THAIRINE MENDES PEREIRA

INSECT-FUNGUS SYMBIOSIS: DIVERSITY AND POTENTIAL ECOLOGICAL ROLES OF THE HYPOCREALEAN FUNGUS *Trichoderma* IN COLONIES OF TERMITES (BLATTODEA: TERMITIDAE)

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

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APROVADA: 27 de julho de 2018.

Eduardo Seiti Gomide Mizubuti

Vinícius Barros Rodrigues

Vinícius Barros Rodrigues

Simon Elli

Simon Luke Elliot
(Orientador)

*"São as nossas escolhas que revelam o que realmente
somos, muito mais do que as nossas qualidades."*
JK Rowling

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Num país como o Brasil, fazer graduação e pós-graduação em uma universidade pública é um enorme privilégio. Privilégio porque nos faltam oportunidades iguais. Desde minha entrada em 2010 na Universidade Federal de Viçosa, pude presenciar e me beneficiar de políticas de desenvolvimento da instituição. E por elas sou muito grata: à UFV pela formação em Bacharelado e Licenciatura em Ciências Biológicas, à Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) pela concessão das bolsas de Iniciação Científica durante a graduação e ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pela concessão da bolsa do programa Ciência Sem Fronteiras na University of Sussex, Brighton, UK e pela minha bolsa de mestrado.

Desde 2016, quando ingressei no Mestrado em Entomologia pela mesma universidade, pude alimentar a vontade de fazer a diferença na Ciência. No entanto, vi também as oportunidades sendo reduzidas, e cada vez ficando mais evidente que a Universidade está longe de cumprir seu papel de inclusão social. Muitas vezes, temos sorte de podermos contar com oportunidades que deveriam ser direito de todos. Optamos por uma educação superior pública, gratuita e de excelente qualidade, que infelizmente não consegue beneficiar todos que ajudam a financiá-la diariamente. Tive a sorte de estar inserida nesse ambiente acadêmico tão instigante, mesmo no contexto tão desfavorável que o Brasil se encontra em relação aos investimentos na Pesquisa. Portanto, sou grata ao Programa de Pós-graduação em Entomologia e à secretaria do Programa por toda ajuda nas questões burocráticas ao longo desses dois anos. Gostaria de agradecer principalmente à sociedade brasileira como um todo. Sem essa colaboração, não teríamos o incentivo necessário para a execução desta dissertação.

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ABSTRACT

PEREIRA, Thairine Mendes, M.Sc., Universidade Federal de Viçosa, July, 2018. **Insect-fungus symbiosis: diversity and potential ecological roles of the hypocrealean fungus *Trichoderma* in colonies of termites (Blattodea: Termitidae).** Advisor: Simon Luke Elliot. Co-advisor: Raquel Gontijo de Loreto.

Insects and microbes have mutualistic and antagonistic symbioses that can drive important processes of hosts and symbionts. We found a symbiosis between termites (Blattodea: Termitidae) and *Trichoderma* (Ascomycota: Hypocreales). This fungus produces cellulolitic and fungistatic compounds, but its ecological role in termites is unclear. We estimated the prevalence and investigated the diversity of *Trichoderma* in three species of termites: *Cornitermes cumulans*, *Diversitermes* sp. and *Termes* sp. Considering *Trichoderma* strategies of habitat exploration, we hypothesized that the fungus would contribute to termite nutrient acquisition and increase their survival. We obtained 136 isolates of *Trichoderma* from workers, soldiers and nest wall, belonging to 12 different phylogenetic species from 3 clades: section Trichoderma, clade Harzianum and clade Virens. We found a low prevalence of *Trichoderma* inside termite bodies, indicating that this termite-fungus interaction is not an obligatory symbiosis. No fungi were isolated from immatures, suggesting that *Trichoderma* may be acquired in later stages of the insect life. To assess termite acquisition of nutrients, we compared residual fat content of workers of *C. cumulans* with or without the fungus. Fat content of workers varied from 0 to 0.5 mg and the presence of *Trichoderma* was not related to residual fat content so it is likely that the fungus is not determinant for termite nutritional status. To evaluate termite survival, we created 24 arenas using macerated fragments of nest walls from 8 nests of *C. cumulans* as substrates (collected from Viçosa/MG). For each nest, three groups of 20 workers and 4 soldiers were exposed to *T. harzianum* or *Lichtheimia brasiliensis*, inoculated on to sterilized sugar cane, or no fungi. Survival was assessed every 8h for 25 days. After 25 days, survival of termites exposed to *T. harzianum* was lower than termites exposed to control or *L. brasiliensis. Trichoderma harzianum* is widely applied in agriculture as antagonist of plant and soil pathogens. Our results showed an unexpected negative effect on termite survival, one that may be neglected in other systems. Our research therefore indicates that

*Trichoderm*a likely has no specific ecological role that benefits termites and might be a potential parasite to termite colonies.

RESUMO

PEREIRA, Thairine Mendes, M.Sc., Universidade Federal de Viçosa, julho de 2018. **Simbiose inseto-fungo: diversidade e possíveis papéis ecológicos do fungo *Trichoderma* em colônias de cupins (Blattodea: Termitidae).** Orientador: Simon Luke Elliot. Coorientadora: Raquel Gontijo de Loreto.

Insetos e microrganismos apresentam simbioses mutualísticas e antagonistas que influenciam processos importantes de hospedeiros e simbiontes. Nós registramos uma simbiose entre cupins (Blattodea: Termitidae) e *Trichoderma* (Ascomycota: Hypocreales). Tal fungo produz compostos celulolíticos e fungistáticos, mas o papel ecológico do fungo em cupins é desconhecido. Estimamos a prevalência e investigamos a diversidade de *Trichoderma* em três espécies de cupins: *Cornitermes cumulans, Diversitermes* sp. e *Termes* sp. Considerando as estratégias de exploração do ambiente que o fungo possui, nós criamos a hipótese de que *Trichoderma* poderia contribuir com a aquisição de nutrientes pelos cupins e afetar sua sobrevivência. Nós obtivemos 136 isolados de *Trichoderma* de operários, soldados e parede interna dos ninhos, pertencendo a 12 espécies filogenéticas de três clados: seção Trichoderma, Harzianum e Virens. Nós encontramos uma baixa prevalência de *Trichoderma* dentro do corpo dos cupins, indicando que essa interação cupim-fungo não é uma simbiose obrigatória. *Trichoderma* também não foi isolado de imaturos, sugerindo que o fungo é adquirido em estágios posteriores do ciclo de vida. Para avaliar a aquisição de nutrientes pelos operários, nós comparamos o conteúdo residual de gordura de operários de *C. cumulans* com e sem a presença de *Trichoderma*. O conteúdo de gordura dos operários variou de 0 a 0,5 mg e a presença de *Trichoderma* não foi relacionada ao conteúdo residual de gordura. Portanto, é provável que o fungo não seja determinante para o estado nutricional dos cupins. Para avaliar a sobrevivência dos cupins, nós criamos 24 arenas usando fragmentos macerados da parede do ninho de 8 colônias de *C. cumulans* como substrato (coletados em Viçosa, MG). Para cada ninho, três grupos de 20 operários e 4 soldados foi exposto a *T. harzianum* ou *Lichtheimia brasiliensis*, inoculados em cana de açúcar esterilizada, ou sem fungo. Sobrevivência foi avaliada a cada 8h por 25 dias. Após 25 dias, a sobrevivência dos cupins expostos a *T. harzianum* foi menor que cupins expostos ao controle ou a *L. brasiliensis*. *Trichoderma harzianum* é amplamente aplicado na agricultura como antagonista de patógenos de plantas e que vivem no solo. Nossos resultados mostraram um efeito negativo na sobrevivência dos cupins, que pode estar sendo negligenciado em outros sistemas. Nossa pesquisa, portanto, indica que *Trichoderma* provavelmente não apresenta papel ecológico específico que beneficie os cupins e pode ser um potencial parasita para a colônia desses insetos.

SUMMARY

Insect-fungus symbiosis: diversity and potential ecological roles of the hypocrealean fungus *Trichoderma* in colonies of termites (Blattodea: Termitidae)

Thairine Mendes Pereira, Simon Luke Elliot, Raquel Gontijo de Loreto

1. Introduction

Insect-microbe symbioses are widespread and can be mutualistic or antagonistic (Vega et al*.,* 2009; Douglas, 2010; Kaltenpoth and Engl, 2014). The type of interaction between such organisms is dynamic and can vary in ecological time or over lifetimes, depending on the outcomes for individual host and symbiont (Aanen and Boomsma, 2005; Douglas, 2010). In mutualistic interactions, fitness of symbionts can be improved by the interaction and these symbioses may be facultative or obligatory. In a facultative mutualism, symbionts can still survive on its absence (Way, 1963; Currie et al*.,* 1999; Kaltenpoth and Engl, 2014). Such benefits might become essential for both organisms and evolve to an obligatory relationship (Oliver et al., 2005; Hölldober and Wilson, 2009; Douglas, 2010). In contrast, symbionts can become a burden for the host if the costs of the interaction overcome its benefits, which, if it persists, can evolve to parasitism. At some point, the evolution of specific strategies that increase the chance of parasite survival can drive the symbiosis to an obligatory parasitism (Read and Taylor, 2001; Alizon et al*.,* 2009; Douglas, 2010).

Regarding to symbioses between insects and fungi, mutualism or parasitism have driven the evolution of both groups at individual, population and community levels (Aanen and Boomsma, 2005; Douglas, 2010). For example, leaf-cutting ants

(Hymenoptera: Attini) and their mutualistic fungus are obligatorily symbiotic: the ant's foraging behaviour is directly shaped by the nutritional or other needs of the fungus, while the fungus' productivity and propagation is strictly dependent on the insect (Hölldobler and Wilson, 2009). On the other hand, some entomopathogenic fungi present diversified strategies of environmental exploitation (Meyling and Eilenberg 2007; Vega et al., 2009), such as some species belonging to the genera *Metarhizium* and *Beauveria* (Ascomycota: Hypocreales). These genera are well known as pathogen of several insect species (Meyling and Eilenberg, 2007) and are also described using alternative nutritional modes by producing cellulolytic enzymes and associating with plant tissue and soil (Meyling and Eilenberg, 2007; Vega et al., 2009; Behie and Bidochka, 2014). Therefore, it is possible to highlight that insects and fungi share diverse habits, in which some filamentous fungi are ubiquitous and have evolved different strategies to obtain nutrients, which has resulted in several ecological roles.

