.55 mm), and control (21.33±0.69 mm) (z=4.33; p<0.001). For *M. anisopliae* Isolate 2, after 12 d, the colony radius in the presence of *T. harzianum* (22.66±0.41 mm) (z=9.78; p<0.001) and *T. virens* (24.82±0.48 mm) (z=2.54, p=0.006) were smaller than the colony radius of *M. anisopliae* observed in the presence of *Fusarium* sp. (24.99±0.59 mm), and the control (25.66±0.25 mm).

3.5. Termite survival when exposed to *Trichoderma*

The survival assay lasted 600 h (25 d). During this period, 368 of 576 (63.8%) insects died. Survival of termites exposed to *T. harzianum* was lower than termites exposed to the other two treatments (p<0.001; Fig. 4C). Meanwhile, termites exposed to *L. brasiliensis* survived longer than those not exposed to fungus (p=0.04; Fig. 4C). After 25 d, 89.1% of termites exposed to *T. harzianum* had died, in contrast to 46.3% of



(caption on next page)

Fig. 2. Phylogenetic tree showing the positions of different *Trichoderma* species isolated from two species of termites: *Cornitermes cumulans*, *Diversitermes* sp. The fungal species were isolated from workers, soldiers, or fragments of nest walls, collected from termite nests from *Mata do Paraíso* Research Station, Viçosa, Minas Gerais, Brazil. Analysis was based on concatenated sequences of *tef1* and *rpb2* markers. This tree was built using the Bayesian Inference, but Maximum Likelihood showed the same topology in most clades. Numbers on branches indicate the posterior probabilities (BI>0.70) followed by number of bootstraps (ML>50%). Bars show nucleotide substitutions per site. The tree includes a total of 138 sequences of *tef1* and 117 sequences of *rpb2*. We included 55 *Trichoderma* sequences from previous studies obtained from GenBank. The 81 isolates identified in this study are indicated with their codes (VIMI 17.0xxx) and species from GenBank are followed by their strain codes. For each *Trichoderma* isolate obtained in this study, we show the substrate (soldiers=orange, nest wall=green, and workers=blue) and termite species (circle=*C. cumulans*, triangle=*Diversitermes* sp.) from which fungi were isolated. The species *Hypomyces aurantius* and *Sphaerostilbella aureonitens* were used as outgroups.

