Original Research Article

Screening of Fungi Isolated from the Brazilian Restinga for Insecticidal Activityt

ABSTRACT

Research on microorganisms for the control of pests and pathogens is growing. Such organisms display antagonistic effects on the pests and pathogens, at the same time, they do not interfere with the sustainable development processes and are environmentally safe for human populations. Thus, bioprospection of fungi from restinga ecosystems is of interest as a novel source of microorganism and a yet unexplored source of chemical structures. This study aimed selected endophytic fungi and fungi from the restinga soil samples to investigate their biological activity against insects. Fifty-three fungal isolates were used in screening bioassays against *Atta sexdens rubropilosa* leaf-cutting ant workers via direct contact of the insects with sporulating fungi cultures. This assay indicated that *Trichoderma* caused the highest mortality. Extracts from *Trichoderma* were then assessed for biological activity via ingestion, contact or exposure to fungal volatiles. Results showed that one *Trichoderma* sp. isolate (TR1) caused 50% mortality in 2, 1.5 and 4 days when ingested, sprayed onto the ants or the ants were exposed to volatiles, respectively. Although this fungus is not known to be entomopathogenic, it could have potential used as an additional tool for pest control as it produces metabolites with antagonistic effects.

Keywords: Biodiversity; toxicity; biological control; bioactive compounds.

1. INTRODUCTION

In general, pests pose constant threats due to their seasonality and outbreaks causing major losses and damage to agriculture and forestry. The use of “synthetic” insecticides, which are extremely toxic, has caused environmental pollution and indiscriminately affected human health. Economically important insect pests, both rural and urban, rapidly become resistant to such products. Therefore, interest in biological control methods has increased, as they represent a promising option in the search for molecules that are less aggressive to humans. From this perspective, our studies have focused in the search for fungi from the Brazilian restinga, a yet poorly studied ecosystem that is a possible source of novel biological control agents and molecules [1,2].

Several studies have highlighted out the high diversity of flora and fauna in restinga areas [3,4,5]. However, little is known about the presence of fungi associated to plant species from such ecosystems, let alone about the potential use of such organisms for biological control [6].

Restingas exhibit a diverse group of biological communities, reflecting the influence of soil conditions and the degree of exposure to the ocean breeze, radiation and salinity, among other factors [7]. In recent years, the LAQUIBIO team has explored this ecosystem’s fungal diversity particularly in northern Rio de Janeiro state [8,9]. Discovering new fungal species or

isolates is important in the tropics as they occupy differentiated ecological niches. Microorganism are important components of the local biodiversity, and they may also contribute to the production of drugs and other natural chemical substances or compounds that are less environmentally toxic, with potential as control agents for urban and agricultural pests, such as leaf-cutting ants.

Leaf-cutting ants are severe pests of agroforestry systems because they occur throughout the year and can cause major losses if not combated. The worker ants carry plant fragments into their underground colonies where they cultivate a mutualistic fungus *Leucoagaricus gongylophorus* (Möller) Singer from which they obtain nutrients [10].

The ants' habits make them, especially *Atta sexdens rubropilosa*, one of the most frequent pests attacking areas dedicated to agricultural, pasture and forestry activities in South, Central and North America [11]. Besides, leaf-cutting ants are considered the dominant herbivores in neotropics: they consume more vegetation than any other animal group such as mammals, homoptera and lepidoptera [12]. Plant damage due to leaf-cutting ant activity is caused by cutting of leaves, shoots, thin branches and flowers, and this leads to further plant damages, making the control of these ants such an important matter [13].

One way to control these ants is by intoxicating the workers by applications of insecticides mainly in the form of baits [14], because they are cheap, practical and easy to disperse. The most common active ingredient in baits is sulfluramid a compound originally stated to offer technological and environmental advances, although it is highly toxic. According to Brazilian Health Regulatory Agency (ANVISA), in compliance to the Stockholm Convention dealing with Persistent Organic pollutants, sulfluramid producers should have observed the date 09/09/2015 as a deadline for disposing of their previous stocks and for adapting their production lines to the new standards removing this active ingredients use thereafter [15]. However, sulfluramid is still being commercialized in Brazil.

Consequently, it is very important to develop alternatives for the control of leaf-cutting ants. Biological control has been studied considering the use of entomopathogenic fungi, which are capable of infecting and killing the ants [16], or via antagonistic action on the symbiont fungus (the ant's food source) [17]. However, ants have their own mechanical and chemical sanitation strategies, which are able to protect them and their symbiont against parasites and competing microorganisms [18].