A group of filamentous fungus that is in evidence because of its range of different roles is the genus *Trichoderma* (Ascomycota: Hypocreales), a complex and diverse group. In this group, the over 250 species described can display different strategies depending on their symbionts (Harman et al., 2004). For example, some species belonging to this genus have been associated with plants, other with soil and other with animals (Druzhinina et al., 2011; Rodrigues et al., 2014; Mevers et al., 2017). In social insects, several species of *Trichoderma* have been reported as component of fungal communities present in nests of wasps (Poulsen, 2011), ants (Rodrigues et al., 2014; Montoya et al., 2016), and termites (Zoberi and Grace, 1990; Sreerama and Veerabhadrappa, 1993; Mevers et al., 2017; Guswenrivo et al., 2018). A few studies have shown co-ocurrence of *Trichoderma* and other fungal symbionts with termites (Zoberi and Grace, 1990; Sreerama and Veerabhadrappa, 1993; Guswenrivo et al., 2018). Even though these studies have shown the diversity of fungi associated with termite nests, the ecological consequences of the *Trichoderma* presence within nests and termites are still unclear. Guswenrivo et al. (2018) recently showed a negative effect of the fungus on survival of the subterranean termite *Globitermes sulphureus* (Blattodea: Termitidae). However, fungal identification was done at genus level. Considering the diverse ecology among the species of *Trichoderma*, it remains important to understand aspects such as species community, prevalence and ultimately the ecological role of the fungus in these termite-fungus symbioses.

Some species of *Trichoderma* described in association with animals have also been reported in symbiosis with plant rhizospheres (Chaverri et al., 2015; Druzhnina et al., 2011), or isolated from living plant tissues (Schuster and Schmoll, 2010). As an endophyte, the fungus can penetrate the first root layers and produce glycolytic, chitinolytic or cellulolytic enzymes, that are able to degrade cellular walls of pathogenic fungus and induce plant resistance to disease (Howell, 2003; Harman, 2006; Chutrakul et al., 2008; Atanasova and Druzhinina, 2010). In some hosts, these enzymes also can mobilize minerals or induce the production of hormones, optimizing plant resource acquisition and stimulating growth (Altomare et al., 1999; Yedidia et al., 2001; Hoyos-Carvajal et al., 2009; Samuels and Hebber, 2015). In other cases, enzymes produced by *Trichoderma* can degrade cellulose and it may benefit different organisms that consume cellulose sub-products (Harman, 2006). Thus, the presence of microorganisms that could ameliorate the acquisition of nutrients due to the decomposition of cellulose could be determinant for their maintenance in the environment. This could be the case of termite-*Trichoderma* association, since these insects consume, but are not able to fully digest cellulose by themselves. So, we can expect that this fungal symbiosis can favor acquisition of nutrients by this social insect.

Trichoderma's secondary metabolites also present fungistatic properties (Harman, 2006; Samuels, 2006; Chutrakul et al., 2008). The production of toxic and antibiotic substances characterizes several species of the genus as efficient antagonists of plant pathogens, including groups of nematodes and bacteria (Howell, 2003; Schuster and Schmoll, 2010; Druzhinina et al., 2011; Samuels and Hebber, 2015). In addition, some strains of *Trichoderma* have been reported parasitizing other fungi and exploiting their resources (e.g. as a mycoparasite; Harman et al., 2004; Schuster and Schmoll, 2010). These mycoparasite species are able to penetrate the host hyphae and secrete chitinases that dilute the parasitized cells' wall and cytoplasm, provoking its disruption (Howell, 2003; Druzhinina et al., 2011). Thus, considering that there are species of *Trichoderma* that can induce suppression of pathogenic fungi and bacterias in soils (Harman, 2006; Chutrakul, 2008), association with such species could potentially enhance hosts defences against pathogens. Considering that other microorganisms have already been reported producing fungistatic substances and inhibiting opportunistic microbes inside termite nests (Rosengaus et al., 1998; Rosengaus et al., 2010; Chouvenc et al., 2013), it is plausible to hypothesize that (i) the presence of microorganisms like *Trichoderma* that could also improve insect defences against entomopathogenic fungi could be favoured in the nest environment or (ii) this fungus can negatively affect termites' health.

This study focused on describing the termite-*Trichoderma* symbiosis and understanding the role of this group of fungus inside termite colonies. We aimed to (1) estimate the prevalence of *Trichoderma* and (2) evaluate whether the fungal prevalence differs among termite species or among groups of insects within the species, as well as to (3) assess the diversity of *Trichoderma* species associated with termites. How termites acquire and maintain *Trichoderma* within their bodies and nest is still unclear.

Therefore, we also (4) investigated the origin of *Trichoderma* acquisition to termite nest walls. Here, we hypothesized that *Trichoderma* is carried and spread in nest walls by a specific group of workers of *C. cumulans* that actively build the walls. In this situation, if the fungus is deposited on the walls for a designated group of workers, we predict that *Trichoderma* prevalence among workers from recent built nest areas would be higher than among workers from older parts of the nest.

Next, we approached the role of the *Trichoderma* associated with termites. Considering the diversity of *Trichoderma*'s strategies to exploit its environment, we hypothesized that it 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 present higher amount of body fat content. Thus, we tested (5) if the fat content in individuals is directly correlated to the presence of *Trichoderma* in body cavities. Lastly, if this symbiosis is beneficial to termites, we also expect positive impacts on termite survival when they are exposed to *Trichoderma*. On the other hand, if this symbiosis is prejudicial to termites, we expect negative impacts on termite survival when they are exposed to *Trichoderma*. Therefore, we aimed to investigate (6) if the fungus will alter the survival of the termite *C. cumulans* when the insects are exposed to this fungus.

2. Material and methods

2.1 Study area, species identification and fieldwork

We conducted the fieldwork at Mata do Paraíso research station of the Universidade Federal de Viçosa (20° 48’ 18” S, 42° 51’ 30” W), at Sítio Bom Sucesso (20º 47’ 34” S, 42º 50’ 31” W), and at Povoado dos Cristais (20° 46’ 58” S, 42° 50’ 25” W), all located at 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 owned by Francisco Cláudio Lopes de Freitas, 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 in the areas that revealed different species of epigeic termites. The nests are semi-spherical structures, fixed on soil, consisting of a mixture of soil, saliva and partly digested organic material (Eggleton, 2010). From each of the 35 nests, we collected 10 workers and 10 soldiers in order to morphologically identify the termite species. We then identified three different genera: *Cornitermes, Diversitermes* and *Termes* (Blattodea: Termitidae). We collected the nests from April 2017 to March 2018. Termite collections were carried out with permanent permission of ICMBio (23915).

2.2 Prevalence of *Trichoderma*

In order to investigate fungi associated with termite body cavities and nest walls, we collected four nests of *Cornitermes cumulans* (Kollar, 1832), four nests of *Diversitermes* sp. and one nest of *Termes* sp. We broke one side of the nest structure using a pickaxe and a hammer, that were washed and sterilized with 70% ethanol between each sample colected. We initially screened the material in the field to guarantee sufficient individuals for the assay. Individuals and fragments were taken to the laboratory for processing.

Fungal isolation

For each of the nine nests, we collected 30 immatures, 30 soldiers, 30 workers, and 30 fragments of internal nest walls, totaling 810 individuals and 270 fragments. For *Diversitermes*, we collected the 2 types of soldiers we found in the nests. We separated these individuals from nest material using flexible forceps, that were sterilized between the samples to avoid external contamination, and transferred them immediately to a -20º C freezer. After 24 hours, we removed the cadavers from the freezer, surface-sterilized them using 70% ethanol for 30 s and 5% sodium hypochlorite for 1 min, then rinsed 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 20%) and 0.3 g of chloramphenicol per litre of medium, and incubated at 25º C. All the insects were inspected daily for 10 days to evaluate the emergence of fungal hyphae.

We also fragmented the internal wall of each nest in 30 arbitrary pieces of approximately 1 cm^2. To avoid external contamination, we separated these fragments using forceps that were sterilized between each sample. We then placed these fragments in Petri dishes (90 mm × 15 mm) containing PDA modified medium (PDA 20% + 0.02 g of rose bengal, 0.3 g of chloramphenicol, 0.02 g of streptomycin sulphate per litre of medium). This is a selective medium used to isolate *Trichoderma* from soil (Gil et al., 2009). The plates were incubated at 25º C and inspected daily for 10 days to evaluate the emergence of fungal hyphae from the nest fragments.

Fungal identification

Once fungi emerged from termites or nest fragments, we transferred all possible strains to new Petri dishes (49 mm × 12 mm, PDA 20%) and incubated these at 25º C.

After fungal growth, we classified the strains according to their morphological features, categorizing them as morphotypes. From each morphotype with similar features to the genus *Trichoderma*, we prepared slides for microscopic inspection and stored them for further molecular confirmation. All strains morphologically identified as *Trichoderma* are preserved in 10% glycerol at 4º C at the mycological collection of the Laboratory of Insect-Microbe Interactions (LIIM-UFV), Viçosa, Brazil. Fungal collections were carried out with permission of ICMBio (23920).

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. We showed the prevalence varying from 0 to 1. Each nest was considered as a biological replicate. As we found only one nest of *Termes* sp., we calculated prevalence just among *C. cumulans* and *Diversitermes* sp. To evaluate whether the prevalence of *Trichoderma* differs within and between the species, we adjusted generalized linear mixed models (GLMM) with binomial distributions and random intercepts. For this, the origin of the fungus in insects (immatures, soldiers, or workers) or fragments were considered as explanatory factors. Insects and fragments were analyzed separately. Termite species and nests were considered as random factors in both analyses. Prevalence of *Trichoderma* was the response factor. Significance was evaluated using χ^2. Analyses were performed in R software (R Core Team, 2018).

2.3. Diversity of *Trichoderma* associated with termites

DNA extraction, PCR and sequencing

To evaluate the diversity of *Trichoderma* associated with termites, we generated monosporic cultures from all isolates and stored these in 10% glycerol. These were grown in PDA 20% medium for 7 days in the dark. We macerated the mycelium in liquid nitrogen, and the genomic DNA from each sample was extracted using the Wizard® Genomic DNA Purification Kit (Promega Corporation, WI, U.S.A.), following the manufacturer's protocol. Although the ITS region is used as a barcode for most *Trichoderma* species (Atanasova et al., 2013), there is low intraspecific ITS resolution for this genus (Druzhinina and Kubicek, 2005; Druzhinina et al., 2006; Atanasova et al*.,* 2013; Chaverri et al., 2015, Montoya et al., 2016). Thus, we opted to amplify two other molecular markers: the partial sequences of the gene coding for the elongation factor 1 alpha (tef1) (ca. 600 bp) and the gene coding for the second subunit of the RNA polymerase II (rpb2) (ca. 900 bp). For the tef1 region, we used the primer pair EF1-728F: 5' CATCGAGAAGTTCGAGAAGG 3' and TEF1R: 5' GCCATCCTTGGAGATACCAGC 3' (Carbone and Kohn, 1999; Samuels et al., 2002; Montoya et al., 2016). For the rpb2 region, we used the primer pair fRPB2-5F: 5' GA(T/C)GA(T/C)(A/C)G(A/T)GATCA(T/C)TT(T/C)GG 3' and fRPB2-7cR: 5' CCCAT(A/G)GCTTG(T/C)TT(A/G)CCCAT 3' (Liu et al., 1999). We performed PCRs 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 94º C/2 min followed by 15 cycles at 94º C/30 s, 65º C/30 s and 72º C/1 min; followed by 35 cycles at 94º C/30 s, 48º C/30 s, 72º C/1 min and final extension at 72º C/10 min.