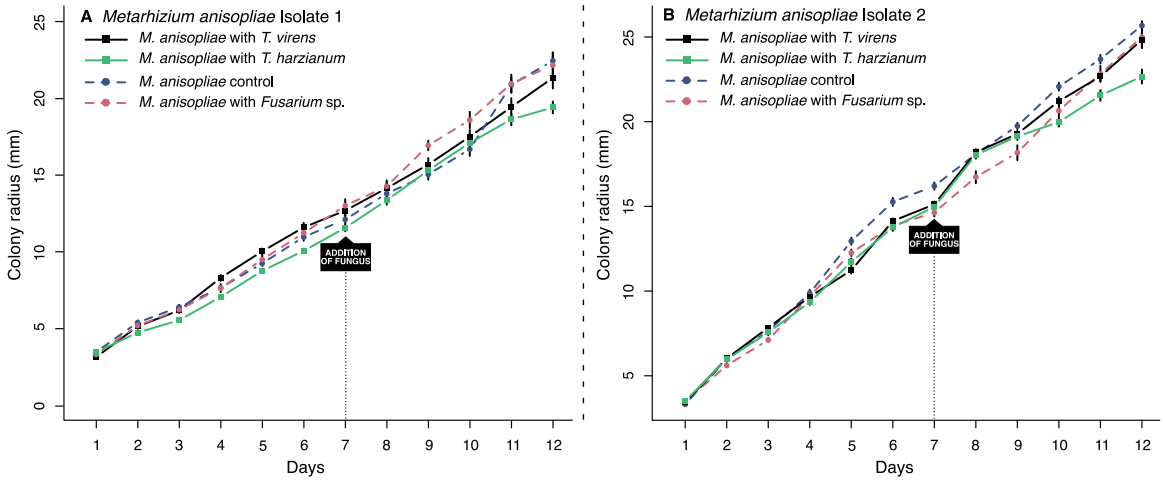


Fig. 3. Growth of the entomopathogenic fungus *Metarhizium anisopliae* in the presence of *Trichoderma harzianum* (green square), *Trichoderma virens* (black square), *Fusarium* sp. (orange circle), and agar (control) (blue circle) over 12 d. Colony radius (mm) of (A) *M. anisopliae* Isolate 1 and (B) *M. anisopliae* Isolate 2 did not show different growth before the inoculation of fungi at the plates after 7 d (black flags). Size of colonies of both *M. anisopliae* were compared in the presence of fungi after 12 d of experiment and were smaller in the presence of *T. harzianum*. Bars show standard errors. Representative photographs can be seen in Fig. S1.

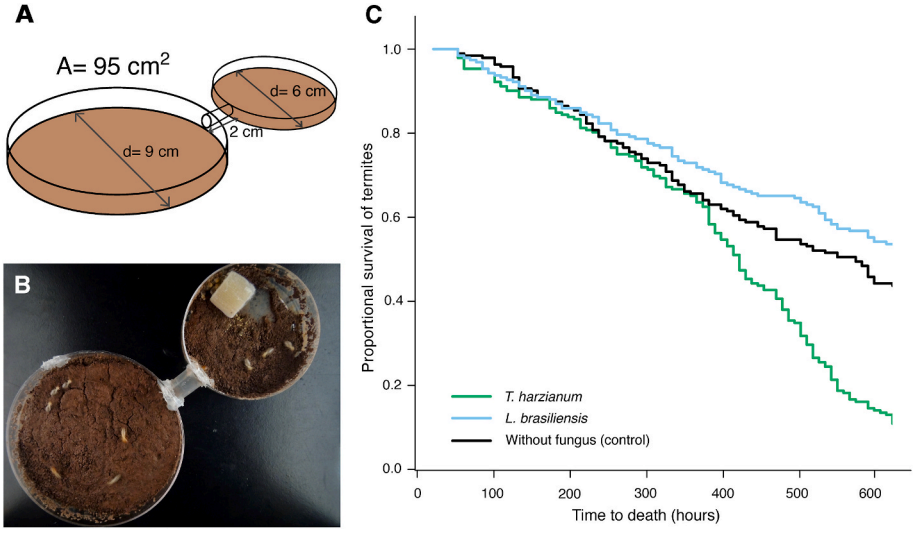


Fig. 4. Survival of termites *Cornitermes cumulans* in arenas. (A) Representation of the arena of 95 cm^2 and (B) one of the arenas used in the experiment, with the termites *C. cumulans* and sugar cane used as baits to inoculate the fungi inside the arenas. (C) Survival of termites *C. cumulans* over 25 d. Eight nests of *C. cumulans* were collected at *Mata do Paraíso* Research Station, Viçosa, state of Minas Gerais, Brazil. From each nest, we set up three arenas containing processed soil, one for each of three treatments. Termites were exposed to the fungus *Trichoderma harzianum* (green line; n=192, 8 arenas), without fungus (black line; n=192, 8 arenas), and *Lichtheimia brasiliensis* (blue line; n=192, 8 arenas). We kept 20 workers and 4 soldiers in each arena with soil from their nest of origin (n=24) and evaluated survival in 8 h intervals over 25 d (600 h).

mortality of termites exposed to *L. brasiliensis* and 56.3% of termites not exposed to fungi. Analysing the data up to the first half of the experiment (300h), termites exposed to *T. harzianum* did not show a difference in survival (p>0.05) when compared to the control, but survival of termites exposed to *L. brasiliensis* was already higher than in the other two groups (p=0.03).

4. Discussion

We found a low prevalence of *Trichoderma* species inside termite bodies, which suggests that this termite-fungus interaction is not an obligatory symbiosis for the termites. Nevertheless, *Trichoderma* species were found in workers, soldiers, and nest walls, indicating that the fungus is ubiquitous. The presence of *Trichoderma* species in termite nests is in line with previous studies that have shown them to be interacting with insects and plants (Harman et al., 2004; Druzhinina et al., 2011; Chaverri and Samuels, 2013; Montoya et al., 2016; Rocha et al., 2017; Vaz et al., 2018; Malinich et al., 2019). Besides, this symbiosis is not exclusive to higher termites (Jayasimha and Henderson 2007; Wen et al., 2020). Species of *Trichoderma* may produce metabolites that are