Although the potential of synthetic and plant-derived products for ant control have been well explored, studies on fungal-produced compounds on leaf-cutting ants are scarce in the literature [19]. This is also true for *Trichoderma* spp., a fungal species that is widely known to produce bioactive secondary metabolites [20], and is frequently found in soils from temperate and tropical regions, and that can inclusively be found in the microbiota of ant colonys [21].

Some fungal isolates, including *Trichoderma*, were obtained from the restinga ecosystem leaf litter and soil samples in the region of northern Rio de Janeiro state, Brazil. The isolates are maintained in LAQUIBIO's (ISECENSA's Chemical and Biomolecules Laboratory) biological collection [9]. The availability of a high species diversity enables one to improve knowledge regarding insect-microorganism interactions, what includes knowledge on insect pest microbial control [22].

The objective of this study was to select and cultivate, in solid and liquid media, endophytic and soil-borne fungi from the restinga and screen these to select the most active isolates and test the extracts they produced against *Atta sexdens rubropilosa* worker ants as a model species.

2. MATERIAL AND METHODS

2.1 Tested Fungi, Cultivation and Inocula Preparation

All fungi tested in this research came from the LAQUIBIO/ISECENSA's Chemistry and Biomolecules Laboratory fungal collection (Table 1). Isolates derived from endophytic colonization and soil samples were collected in restinga ecosystems from Rio de Janeiro state's northern region, and identification was confirmed by morphological and/or molecular analyses.

Each fungus was cultivated on potato-dextrose-agar (PDA) and maintained via periodic transfer to fresh media throughout the whole test period.

Initial screening was performed on 53 fungi isolates aiming to observe any possible direct contact effects between fungal cultures and the ants, enabling selection of the most promising isolates regarding their effect in reducing ant survival rates.

2.2 Ant Collection and Laboratory Maintenance

The worker ants, displaying an average width of cephalic capsule of 2.9 mm, were obtained from active *Atta sexdens rubropilosa* nests foraging trails in the municipality of Campos dos Goytacazes, RJ. The ants were then kept in 250 mL plastic containers whose edges were impregnated with inert talc to avoid escape. After collection, they were taken to the laboratory (average temperature, 25 ºC ± 1 ºC; relative humidity, 60%). For experiments, ten workers were placed in each dish (cohort), and the ants were fed on 10% sucrose solution soaked in cotton wools. This diet was changed every two days and the effects of exposure to fungi on ants survival was observed through the bioassays.

2.3 Bioassays

2.3.1 Screening for lethal effects by contact with fungal colonies

Each of the 53 fungal colonies cultivated on PDA provided one test disc (3 cm in diameter). In order to promote direct contact between workers and fungi, those disks were placed at the bottom of each 250 mL container and ants were allowed to move freely within the container. In the negative control treatment, a PDA disc without fungus was placed at the bottom of the container. A positive control, was used which had discs of entomopathogenic fungi, *Beauveria bassiana* (Bals-Criv.) Vuill. (isolate LPP2) or *Metarhizium anisopliae* (Metschn.) Sorokīn., (isolate LEF 2000) These isolates were obtained from the Insect Pathology Sector fungal collection at UENF.

2.3.2 Bioassays for ant infection via contact with fungal spores

For this bioassay, a sterilized filter paper disc (7 cm in diameter) soaked in 2 mL of a spore suspension (107 conidia/mL) each with one of the isolates shown in Table 1 was used.

Suspensions were prepared from sporulating cultures in Petri dishes, washed with 10 mL sterile distilled water; spores were then brushed from the surface. After agitating in a vortex mixer for 30 s, the concentration of the suspensions was estimated using a Neubauer chamber. For the control treatment, only sterile distilled water was added to the filter paper.

Dead ants were removed daily during the bioassays (A and B). They were counted; then superficially disinfected in alcohol 70% (1 min.), sodium hypochlorite (1000 ppm for 1 min.), rinsed again in alcohol 70% (30 s), washed in sterile distilled water (1 min.) and individualized in Elisa plate wells (96 well plates). A sheet of filter paper soaked in sterile distilled water was placed between the wells and the lids in order to increase the humidity and induce conidiogenesis.

As conidia appeared on the cadavers, fungi were identified and compared to the inocula used in each treatment to indicate isolate recovery or pathogenicity.