PCR products were purified and sequenced by Macrogen®, South Korea (http://www.macrogen.com).

Phylogenetic analyses

We edited and assembled *Trichoderma* sequences with SeqAssem (Hepperle, 2004). Tef1 and rpb2 contigs were compared to homologous sequences using NCBI nucleotide database BLASTn to ensure that all the sequences were from *Trichoderma* (Altschul et al., 1990). We also included in our phylogenetic analyses sequences of other studies (Table S2) that were selected from the NCBI-GenBank database. Sequences from previous studies were selected by analyzing 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 112 partial tef1 sequences and 103 partial rpb2 sequences.

Individual gene alignments were generated by MEGA v. 7 (Kumar et al., 2016). The alignment of every gene was improved manually, annotated and concatenated into a single combined dataset using Geneious version 8.1.8 (Kearse et al., 2012). Gaps were treated as missing data and ambiguously aligned regions were excluded from phylogenetic analyses. The final alignment length was 1,727 bp: 689 bp for tef1 and 1,038 bp for rpb2. Maximum Likelihood and Bayesian Inference analyses were performed to reconstruct our phylogenetic trees on the independent regions tef1 and rpb2. Later, we performed the analyses using the concatenated dataset of tef1 and rpb2. For all analyses, the nucleotide substitution model was established based on the Akaike information criterion (AIC) and likelihood ratio of MrModelTest (Nylander, 2004). Maximum Likelihood was performed with RAxML v. 8.2.4 (Stamatakis, 2006) on the independent regions tef1 and rpb2. We then performed it again on a concatenated dataset containing the two loci. The dataset consisted of six data partitions, including three 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 1,000 bootstrap replicates. Bayesian Inference was performed with MrBayes v. 3.2.6 (Ronquist et al., 2012), using the GTR+I+G nucleotide substitution model for tef1 and SYM+I+G for rpb2. For Bayesian Inference, a parallel run, consisting of four chains, was subjected to Markov Chain Monte Carlo (MCMC) analysis until the runs converged with a split frequency of <0.01. The MCMC analysis started with a heating parameter 0.1 from a random tree topology and lasted 10,000,000 generations. Trees were saved every 1,000 generations, resulting in 10,000 saved trees. Finally, the first 2,500 trees were discarded as the burn-in phase. Phylogenetic trees were visualized using Dendroscope v. 3.5.9 (Huson, 2017) and edited using Inkscape (www.inkscape.org). The species *Protocrea pallida* was used to root the trees (Jaklitsch et al., 2008; Montoya et al., 2016).

2.4. Investigating how *Trichoderma* arrives at termite nest walls

To test whether *Trichoderma* is deposited on the walls by a specific group of workers, we assessed the prevalence of the fungus at genus level in termite workers collected from old areas of the nests and from areas recently built or in construction (in the same nest). We selected seven nests of *C. cumulans* and calculated their approximate nest volumes (Methods S1 and Figure S1). We then removed 10% of the nest volume in the field. After 48h hours, we collected the recently built nest area and the termites that were in the recently built part of the nest. Moreover, we located six nests of *C. cumulans* that were naturally expanding their volume. Natural expansion is characterized by the wet soil in the surface of nests. From these nests, we also measured their volume and collected 10% of the recently built area. We then took 10% of the nest in the opposite side of first collection area (recently built) in order to collect the old constructed walls and workers which were in that part of the nest. Nest walls and insects collected in these steps were taken to the laboratory for processing. We separated 60 individuals from each nest (30 workers from old and 30 from recently built walls), totaling 780 individuals (13 nests: 7 artificially damaged; 6 naturally expanding). Fungal isolation from the termite's internal body cavities and identification were further performed using the same protocol described in section 2.2 above.

We compared *Trichoderma* prevalence between workers from old areas and workers from recently built areas belonging to the same nest of *C. cumulans*. For the analysis, we adjusted a generalized linear mixed model (GLMM) with binomial distribution and random intercepts. We considered the nature of the fungus (workers from old nest walls or recently built walls) and type of expansion (artificially damaged or naturally expanding) as explanatory variables. Prevalence of *Trichoderma* was considered as the response variable, with a binomial distribution. Termite nests were considered as a random factor. Significance was evaluated using χ^2. Analysis was performed in R software (R Core Team, 2018).

2.5. Influence of *Trichoderma* on termite fat content

To assess if the fat content in individuals is positively correlated to the presence of *Trichoderma* in body cavities, we compared the amount of fat content of workers that presented the fungus. For this, we collected 226 workers from 5 colonies of *C. cumulans* which presented higher prevalence of *Trichoderma* in our prevalence study (section 2.2). These termites were frozen at – 20º C for 24 h. We weighed these on an analytical balance (Shimadzu ATX224, 0.0001g precision). To evaluate the emergence of fungus from worker's internal body cavities, we processed the individuals as described above (section 2.2). After 48 h of incubation, we removed all termites from Petri dishes and kept the plates incubated at 25º C to verify the fungi strains which emerged from the insects. To evaluate whether the time spent in Petri dishes was relevant for termites' body mass loss, we then weighed the termites again and compared with fresh body mass. After emergence of fungi in the plates we kept in the incubator, we separated the samples in three groups: no fungi, *Trichoderma*, and other fungi.

We assessed the amount of fat content in the termite body of 226 workers. For this, we dried the workers we removed from Petri dishes in an oven at approximately 50º C for 3 hours before weighing them again. Later, we placed each termite in 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; Lopes-Junior and Peixoto, 2013). After extraction, we dried the individuals in an oven at approximately 50º C for an additional 3 hours 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; Lopes-Junior and Peixoto, 2013).

To estimate values of fat content regardless of 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 body mass and body length were correlated (F=7.75, d.f.=55, P<0.001). To test whether fat body mass content is dependent of the presence of *Trichoderma*, 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 software (R Core Team, 2018).

2.6. Termite survival when exposed to *Trichoderma*

Arena set-up

We created 24 arenas, composed of one 9 cm × 15 cm Petri dish connected to a 6 cm × 15 cm Petri dish by a 2 cm-length cylindrical tube (Figure S2), summing approximately 95 cm^2 inside the arena. Prior to the experiment, the arenas were washed with neutral detergent, immersed in 5% sodium hypochlorite solution for 24 h and rinsed 2 times with sterile water.

We collected fragments of nest walls from 8 colonies of *C. cumulans,* macerated and sifted the fragments to homogenize the size of soil particles. Soil was used in natural conditions (not sterilized) because a previous assay using sterile soil resulted in lower survival of termites exposed to sterile than natural soil (P=0.005, Methods S2). We then filled three arenas with soil of each colony. In each arena, we inserted 12 g of processed soil in the larger plate and 5 g in the smaller plate, and added 5 ml of sterile water to moisten the soil. 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 sterile water daily in each arena to keep them moistened.

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

Fungal selection and inoculation

To test the impact of the fungus *Trichoderma* on termite survival, we selected one strain of *T. harzianum* (VIMI-17.0133) that we isolated from a termite internal body cavity in section 2.2. We also selected the most prevalent fungus from the internal body cavity of insects we used in section 2.5, *Lichtheimia brasiliensis* (Zigomycota: Mucorales) (VIMI-18.0001), to use as control for fungus presence. This is a non-pathogenic fungus (Schwartze et al., 2014) and was found in 36.9% of the samples. Both strains are preserved in 10% glycerol at 4º C at the mycological collection of the Laboratory of Insect-Microbe Interactions (LIIM-UFV), Viçosa, Brazil, and were identified using ITS and tef1 region.

To introduce the treatment inside the arena, we used fragments of approximately 1 cm^3 of sugar cane as baits. We sterilized fragments of sugar cane daily and kept them untouched, or inoculated them with the fungi *T. harzianum* or *L. brasiliensis* (inoculation was done with a needle and plates containing the untouched cubes were opened for the same time as those inoculated). All fragments were incubated at 25º C. Once the fungi had grown for 5 days, we estimated fungus growth 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% (Figure S3). The designated treatment was 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 no fungi. The first fragments were placed 24 h after termites were inserted in the arenas and were replaced every 24 hours.

Termite survival

The 24 arenas were maintained in the dark and at 25º C during the experiment, except for the evaluation periods. Termite mortality was assessed by opening the arenas and removing the cadavers every 8h, totaling 75 monitoring events along 25 days. Dead termites (death was determined by a lack of a movement reaction) were removed and surface sterilized as described in earlier sections. To verify acquisition of the fungi by the termites, we placed the sterile cadavers in Petri dishes containing PDA modified medium and incubated these at 25º 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. For the analysis, we performed a survival analysis with Weibull distribution. The colony of origin was included as a frailty factor. Frailty model is used when survival is correlated or clusted. In this case, data is correlated to colony of origin. So, we used the shared frailty gamma model (Rondeau et al*.,* 2003), adding a random effect (colony) which act as a multiplicatively on the survival function (Rondeau et al*.,* 2012). Analysis was performed in R software (R Core Team, 2018).

3. Results

3.1 Prevalence of *Trichoderma*

We compared prevalence of *Trichoderma* among insect groups and nest fragments separately. Among insect groups, in *C. cumulans*, the fungus prevalence varied from 0 to 0.27, with a global mean of all nests being 0.058±0.022 (mean±SE) (n=4; Fig 1A). *Diversitermes* showed a variation from 0 to 0.1, with a *Trichoderma* global mean of 0.015±0.009 (n=4). *Trichoderma* prevalence in *C. cumulans* was higher than in *Diversitermes* sp. (χ^2_5=5.08, n=8, P=0.02, Fig 1A). *Trichoderma* prevalence among workers of *C. cumulans* and *Diversitermes* sp. was higher than among soldiers (χ^2_5=10.04, n=8, P=0.002, Fig 1A). Additionally, for all the three species of termites used in this study, no fungi were isolated from immatures.

Among nest fragments, *Trichoderma* prevalence was also higher in *C. cumulans* (0.57±0.06; Fig 1B) than in *Diversitermes* (0.14±0.05; χ^2_4=7.10; n=8; P<0.01; Fig 1B).