attractive to lower termites or suppress termite infection by entomopathogenic fungi such as *M. anisopliae* (Wen et al., 2020), while higher termites tend to avoid contacting sporulating *Trichoderma* (Bodawatta et al., 2019). This diversity of ecological functions of *Trichoderma* species (Loreau, 2001; Balvanera et al., 2006), in which the same species of the fungus can result in positive and negative effects on different species of termites, can indicate that the effect of some species of *Trichoderma* (such as *T. harzianum*) on termites may be double-sided. Therefore, we cannot rule out the possibility that this termite-fungus symbiosis within the nest is a consequence of the ubiquity of *Trichoderma* in soil and related environments, and its diversity of strategies for environmental colonization.

Trichoderma was not isolated from immature termites of either species, even though immature termites interact with adults inside the colony (Cremer et al., 2007; Rosengaus et al., 2010). Adults participate actively in different tasks inside and outside the nests (Schmid-Hempel, 1998; Miura and Scharf, 2010). Interactions between immature and adult termites may lead to indirect exposure of the former to components of outside the nest, such as microorganisms (Rosengaus et al., 2010; Chouvenc et al., 2018, and references therein). The lack of immature termites with *Trichoderma* suggests that some factor limits the exposure of the former to environmental microbes via interaction with workers. When moulting, for instance, termites discard their symbionts and potential pathogens with the old exocuticle and hindgut epithelium (Brune and Dietrich, 2015; Nalepa, 2017). The short periods between moults in the immature termites may limit their colonization. Moreover, immature termites do not forage and their access to food and acquisition of gut symbionts depend on trophallaxis with adult workers (Cremer et al., 2007; Eggleton, 2010; Ohkuma and Brune, 2010). These microbes can be recovered and lost frequently via trophallaxis, as this is common among immature and adult termites (Nalepa et al., 2001; Ohkuma and Brune, 2010). Despite the possibility of contact between immature termites and fungi from outside the nest (including *Trichoderma* species but also potentially entomopathogens), the lack of *Trichoderma* among immature termites might be explained by the age-based division of tasks. For example, immatures are involved in nursing and nest maintenance tasks (Miura and Scharf, 2010) while adults are responsible for foraging (Rosengaus et al., 2010). This question remains open as does that of exactly how *Trichoderma* species are acquired once termites are adults.

Our phylogenetic reconstruction demonstrated that closely related *Trichoderma* species did not necessarily share the same insect-associated substrate. This means that the same species of *Trichoderma* can persist in different substrates within the nest. This is not surprising considering the diversity of niches described for *Trichoderma* species (Harman et al., 2004; Druzhinina et al., 2011; Chaverri and Samuels, 2013; Samuels and Hebbar, 2015). For instance, *T. virens* was originally described from soil (Chaverri et al., 2001) and all strains in our study were found in nest walls, of which the main component is soil particles. Another case, *T. koningiopsis*, a species described as endophytic (Samuels, 2006), was found in the present study in termite nest walls and workers. Considering the lack of substrate specificity, it is unlikely that the symbiosis between *Trichoderma* species and termites is obligatory for the fungi. *Trichoderma afroharzianum*, for example, has previously been found in soil and as a mycoparasite (Bissett, 1991; Chaverri et al., 2015), *T. ghanense* and *T. harzianum* are commonly isolated from soil and decaying wood (Samuels and Hebbar, 2015), and *T. hamatum* in soil and as an endophyte (Bissett 1991; Samuels and Hebbar, 2015). In our study, they were isolated from insects and from nest walls. We have therefore added new niches for some *Trichoderma* species, such as *T. afrohazianum, T. hamatum, T. harzianum,* and *T. koningiopsis*, since they were also isolated from insects (Table 2). The lack of phylogenetic basis for the pattern of termite-*Trichoderma* symbiosis may be a consequence of the habitats of the fungal genus. Most *Trichoderma* species found here were initially described as soil-borne and identified in the termite nests, so perhaps have no different function within the termite nests than in soil. Alternatively, there may not yet have sufficient evolutionary time to differentiate traits that lead to specialization of functions within some recent clades. The insufficient evolutionary time could result in species within the same clade belonging to different functional groups and presenting different phenotypic traits, such as the species *T. harzianum* and *T. afroharzianum* (Kubicek et al., 2019).