2.3.3 Toxicity via ingestion of fungal extracts by worker ants

In order to assess the effects of fungal extracts on ants, a cotton pad was immersed in the extracts were mixed with a 10% sucrose solution and then placed in each dish. Extracts were prepared from 50 mL of potato-dextrose (PD) medium to which three discs from BDA cultivated fungal colonies had been added. Flasks were kept at 40 rpm agitation for 15 days. After this time, each extract was filtered using nitrocellulose membrane (0.22 µm) and the filtrate was used in the tests.

For the *Trichoderma*, the toxicity assays, worker ants were divided in groups of 10 individuals each and exposed to different *Trichoderma* undiluted extracts; mortality percentages were determined every 24 hours for 7 days. Each assay was performed three times. For control tests, ants were fed 10% sucrose solution soaked in a cotton pad. This diet was replaced evey two days. The assessment methodology was repeated in the following assays.

2.3.4 Contact toxicity of fungal extracts

For each replicate, ten ants were sprayed with 3 mL extract using a manual hand sprayer. Control treatment insects were sprayed with sterile distilled water. Mortality was evaluated every 24 hours for 7 days. Experimental treatments and controls were performed three times each.

2.3.5 Toxicity via exposure to volatile compounds from fungal extracts

Five mL of each fungal extract was applied to a cotton pad placed inside a small container with a perforated lid. The container was, then, placed inside a larger container so that the worker ants did not have direct contact with the liquid extract, but only with the volatile compounds produced by the extract therein. In the control treatment, sterile distilled water was used. For 10 days, at 24-hour intervals, the number of dead ants was verified, and the diet was replaced every two days.

2.4 Experimental Design and Statistical Analyses

We employed the total randomized experimental design for all assays. Treatments were fungi isolates (from culture dishes, spore suspensions, liquid extracts or volatile compound extracts); 3 repetitions with 10 ants each with a total of 30 individuals per treatment.

Evaluations were performed every 24 hours and dead ants were removed every day up to 10 days. Survival data was collected and survival curves were generated by the Kaplan-Meier method [23]. Curves were compared by the Log-Rank test [24,25], with P=0.05 used to verify significant differences between treatments. Analysis of variance and Tukey multiple comparison tests were also performed. We also assessed an LT_{50} (Lethal Time for 50% of

tested individuals) estimate and made trend adjustments using the polynomial model. All analyses were performed with the aid of GraphPad Prism 5.0 and Excel software.

3. RESULTS AND DISCUSSION

Endophythic fungi are microorganisms that live within plants without harming their hosts and may constitute an alternative source of secondary metabolites. Similarly, soil-borne fungi, particularly from uninhabited areas such as restingas in northern Rio de Janeiro state, are also capable of producing structurally heterogeneous molecules that exhibit low molecular mass, with potential as bioactive compounds [26] and as potential biological control agents [27]. Table 1 shows the host plants, soils and substrates of fungi which could produce secondary metabolites that were tested against *Atta sexdens rubropilosa* leaf-cutting ants.

3.1 Screening for Contact Effect when Exposing Ants to Fungal Colonies

Ants were directly exposed to 53 fungal isolates (screening) either by walking on the colony surfaces or, in some cases, by cutting and ingesting disc fragments, both of which caused mortality. This fact can be attributed to metabolites produced by the fungi in culture media [20]. Ants that fed on *Trichoderma* fungal colonies displayed the highest levels of mortality. From this result we decided to use this fungus for the production of the extracts used in the subsequent assays. Recently, *Trichoderma* has been tested as an antagonist of the leaf-cutting ants symbiotic fungus. This fungus also produces substances that display potential inhibitory activity of the ants normal immune responses [28].

The so called positive control treatments, using the entomopathogenic fungi *B. bassiana* and *M. anisopliae*, caused mortality rates of 97% to 100%, respectively on the fifth day of evaluation. This time range was inferior to the one reported by [29], who have estimated average lethal time of *B. bassiana* against *Atta* between four and five days, but *M. anisopliae* when tested against *Acromyrmex* killed 100% in around six days when inoculated by spores suspension.