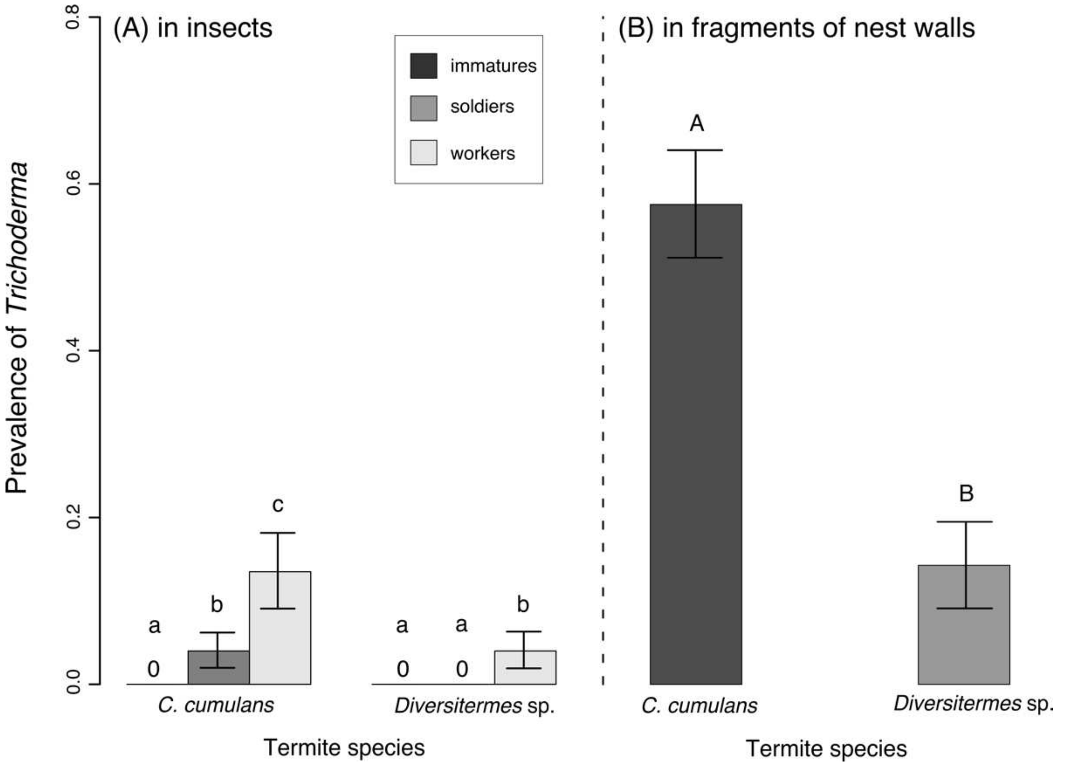


Figure 1. Mean prevalence of the fungus *Trichoderma* in termites (mean±SE). The prevalence of *Trichoderma* was estimated (A) according to termite species and insect of origin, and (B) according to termite species and nest wall. Eight nests of termites (*Cornitermes cumulans*, n=4; *Diversitermes* sp., n=4) were collected at Mata do Paraíso Research Station, Viçosa, state of 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 the fungus *Trichoderma*. (A) Prevalence of *Trichoderma* in *C. cumulans* was higher than in *Diversitermes* sp. (χ^2_5=5.08, n=8, P=0.02), and *Trichoderma* prevalence in workers of *C. cumulans* and *Diversitermes* sp. was higher than in soldiers (χ^2_5=10.04, n=8, P=0.002). (B) Prevalence of *Trichoderma* in nest walls was again higher in *C. cumulans* than *Diversitermes* sp. (χ^2_4=7.10; n=8; P<0.01).

3.2. Diversity of *Trichoderma* associated with termites

We obtained 136 isolates of the fungus *Trichoderma* from insects and fragments of 9 nests of termites (Table S1). We then molecularly identified 113 of these isolates, which comprise 17 distinctly species. The distribution of the species according to nest, substrate of origin and termite species is described in Table 1. *Trichoderma harzianum,* the most abundant species, and *T. atroviride* were found in nest walls, soldiers and workers (Table 1 and Figure S4). *Trichoderma koningiopsis* and *T. hamatum* were present in nest walls and workers, and *T. lentiforme* was isolated from nest walls and soldiers. Another nine *Trichoderma* species were present only in nest walls: *T. virens, T. lixii, T. longibrachiatum*, *T. ovalisporum, T. sparsum, T. spirale*, *T. simplex*, *T. strigosellum,* and *T. subviride.* Meanwhile, *T. afroharzianum* and *T. koningii* were isolated only from soldiers, and *T. inhamatum* was found only in workers.

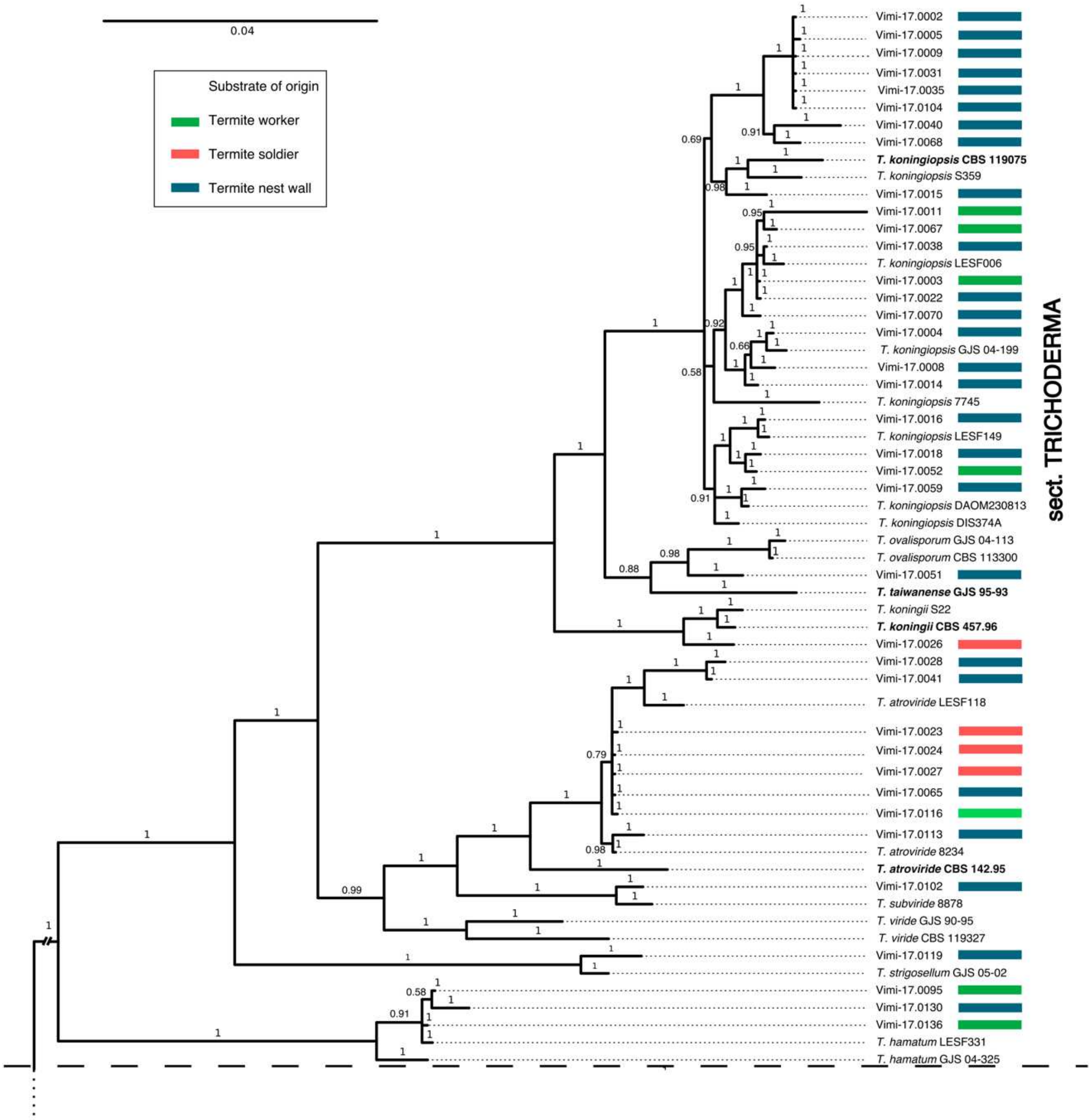
Five species of *Trichoderma* were found in all three termite species (Table 1 and Figure S4): *T. harzianum, T. koningiopsis, T. lentiforme, T. hamatum* and *T. virens. Trichoderma atroviride* was found in *C. cumulans* and *Termes* sp., and *T. lixii* was found in *C. cumulans* and *Diversitermes* sp. Two species of *Trichoderma* were unique for *Diversitermes* sp.: *T. spirale* and *T. subviride.* Seven species of the fungus were unique for *C. cumulans: T. afroharzianum, T. longibrachiatum*, *T. ovalisporum*, *T. simplex*, *T. strigosellum, T. koningii* and *T. inhamatum. Trichoderma sparsus* was found only in *Termes* sp.

Table 1. *Trichoderma* isolates from each substrate

Species	Worker	Soldier	Immature	Nest wall	Hosts	# nests	Total of isolates
T. harzianum	10	1	0	29	all	9	40
T. koningiopsis	4	0	0	28	all	7	32
T. atroviride	1	3	0	5	*Cornitermes cumulans, Termes* sp.	2	9
T. lentiforme	0	1	0	3	all	4	4
T. hamatum	2	0	0	5	all	3	7
T. afroharzianum	0	1	0	0	*C. cumulans*	1	1
T. virens	0	0	0	9	all	4	9
T. lixii	0	0	0	2	*C. cumulans, Diversitermes* sp.	2	2
T. longibrachiatum	0	0	0	1	*C. cumulans*	1	1
T. ovalisporum	0	0	0	1	*C. cumulans*	1	1
T. sparsum	0	0	0	1	*Termes* sp.	1	1
T. spirale	0	0	0	1	*Diversitermes* sp.	1	1
T. simplex	0	0	0	1	*C. cumulans*	1	1
T. strigosellum	0	0	0	1	*C. cumulans*	1	1
T. subviride	0	0	0	1	*Diversitermes* sp.	1	1
T. koningii	0	1	0	0	*C. cumulans*	1	1
T. inhamatum	1	0	0	0	*C. cumulans*	1	1

Phylogenetic analyses

Seventy-six isolates were used for the phylogenetic analyses (Figure 2). These 76 isolates grouped into 12 different phylogenetic species (Table S2). These 12 phylogenetic species belong to three different clades, according to the division proposed by Atanasova et al. (2013): (a) **sect. Trichoderma**: *T. atroviride* (8 isolates), *T. hamatum* (3 isolates), *T. koningii* (1 isolate), *T. koningiopsis* (22 isolates), *T. strigosellum* (1 isolate), *T. subviride* (1 isolate), *T. ovalisporum* (1 isolate); (b) **Clade Harzianum**: *T. afroharzianum* (1 isolate), *T. harzianum* (31 isolates), *T. inhamatum* (1 isolate), *T. lentiforme* (1 isolate); (c) **Clade Virens**: *T. virens* (5 isolates).