The presence of *Trichoderma* species in termite workers was not related to residual fat content. It is possible that acquisition of nutrients from substrates and fat storage are optimized by termites' gut symbionts by mechanisms that do not depend on the metabolites of *Trichoderma*. Digestion of cellulose in higher termites is mediated by the insects' own cellulases in combination with a range of gut microbes (Lo et al., 2010; Ohkuma and Brune, 2010). The role of gut microbes in partial metabolization of nitrogen, carbohydrates, and lignocellulose are well documented (Benemann, 1973; Breznak et al., 1973; Brune and Ohkuma, 2010; Brune, 2014). Although *Trichoderma* species are well known for producing cellulolytic compounds, we found no evidence for a role of these fungi on termite nutrition. If there is such a role, they are not essential for the fat body content.

The colony radius of the entomopathogenic fungus *M. anisopliae* in the presence of *T. harzianum* was smaller than in its absence, indicating that this strain of *T. harzianum* is able to inhibit the entomopathogen's growth prior to physical contact. Only one isolate of *M. anisopliae* suffered a negative effect on its growth when exposed to *T. virens*, which suggests that this strain of *T. virens* might not be a relevant antagonist to *M. anisopliae in vitro*. Since *M. anisopliae* is a generalist entomopathogen (Meyling and Eilenberg, 2007; Vega et al., 2009), it is likely that this fungus has not evolved specialised defences against other fungi that do not represent a major threat to it. This might explain why *T. harzianum* and *T. virens* were able to grow and reach *M. anisopliae* colonies *in vitro* (Figs. S1A and B) and that these did not show any visible antagonistic response against the mycoparasites, such as inhibition zones. Even though mycoparasitism is an innate characteristic of both *T. harzianum* and *T. virens* (Druzhinina et al., 2011; Atanasova et al., 2013b), species from this genus have variations in their strategies during antagonistic interactions, such as differential expression of genes related to parasitism, different pathogenicities, or surface colonization (Chutrakul et al., 2008; Xie et al., 2014; Lima et al., 2016; Malinich et al., 2019). Additionally, different fungal strains can display distinct effects in interaction with the same substrate (Atanasova et al., 2013a; Malinich et al., 2019). This range of strategies might explain the differences in growth inhibition induced by *T. virens* and *T. harzianum* on *M. anisopliae*. Thus, the negative effect of some strains of *T. harzianum* and *T. virens* towards *M. anisopliae* can indirectly benefit termites as an extended defence against entomopathogenic fungi within the nest environment, as we initially hypothesized.

When we experimentally exposed *C. cumulans* to *T. harzianum*, the most abundant species found in our systematic survey, termite survival decreased. It is intriguing that, despite the high prevalence in nest walls and low prevalence within termite bodies, constant exposure to *T. harzianum* is prejudicial to the insects. Guswenrivo et al. (2018) found that the mortality of the termite *G. sulphureus* in contact with *Trichoderma* reached 50–100% after 14 d and suggested that high termite mortality is related to fungal compounds released during sporulation (Guswenrivo et al., 2018). In our experiment, we exposed termites to sugar cane baits with sporulating fungi, which could explain their higher mortality. Indeed, investigating fungal sporulation and volatiles released by the fungus is the next step to understand the negative effects of *T. harzianum* on termites. The use of another sporulating fungus isolated in our assays, *L. brasiliensis*, allows us to infer that the lower survival upon *T. harzianum* exposure was not due the presence of another organism (*i.e.* any fungus) in the experimental arena. Although the sugar cane baits were covered with hyphae and spores, workers and soldiers cut and carried fragments during the experiment (T.M.P personal observations). It may be that the putative harm caused by *T. harzianum* to the termites depends on the presence of spores and that the fungus does not normally

sporulate inside the nests; if so, however, we found no apparent immediate behavioural avoidance of this material. Moreover, keeping termites confined in arenas for 25 d imposed different conditions from natural colonies, which may have caused an additional stress for the insects. Rearing colonies of higher termites in the laboratory has been a challenge (Li et al., 2015; Xiong et al., 2018) that may help to explain the difference in mortality of termites from the control. Surprisingly, the survival of insects exposed to *L. brasiliensis* was greater than of those not exposed to fungi. As this fungus is described as non-pathogenic (Schwartze et al., 2014), this exemplifies the important gap in our knowledge as regards the microbial community and its ecological role associated with termites. In the conditions here tested, we confirmed that *T. harzianum* can be antagonistic to the higher termites.