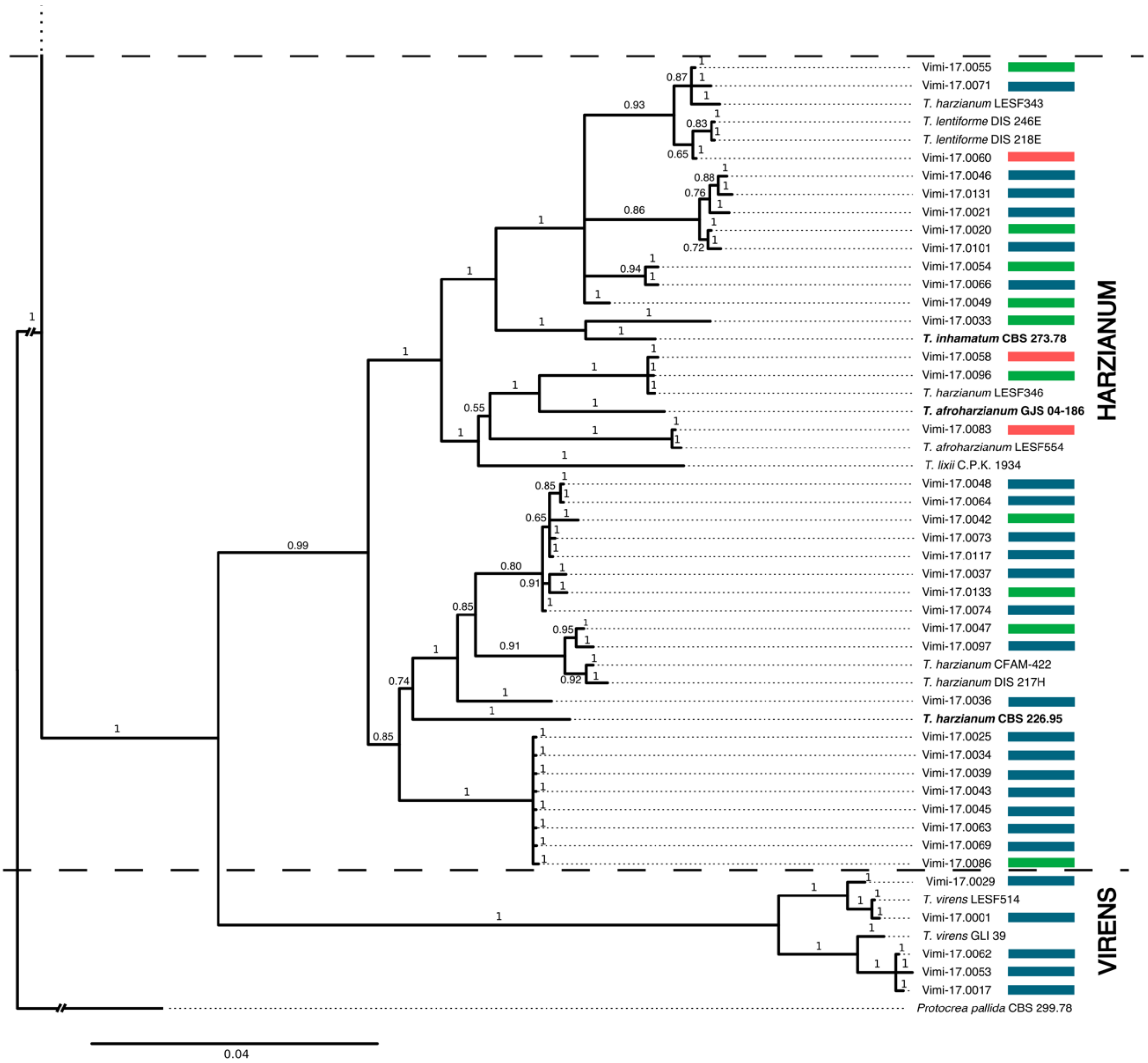


Figure 2. Phylogenetic tree showing the position of different *Trichoderma* species isolated from three species of termites: *Cornitermes cumulans*, *Diversitermes* sp. and *Termes* sp. The species were isolated from internal cavity of workers, soldiers or fragments of nest walls. Analysis was based on concatenated sequences of tef1 and rpb2 markers. This tree was built using the Bayesian Inference and the numbers on branches indicate the posterior probabilities. Bars show nucleotide substitutions per site. The tree includes a total of 112 sequences of tef1 and 103 sequences of rpb2. We included 36 *Trichoderma* sequences from previous studies obtained from GenBank. The final alignment had 1,727 base pairs. The 76 isolates identified in this study are indicated with their codes (Vimi-17.xxxx) and species from GenBank are followed by the strain codes. For each *Trichoderma* isolate obtained in this study, we show the substrate from which fungi were isolated (soldiers = red, fragments = blue and workers = green squares). The fungus *Protocrea pallida* was used as an outgroup. Names and strain ID in bold indicates type species. Signals of // in three branches indicate that they were edited to fit the image.

3.3. Investigating how *Trichoderma* arrives at termite nest walls

The prevalence of *Trichoderma* among workers from older nest areas (0.19±0.04) did not differ from workers that were collected in the recently built part of the nest (0.17±0.03) (χ_1^2=0.2, P=0.63; Figure 3) The type of expansion of nests (artificially damaged or naturally expanding) did not affect prevalence of *Trichoderma* in workers (χ_1^2=0.002, P=0.96).

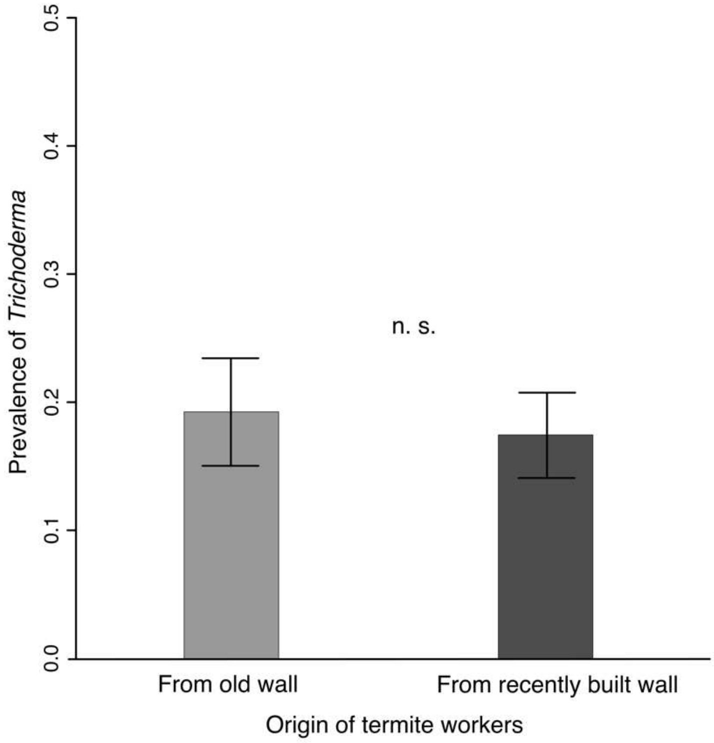


Figure 3. Prevalence of the fungus *Trichoderma* among termite workers of *Cornitermes cumulans* collected from old (n=390) and recently built (n=390) nest areas from naturally expanding (n=6) or artificially damaged nests (n=7). Prevalence of *Trichoderma* was not related to the construction of the walls (χ_1^2= 0.223, P=0.63) or type of nest expansion (χ_1^2=0.002, P=0.96). Nests of *C. cumulans* used in this assay were collected at Mata do Paraíso Research Station, Povoado dos Cristais and Sítio Bom Sucesso, Viçosa, state of Minas Gerais, Brazil.

3.4. Influence of *Trichoderma* on termite fat content

Of the 226 workers for which we measured fat content, 26 presented the fungus *Trichoderma* in body cavities, 165 presented other fungi, and 35 no fungi. Fat content varied from 0 to 0.5 mg and the presence of *Trichoderma* was not related to residual fat content (χ_1^2=0.001, P=0.96; Figure 4).

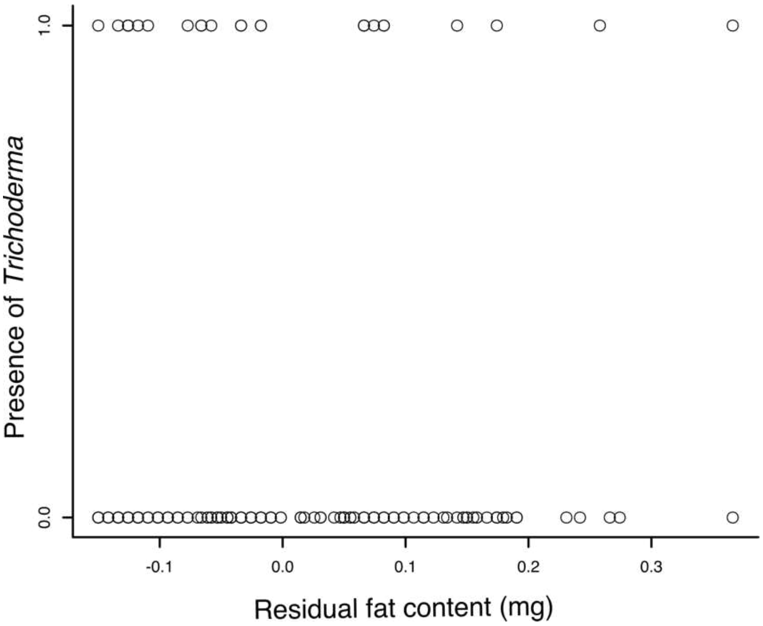


Figure 4. Relation between presence of the fungus *Trichoderma* and residual fat content (fat × dry weight) of workers of the termite *Cornitermes cumulans*. Workers were collected from five nests of *C. cumulans* at Sítio Bom Sucesso, Viçosa, state of Minas Gerais, Brazil. Presence of *Trichoderma* was determined after placing the insects for 48 h in Petri dishes for fungi emergence. The fat content was calculated after submersion of insects in chloroform for 72 h. Fat content was not related to presence of the fungus in body cavities of termite workers (χ_1^2=0.001, n=226, P=0.96).

3.5 Termite survival when exposed to *Trichoderma*

The survival assay lasted for 600 hours (25 days). 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; Figure 5). In addition, termites exposed to *L. brasiliensis* survived longer than termites not exposed to any fungus (P=0.04; Figure 5). After 25 days of observations, 89.1% of termites exposed to *T. harzianum* had died, in contrast to 46.3% of mortality of termites exposed to *L. brasiliensis* and 56.3% of termites not exposed to fungi. Also, until the first half of the experiment (300h), termites exposed to *T. harzianum* did not show difference in survival (P>0.05) when compared to control, but survival of termites exposed to *L. brasiliensis* was already higher than the other groups (P=0.03).

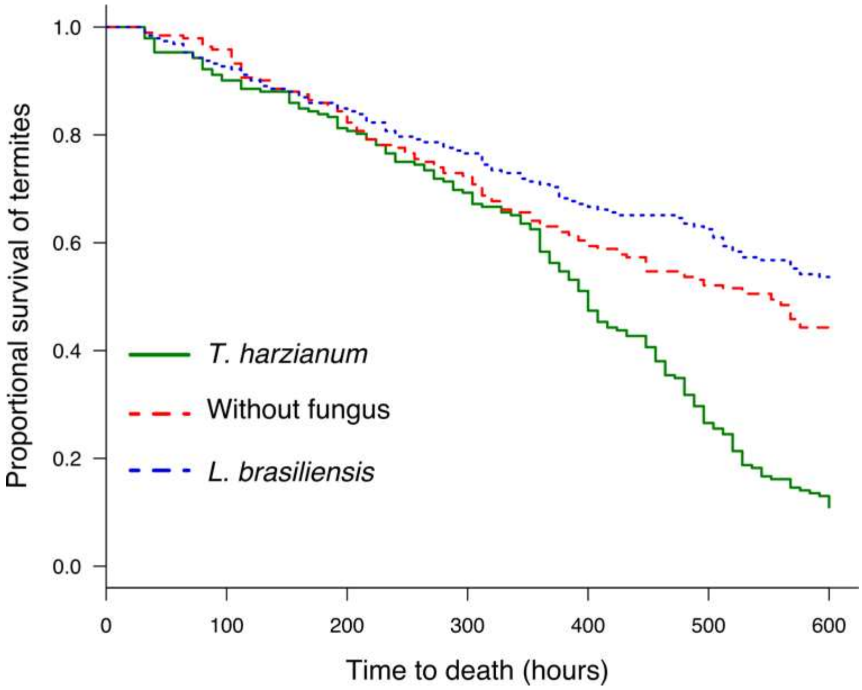


Figure 5. Survival of termites *Cornitermes cumulans* in arenas. Eight nests of *C. cumulans* were collected at Mata do Paraíso Research Station, Viçosa, state of Minas Gerais, Brazil. From each nest, we set up three arenas containing processed soil, one for each of three treatments. Termites were exposed to the fungus *Trichoderma harzianum* (green line; n=192, 8 arenas), without fungus (red 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 hour intervals along 25 days (600 hours). Termite survival of individuals exposed to *T. harzianum* was the lowest (P<0.001). However, survival of termites exposed to *L. brasiliensis* was higher than control (without fungus, P=0.04) and *T. harzianum* (P<0.01).

4. Discussion

We found a low prevalence of *Trichoderma* inside termite bodies, which suggests that this termite-fungus interaction is not an obligatory symbiosis for the parties. However, *Trichoderma* was found in workers, soldiers and nest walls, indicating that the fungus is ubiquitous within termite colonies. Evidence of

Trichoderma in almost all substrates of termite nests is in line with previous studies that reported the occurrence of *Trichoderma* species associated with soil, insects and plants (Harman et al., 2004; Schuster and Schmoll, 2010; Druzhinina et al., 2011; Chaverri and Samuels, 2013; Montoya et al., 2016). So, we cannot rule the possibility of this termite-fungus co-existing within the nest to be a consequence of *Trichoderma* ubiquity and its diversity of strategies on environmental colonization.

For all the three species of termites, *Trichoderma* were not isolated from immatures. In social insects, most immatures are generally confined to specific chambers within the nest, where interactions with other individuals are limited (Schmid-Hempel, 1998; Cremer et al., 2007; Hölldobler and Wilson, 2009; Rosengaus et al., 2010). However, termite immatures participate to tasks inside the colony, enhancing the exposition of immatures to exogenous factors of the nest, such as microorganisms (Rosengaus et al., 2010 and references therein). Additionally, immatures are not able to forage and their access to food depend on trophalaxia with workers (Cremer et al., 2007; Eggleton, 2010), although *Trichoderma* was found in workers and nest walls. Thus, contact of immatures with *Trichoderma* via trophalaxia during feeding is less likely, which might justify the absence of the fungus in immatures and indicate that *Trichoderma* is acquired in the later stages of the insect life.

Our phylogenetic reconstruction demonstrated that closely related species did not necessarily share the same insect-associated substrate. This means that the same species of *Trichoderma* is able to persist in different substrates within the nest. This is not surprising considering the diversity of niches described for *Trichoderma* species (Howell, 2003; Harman et al., 2004; Druzhinina et al., 2011; Chaverri and Samuels, 2013; Samuels and Hebber, 2015). For example, *T. virens* was originally described from soil (Bisset, 1991; Chaverri et al., 2001) and all strains in our study were found in nest walls, that contain soil particles in its composition, remaining in line with previous descriptions. Some species with previous habitat described as endophytic (e.g. *T. koningiopsis*) (Samuels et al., 2006) were here found in termite nest walls. In addition, many species were found in several substrates, agreeing with previous descriptions of their cosmopolitan occurrence and diverse mode of environmental exploitation (Harman et al., 2004; Druzhinina et al*.,* 2011). To illustrate, *T. atroviride, T. harzianum* and *T. hamatum* were previously described in soil and as mycoparasites (Bisset, 1991), in soil and wood (Chaverri et al*.*, 2015), and in soil and as endophytes (Bisset, 1991), respectively. In our research, they were isolated from insects and from nest walls. We therefore added new niches for some of *Trichoderma* species here isolated from termite nests. We also investigated whether there is a phylogenetic basis for a range of termite-associated subtract (e.g. soil nest, insect bodies). As closely related strains tend to present similar ecological roles (Samuels and Hebber, 2015), it is plausible that we have the same origin of some strains described here in a new substrate. It can be a consequence of the habitat of the fungus - initially found in the soil that is incorporated to the termite nests, the conservation of different traits by the same lineage or insufficient evolutionary time to differentiate traits that lead to specialization of functions within the genera.

Prevalence of *Trichoderma* among workers from older nest areas was similar to the prevalence among workers from recently built parts of the nest. Although it has been suggested that the fungus arrives to the nest in the soil used to build the nest galleries (Rosengaus et al., 1998; Rosengaus et al., 2010; Chouvenc et al., 2013), our results indicate that the acquisition of *Trichoderma* by termite workers is not limited to workers directly related to the task of construction, contrary to our initial hypothesis. This corroborates with the observation that *Trichoderma* were also present in 70% of the samples from adjacent soil of *C. cumulans* nests (T.M-P., personal observation). Considering that a social insect performs different tasks inside the colonies during their lifetime (Gullan and Cranston, 2010), they can be exposed to *Trichoderma* in distinct environments. As long as workers are allocated in construction, they are more likely to be in contact with the fungus when they manipulate soil particles to build the walls. Additionally, when workers are foraging, they are exposed to plant and soil particles that might host the fungus. Therefore, the deposition of *Trichoderma* in nest environment might be consequence of its presence in soil and the termite habits of construction and foraging.

Presence of *Trichoderma* in termite workers was not related to residual fat content. However, since we did not experimentally manipulate the presence of the fungus, we cannot completely eliminate possible indirect effects of the presence of the fungus on termite nutrition. Digestion of cellulose in higher termites is mediated by the insect's own cellulases, that are insufficient to degrade cellulose completely (Lo et al., 2010), and gut microbes. Because of their huge diversity, the identity of gut microbes responsible for digestive processes is still being explored (Ohkuma and Brune, 2010). Despite that, their role in nitrogen fixation (Benemann, 1973; Breznak et al*.*, 1973; Brune, 2014), in addition to partial metabolization of carbohydrates, fibres (Brune and Ohkuma, 2010), and lignocellulose (Brune, 2014), in which sub-products can be used by termites and other symbionts (Rouland-Lefèvre and Bignell, 2001) are well documented. For termites, digestion of celluloses and lignocelloses probably is mediated by different mechanisms that do not depend on the *Trichoderma*'s metabolites. Considering that, it is possible that acquisition of nutrients from substrates and fat storage by termites are optimized by their gut symbionts, and the presence of *Trichoderma* is not determinant for termite nutritional status.

The termite survival decreases on the experimental exposure to *T. harzianum*, the most abundant species found in our systematic survey. It is intriguing that, despite the high prevalence in nest walls, low prevalence within insect body and no evidence of effect on termite nutrition, constant exposure to *T. harzianum* is prejudicial to the insects. Keeping termites confined in arenas for 25 days inserted a different condition from colonies, which may have caused a stress for the insects. So, it is possible that antagonistic effects of the fungus when insects are under stress conditions, are more relevant for termites. Guswenrivo et al. (2018) found that the mortality of the termite *G. sulphureus* in contact with *Trichoderma* reached 50-100% at 14 days. They suggested that differences at termite mortality when exposed to the fungus are related to fungal compounds released during sporulation, since their treatments involved fungi in different stages of its life cycle. In our experiment, we exposed the termites to sugar cane baits with sporulating fungi, which could explain their higher mortality. The use of another sporulating fungus, isolated from previous assays, *L. brasiliensis*, allows us to state that the lower survival upon *Trichoderma* exposure was not due the presence of another organism (e.g. any fungus) in the experimental arena. Surprisingly, the survival of insects exposed to *L. brasiliensis* was higher than those exposed to no fungi. As the fungus is described as non-pathogenic (Schwartze et al., 2014), this exemplify that we still have an important gap on the microbial community and its ecological role associated to termites. The negative effect of *Trichoderma* sporulation on termites remain to be investigated. In the conditions here tested, we confirmed that *Trichoderma* can be antagonistic to the insects.

In summary, our results evidence the diversity of a genus of mycoparasite and plant symbiont fungi in a new environment, where multiple unknown microbial interactions are occurring (Aanen and Boomsma, 2005; Brune, 2014). At the colony level, we demonstrated a low prevalence of the fungus among insects and that the presence of *Trichoderma* in the termite nest wall is common. Since the fungus is in almost every substrate, this indicates that there are no specific strategies to avoid fungal spread to the nest. Similar prevalence in workers from old and recently built walls also suggests that there is no a group of workers specialized to spread the fungus in nest walls. Also, the lack of variation in worker fat content suggests that fungal mechanisms of exploitation in nest environment do not benefit termite nutrition directly. We demonstrated in our experimental arenas that termite survival was lower in presence of *T. harzianum*, a fungus commonly used in agriculture for biological control. Investigating how the metabolites naturally produced by the fungus is affecting termites could lead to a better understanding of the costs of this cosmopolitan fungus associated with insects. It is possible that by using soil for constructing their nest the insects simply cannot avoid the fungus to enter their colonies. If the sporulation of *Trichoderma* decreases insect survival, as suggested by Guswenrivo et al. (2018), it is plausible that termites, directly or indirectly, suppress the fungus growth and sporulation inside the nest. To conclude, our research indicates that *Trichoderm*a likely has no specific ecological role that benefits termites and might rather be a potential opportunistic parasite in this insect colonies.