The presence of fungi inside the colony can trigger defensive behaviours in the termites. For instance, insects can perform individual defences, such as hygienic behaviours (Zhukovskaya et al., 2013), removal of infected individuals from the colony (Cremer et al., 2007), production of glandular secretions (Rosengaus et al., 2010), or interacting with other symbionts in third-party defences (Chouvenc et al., 2013). If some species of *Trichoderma* represents a threat to the termites, these defences might explain the low prevalence we found in insects. In this sense, it is possible to highlight that the combination of diverse defensive strategies can result in a plastic response that is more efficient against colony parasitism by fungi than a single strategy, mitigating pathogen spread through the nests.

In summary, our results show evidence of the diversity of a genus of mycoparasitic and plant-symbiotic fungi in a new environment. It is possible that by using soil to construct their nests, the insects simply cannot avoid the fungus entering their colonies. Despite that, we could not find records of prevalence in samples of nests or termite individuals in the literature. We experimentally demonstrated that *T. harzianum* could provide an indirect benefit to the termites as it inhibits the growth of the entomopathogenic fungus *M. anisopliae*, yet it also decreased termite survival. At the colony level, we demonstrated a low prevalence of the fungus among termites even though the presence of *Trichoderma* species in the nest wall was frequent, which may be a consequence of termite life history. Termites are found in large numbers within colonies, which influences how they can control pathogen spread by employing specific social behaviours. If fungal sporulation is specifically responsible for decreasing insect survival, it is plausible that termites directly (*e.g.* removing fungal structures, Yanagawa and Shimizu, 2007) or indirectly (*e.g.* producing fungistatic compounds, Rosengaus et al., 1998) suppress *Trichoderma* growth and sporulation inside the nest. Thus, investigating how the metabolites naturally produced by the fungus affects termites could lead to a better understanding of the costs of this cosmopolitan fungus to termites. Our research indicates that *T. harzianum,* in natural conditions, likely has no specific ecological role that benefits termite colonies and might rather be a potential opportunistic parasite of higher termites.

Funding

This work was supported by the Minas Gerais State Agency for Research and Development – FAPEMIG, Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (TMP received a scholarship and SLE received productivity grants 309221/2013-7 & 309845/2016-5). This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Finance Code 001.

Availability of data and material

Dataset and scripts are available from the corresponding author upon request.

Author's contributions

T.M.P, S.L.E, C.C.M, R.G.L conceived and designed the experiments. T.M.P, S.L.E, R.G.L wrote the paper. T.M.P, C.C.M, T.G.K and R.G.L performed the experiments. T.M.P, C.C.M, T.G.K., and P.L.C.F analysed the data. All the authors revised the manuscript and have approved its final version.

Consent for publication

All the authors revised the manuscript and have approved its final version for publication.

Declaration of competing interest

We declare that we have no competing interests.

Acknowledgements

We would like to thank Gumercindo Souza Lima for permission to sample at *Mata do Paraíso*, Universidade Federal de Viçosa (UFV) in addition to the owners of the private properties for access to conduct this study. We thank the members of the Laboratory of Insect-Microbe Interactions, UFV, and José Augusto M. Rouxinol É for assistance in fieldwork and the laboratory, Og de Souza for identifying the termites, Rebeca B. Rosengaus for discussions about methodology at an early stage, and Carlos F. Sperber, João P. M. Araújo and Paulo Enrique C. Peixoto for their contributions to statistical and phylogenetic analyses. We are also grateful to Eduardo S. G. Mizubuti and Vinícius B. Rodrigues for comments on earlier versions of the manuscript. We Thank the editors Nicolai Meyling and Lynne Boddy, and an anonymous referee for constructive comments on this paper. We are grateful for the financial support provided by Minas Gerais State Agency for Research and Development – FAPEMIG, Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq, and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2022.101152.

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