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6. Supplementary material

Table S1. Isolates of the genus *Trichoderma* from this study

ISOLATE ID	NEST OF ORIGIN	SUBSTRATE	SPECIES
Vimi-17.0001	*Cornitermes cumulans* N17	Nest wall	*T. virens*
Vimi-17.0002	*Termes* sp. N1	Nest wall	*T. koningiopsis*
Vimi-17.0003	*Termes* sp. N1	Termite worker	*T. koningiopsis*
Vimi-17.0004	*Cornitermes cumulans* N20	Nest wall	*T. koningiopsis*
Vimi-17.0005	*Diversitermes* sp. N19	Nest wall	*T. koningiopsis*
Vimi-17.0008	*Cornitermes cumulans* N20	Nest wall	*T. koningiopsis*
Vimi-17.0009	*Termes* sp. N1	Nest wall	*T. koningiopsis*
Vimi-17.0011	*Cornitermes cumulans* N17	Termite worker	*T. koningiopsis*
Vimi-17.0014	*Cornitermes cumulans* N20	Nest wall	*T. koningiopsis*
Vimi-17.0015	*Cornitermes cumulans* N2	Nest wall	*T. koningiopsis*
Vimi-17.0016	*Cornitermes cumulans* N17	Nest wall	*T. koningiopsis*
Vimi-17.0017	*Cornitermes cumulans* N2	Nest wall	*T. virens*
Vimi-17.0018	*Cornitermes cumulans* N17	Nest wall	*T. koningiopsis*
Vimi-17.0020	*Diversitermes* sp. N18	Termite worker	*T. harzianum*
Vimi-17.0021	*Cornitermes cumulans* N20	Nest wall	*T. harzianum*
Vimi-17.0022	*Cornitermes cumulans* N17	Nest wall	*T. koningiopsis*
Vimi-17.0023	*Termes* sp. N1	Termite soldier	*T. atroviride*
Vimi-17.0024	*Termes* sp. N1	Termite soldier	*T. atroviride*
Vimi-17.0025	*Diversitermes* sp. N18	Nest wall	*T. harzianum*
Vimi-17.0026	*Cornitermes cumulans* N16	Termite soldier	*T. koningii*
Vimi-17.0027	*Termes* sp. N1	Termite soldier	*T. atroviride*
Vimi-17.0028	*Termes* sp. N1	Nest wall	*T. atroviride*
Vimi-17.0029	*Cornitermes cumulans* N17	Nest wall	*T. virens*
Vimi-17.0031	*Termes* sp. N1	Nest wall	*T. koningiopsis*
Vimi-17.0033	*Cornitermes cumulans* N16	Termite worker	*T. inhamatum*
Vimi-17.0034	*Cornitermes cumulans* N16	Nest wall	*T. harzianum*
Vimi-17.0035	*Termes* sp. N1	Nest wall	*T. koningiopsis*
Vimi-17.0036	*Cornitermes cumulans* N16	Nest wall	*T. harzianum*
Vimi-17.0037	*Cornitermes cumulans* N17	Nest wall	*T. harzianum*

Vimi-17.0038	*Cornitermes cumulans* N17	Nest wall	*T. koningiopsis*
Vimi-17.0039	*Cornitermes cumulans* N2	Nest wall	*T. harzianum*
Vimi-17.0040	*Termes* sp. N1	Nest wall	*T. koningiopsis*
Vimi-17.0041	*Cornitermes cumulans* N2	Nest wall	*T. atroviride*
Vimi-17.0042	*Cornitermes cumulans* N2	Termite worker	*T. harzianum*
Vimi-17.0043	*Cornitermes cumulans* N2	Nest wall	*T. harzianum*
Vimi-17.0045	*Cornitermes cumulans* N2	Nest wall	*T. harzianum*
Vimi-17.0046	*Cornitermes cumulans* N20	Termite worker	*T. harzianum*
Vimi-17.0047	*Cornitermes cumulans* N20	Termite worker	*T. harzianum*
Vimi-17.0048	*Cornitermes cumulans* N20	Nest wall	*T. harzianum*
Vimi-17.0049	*Diversitermes* sp. N7	Termite worker	*T. harzianum*
Vimi-17.0051	*Diversitermes* sp. N18	Nest wall	*T. ovalisporum*
Vimi-17.0052	*Cornitermes cumulans* N17	Termite worker	*T. koningiopsis*
Vimi-17.0053	*Cornitermes cumulans* N2	Nest wall	*T. virens*
Vimi-17.0054	*Diversitermes* sp. N7	Termite worker	*T. harzianum*
Vimi-17.0055	*Diversitermes* sp. N7	Termite worker	*T. harzianum*
Vimi-17.0058	*Cornitermes cumulans* N20	Termite soldier	*T. harzianum*
Vimi-17.0059	*Cornitermes cumulans* N20	Nest wall	*T. koningiopsis*
Vimi-17.0060	*Cornitermes cumulans* N20	Termite soldier	*T. lentiforme*
Vimi-17.0062	*Termes* sp. N1	Nest wall	*T. virens*
Vimi-17.0063	*Cornitermes cumulans* N2	Nest wall	*T. harzianum*
Vimi-17.0064	*Cornitermes cumulans* N16	Nest wall	*T. harzianum*
Vimi-17.0065	*Termes* sp. N1	Nest wall	*T. atroviride*
Vimi-17.0066	*Cornitermes cumulans* N20	Nest wall	*T. harzianum*
Vimi-17.0067	*Cornitermes cumulans* N17	Termite worker	*T. koningiopsis*
Vimi-17.0068	*Cornitermes cumulans* N2	Nest wall	*T. koningiopsis*
Vimi-17.0069	*Cornitermes cumulans* N20	Nest wall	*T. harzianum*
Vimi-17.0070	*Cornitermes cumulans* N17	Nest wall	*T. koningiopsis*
Vimi-17.0071	*Diversitermes* sp. N6	Nest wall	*T. harzianum*
Vimi-17.0073	*Diversitermes* sp. N6	Nest wall	*T. harzianum*
Vimi-17.0074	*Diversitermes* sp. N18	Nest wall	*T. harzianum*
Vimi-17.0083	*Cornitermes cumulans* N20	Termite soldier	*T. afroharzianum*
Vimi-17.0086	*Cornitermes cumulans* N17	Termite worker	*T. harzianum*

Vimi-17.0095	*Cornitermes cumulans* N16	Termite worker	*T. hamatum*
Vimi-17.0096	*Cornitermes cumulans* N17	Termite worker	*T. harzianum*
Vimi-17.0097	*Cornitermes cumulans* N16	Nest wall	*T. harzianum*
Vimi-17.0101	*Cornitermes cumulans* N16	Nest wall	*T. harzianum*
Vimi-17.0102	*Diversitermes* sp. N19	Nest wall	*T. subviride*
Vimi-17.0104	*Termes* sp. N1	Nest wall	*T. koningiopsis*
Vimi-17.0113	*Cornitermes cumulans* N2	Nest wall	*T. atroviride*
Vimi-17.0116	*Termes* sp. N1	Termite worker	*T. atroviride*
Vimi-17.0117	*Diversitermes* sp. N19	Nest wall	*T. harzianum*
Vimi-17.0119	*Cornitermes cumulans* N20	Nest wall	*T. strigosellum*
Vimi-17.0130	*Cornitermes cumulans* N17	Nest wall	*T. hamatum*
Vimi-17.0131	*Cornitermes cumulans* N2	Nest wall	*T. harzianum*
Vimi-17.0133	*Cornitermes cumulans* N2	Termite worker	*T. harzianum*
Vimi-17.0136	*Termes* sp. N1	Termite worker	*T. hamatum*

Table S2. *Trichoderma* strains used in phylogenetic analyses and their associated metadata. Species and strain ID in bold indicates type species.

SPECIES	STRAIN ID	GenBank NUMBER		REFERENCE
		TEF1	RPB2	
Protocrea pallida	CBS 299.78	EU703900	EU703947	Jaklitsch *et al.* (2008)
T. afroharzianum	**GJS 04-186**	FJ463301	FJ442691	Chaverri *et al.* (2015)
T. afroharzianum	LESF554	KT279017	KT278952	Montoya *et al.* (2016)
T. atroviride	8234	KJ634780	KJ634747	Zhu & Zhuang (2015)
T. atroviride	LESF118	KT278990	KT278922	Montoya *et al.* (2016)
T. atroviride	**CBS 142.95**	AF456891	EU341801	Degenkolb *et al.* (2008)
T. hamatum	LESF331	KT278993	KT278925	Montoya et *al.* (2016)
T. hamatum	GJS 04-325	EU856318	FJ150783	Samuels & Ismaiel (2009)
T. harzianum	CFAM-422	MG986725	MG986724	de Souza *et al.* (2018)
T. harzianum	LESF346	KT279015	KT278953	Montoya *et al.* (2016)
T. harzianum	LESF343	KT279021	KT278948	Montoya *et al.* (2016)
T. harzianum	DIS 217H	FJ463314	FJ442721	Chaverri *et al.* (2008)

				Unpublished
T. harzianum	**CBS 226.95**	AF348101	-	Samuels *et al.* (2002)
T. harzianum	**CBS 226.95**	-	AF545549	Chaverri *et al.* (2003)
T. inhamatum	**CBS 273.78**	AF348099	FJ442725	Chaverri *et al.* (2008) Unpublished
T. koningii	S22	KC285595	KC285749	Jaklitsch *et al.* (2013)
T. koningii	**CBS 457.96**	AF456909	FJ442761	Dodd *et al.* (2003)
T. koningiopsis	**CBS 119075**	DQ284966	EU241506	Hanada *et al.* (2008)
T. koningiopsis	7745	KJ634755	KJ634722	Zhu & Zhuang (2015)
T. koningiopsis	DAOM 230813	FJ463278	FJ442780	Chaverri *et al.* (2008) Unpublished
T. koningiopsis	DIS 374A	FJ463288	FJ442730	Chaverri *et al.* (2008) Unpublished
T. koningiopsis	GJS 04-199	FJ463268	FJ442789	Chaverri *et al.* (2008) Unpublished
T. koningiopsis	LESF149	KT278982	KT278915	Montoya *et al.* (2016)
T. koningiopsis	S359	KJ665546	KJ665285	Jaklitsch & Voglmayr (2015)
T. koningiopsis	LESF006	KT278983	KT278918	Montoya *et al.* (2016)
T. lentiforme	DIS 218E	FJ463310	FJ442793	Chaverri *et al.* (2008) Unpublished
T. lentiforme	DIS 246E	FJ851883	FJ442760	Chaverri *et al.* (2015)
T. lixii	C.P.K. 1934	FJ179573	FJ179608	Jaklitsch *et al.* (2008)
T. ovalisporum	GJS 04-113	FJ463281	FJ442781	Chaverri *et al.* (2008) Unpublished
T. ovalisporum	CBS 113300	AY376037	FJ442742	Holmes *et al.* (2004)
T. strigosellum	GJS 05-02	EU248631	EU248607	Hanada *et al.* (2008)
T. subviride	8878	KU529132	KU529143	Qin *et al.* (2016)
T. taiwanense	**GJS 95-93**	DQ284973	-	Samuels *et al.* (2008) Unpublished
T. taiwanense	CPK 416	-	JN715608	Jaklitsch *et al.* (2012)

T. virens	LESF514	KT279004	KT278935	Montoya *et al.* (2016)
T. virens	GLI 39	AY750891	AF545558	Chaverri *et al.* (2003)
T. viride	GJS 90-95	DQ307535	EU248596	Samuels *et al.* (2006)
T. viride	CBS 119327	DQ672617	EU711362	Jaklitsch *et al.* (2008)

Methods S1. Estimating nest volumes

To estimate the volume of nests of *Cornitermes cumulans* in section 2.5, we selected points in the nest that height and width changes. These points were considered the limit of cylinders to estimate the volume of each part of the nest. Each cylinder (c) had its height and width measured independently. The quantity of cylinders per nest varied according to the shape and size of the nest (Figure S1). The volume of each cylinder was calculated by the following equation:

$$Vc = \pi {r_c}^2 h_c$$

Where r_c is the largest radius at the base of the cylinder and h_c is the distance between the base and the top of the cylinder (Figure S1).

The top of the nest is similar to a half sphere (e), therefore we calculated the volume of the top using the volume of a half sphere. The volume of the half sphere is calculated by the following equation:

$$Ve = \frac{2\pi \ r_1 r_2 h_e}{3}$$

Where r_1 is the largest radius at the base of the half sphere, r_2 is the perpendicular radius to r_1, and h_e is the half sphere height, measured from the top of the last cylinder (Figure S1).

To calculate the volume of the entire nest, we added the volume of cylinders (calculated separately) and the volume of a half sphere using measurement from the top of the nest (Figure S1).

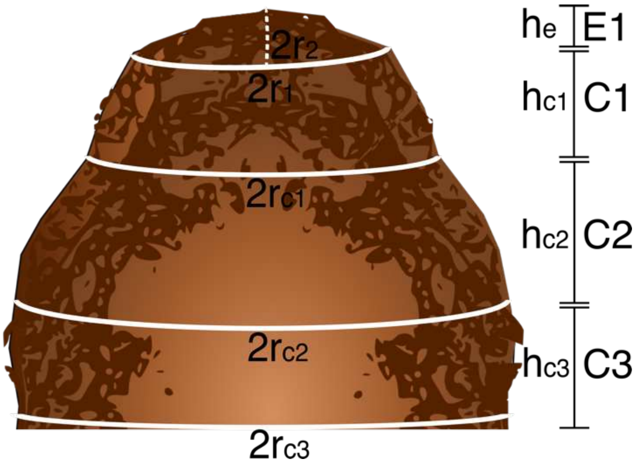

Figure S1. Diagram of the measurement of the termite nest volume
We included the points of measure to calculate the nest volume, according to its shape. C correspond to cylinders that we stipulated where the shape of nest present a visual change. For each cylinder, we measured the height (h) and radius (r). On the top of the nest we measured two perpendicular radii (r_1 and r_2) and the height, that we used to calculate half sphere (E) volume. Each nest total volume was estimated by the sum of cylinder and half sphere volumes.

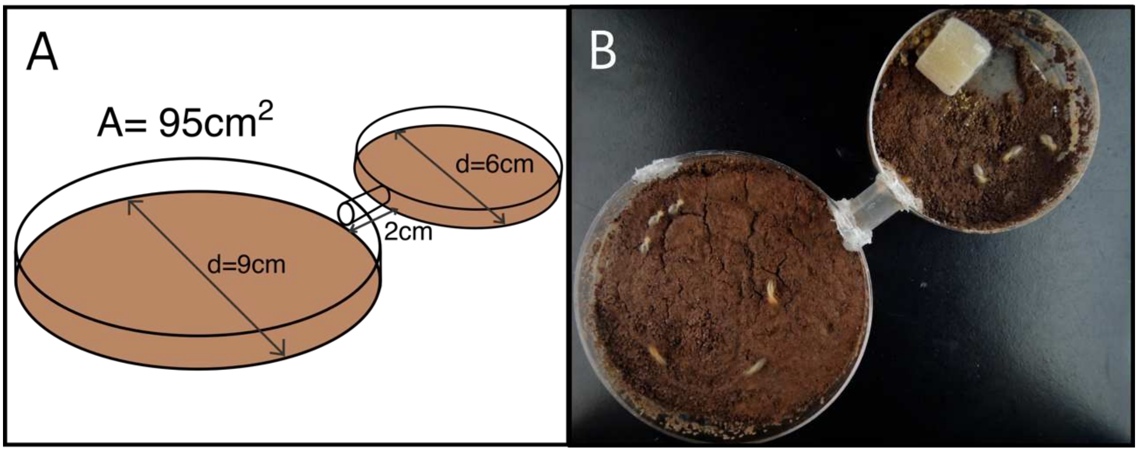

Figure S2. Arena set-up created for survival assays
We used 2 acrylic Petri dishes of 2 different sizes: 9 cm-diameter × 1,5 cm-height and 6 cm-diameter × 1,5 cm-height. We punctured a hole of 1 cm-diameter in the lateral of each plate and connected them with a 2 cm-cylinder. The arena was filled with 17 g of processed soil of termite nests before we added the termites. (A) is the representation of arena and (B) is one of the arenas used in the experiment.

Methods S2. Termite survival exposed to soil with different conditions

Prior to our main experiment, we tested whether the process of sterilizing the soil of termite nest affects termite survival. For this, we collected fragments of nest walls from two colonies of *Cornitermes cumulans,* macerated and sifted the fragments to homogenize the size of soil particles. We then sterilized half of the soil of each colony three times. Each process of sterilization was performed in intervals of 24 h, at 140º C for 20 min. Later, we inserted 12 g of processed soil in the larger plate and 5 g in the smaller plate of the arenas constructed for survival analysis (Figure S2). Thus, we had 5 arenas with sterile soil and 5 arenas with natural soil. We collected groups of 30 workers and 3 soldiers and placed these in each arena filled with soil from their respective nests. The 10 arenas were maintained in the dark and at 25º C during the experiment, except for periods of evaluation. Termite survival was evaluated once a day for 10 days. Dead termites, determined by lack of a movement reaction, were removed from the arenas in every evaluation. For the analysis, we performed a survival analysis with Weibull distribution. The colony of origin was included as a frailty factor. After 10 days, 192 of 330 insects had died, comprising 58.2% of the termites. In the end, 32.7% of termites exposed to sterile soil has died, in contrast to 25.5% of mortality of termites exposed to natural soil. Surprisingly, survival of termites exposed to sterile soil was lower than termites exposed to natural soil (P=0.005). Therefore, for our main experiment, we opted to use natural soil.

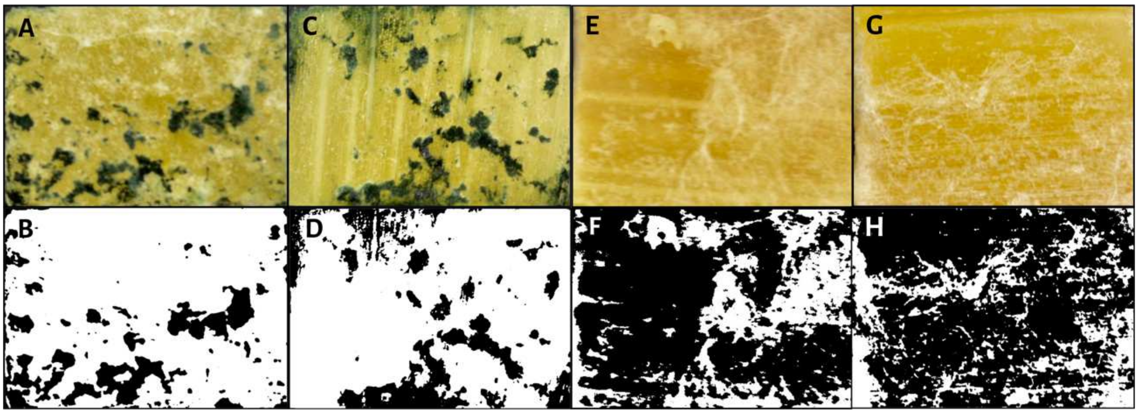

Figure S3. Estimating surface area covered by fungi

Original (A, C, E, G) and edited pictures (B, D, F, H) of sugar cane cubes inoculated with *Trichoderma harzianum* and *Lichtheimia brasiliensis.* We inoculated the fungi on sugar cane cubes of 1 cm^3. After 5 days, we estimated fungus growth calculating the proportional area covered by fungi in sugar cane for each treatment. To measure the surface area covered by fungi, we photographed the 6 faces of the sugar cane cube. Photographs were used to measure the contrast of fungal hyphae and sugar cane surface using Image J 1.X. The contrast of black and white was adjusted in each picture and the percentage of surface covered by fungi was calculated. *Trichoderma harzianum* hyphae is the green region (A and C) and black region (B and D). *Lichtheimia brasiliensis* hyphae is the grey region (E and G) and white region (F and H).

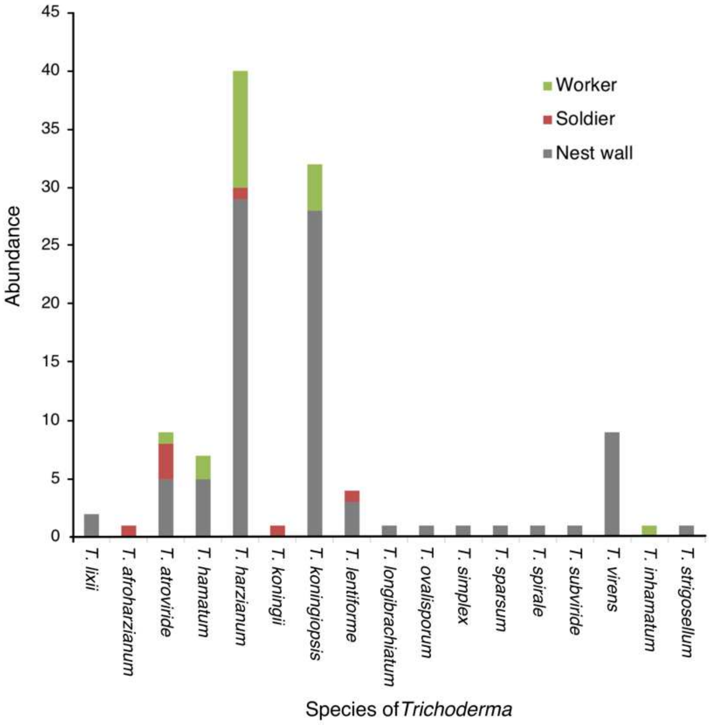

Figure S4. Distribution of the species of fungus *Trichoderma* according to substrate

The 116 strains of *Trichoderma* molecularly identified were isolated from substrate of termite nests: nest walls (grey bars), soldiers (red bars), and workers (green bars). There was no record of *Trichoderma* from immatures. We recorded 17 different species from 9 termite nests. *T. harzianum* was the most abundant species and nest walls was the substrate with the highest number of isolates of the genus.

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