Table 1. Endophytic and soil-borne fungi from the Restinga used for in vitro assessments against *Atta sexdens rubropilosa* leaf-cutting worker ants

Fungus	Isolate Codes	Origin: Host Plant/Soil/Substrate	
		Common Name	Scientific Name
Acremonium sp.	ACR1	Abaneiro	*Clusia hilariana*
	ACR2	Trapoeraba	*Commelina erecta*
	ACR3	Pinheirinho-da-praia	*Remirea maritima*
	ACR5	Cambucazinho	*Eugenia punicifolia*
	ACR6	Massaranduba	*Manilkara subsericea*
	ACR8	Bolo	*Coccoloba alnifolia*
	ACR9	Pêro	*Psidium cattleyanum*
	ACR10	Malva branca	*Sida cordifolia*
	ACR11	Rabo-de-macaco	*Machaerium lanceolatum*
	ACR12	Vassoura	-
Pestalotiopsis sp.	PES1	Gurirí	*Allagoptera arenaria*
	PES2	Fungo liquenizado	-
	PES3	Feijão-de-porco	*Canavalia rosea*
	PES4	Arco-de-pipa	*Erythroxylum ovalifolium*
	PES5	Capororoca-de-folha-larga	*Myrsine umbellata*
	PES6	Bolo	*Coccoloba alnifolia*

	PES7	Almescla	*Protium heptaphyllum*
	PES8	Bolo	*Coccoloba alnifolia*
	PES9	Pitanga	*Eugenia uniflora*
	PES10	Cipó-sangue	*Paullinia weinmanniifolia*
	PES11	Salsa-da-praia	*Ipomoea imperati*
	PES12	Cambucazinho	*Eugenia punicifolia*
	PES13	Fruto-de-guaxo	*Cupania emarginata*
	PES14	Papagaio	*Maytenus obtusifolia*
	PES15	Jenipabinho	*Tocoyena bullata*
	PES16	Massaranduba	*Manilkara subsericea*
	PES17	Murici	*Byrsonima sericea*
	PES18	Cipó-sangue	*Paullinia weinmanniifolia*
	PES19	Capororoca-do-brejo	*Myrsine rubra*
	PES20	Juramento	*Cynophalla flexuosa*
Trichoderma sp.	TR1	Soil	
	TR2	Soil	
	TR3	Soil	
	TR4	Soil	
	TR5	Soil	
	TR6	Soil	
	TR7	Basidimycota (Mushroom)	
	TR8	Basidimycota (Mushroom)	
	TR10	Decomposing leaves on soil surface	
	TR11	Soil	
Alternaria sp.	5B1.1	Juramento	*Capparis flexuosa*
Alternaria sp.	61 A	Batateira-da-praia	*Ipomoea pes-caprae*
Cladosporium perangustum	38C2	Cipo de são joão	*Pyrostegia venusta*
Colletotrichum brevisporum	24B2	-	*Passiflora* sp.
Diaporthe perseae	35C1	Rabo-de-macaco	*Machaerium lanceolatum*
Lasiodiplodia theobromae	5 A2	Juramento	*Capparis flexuosa*
Pestalotiopsis protearum	17B3	Aroeira	*Schinus terebinthifolius*
Phomopsis phyllanthicola	34C1	Calombo	*Pera glabrata*
Rhinocladiella similis	8B2	Aroeira	*Schinus terebinthifolius*
Setosphaeria rostrata	10B1	Calombo	*Pera glabrata*
Stenella musae	20B	Mololô	*Annona glabra*
Talaromyces verruculosus	15 A2	Dormideira	*Mimosa pellita*
Trichoderma atroviridae	7 A2 (TR9)	Cipó-sangue	*Paullinia weinmanniifolia*

3.2 Effects of Contact Between Ants and Fungal Spores

When ants were inoculated with fungi spores, no direct effect on their mortality was observed until 10 days after inoculation. Mortality occurred, but it was probably due to normal causes under the current experimental conditions, and not by infection [30].

Only nine of the 53 inoculated fungi were recovered from superficially disinfected corpses placed in the humidity chambers (Table 2). Among the 20 isolates tested, no *Acremonium* were re-isolated and just one *Pestalotiopsis* isolate was re-isolated.

Despite fungal proliferation on the ants corpses, death could not be exclusively attributed to the re-isolated fungi because we cannot discount the fact that the ants might have died by other reasons, like stress or as a consequence of their manipulation in experimental conditions [31]; therefore, caution is needed when attributing pathogenicity.

Trichoderma exhibited the highest correlation between fungal inoculation and re-isolation: of eleven isolates were used, seven were recovered from dead ants. As endophytic, saprophytic or soil-borne fungi, *Trichoderma* might have developed within the ants, before or after their deaths, without provoking an immune response [32]. Such internal growth can be seen in plants when fungi are termed endophytes to indicate that they can grow within the

plants apparently without harming them. This association seems to be intimate enough not to activate the plants' enzymatic defense arsenal: becoming an ecological niche for the fungi, which is beneficial for the host plant and the fungus.

[33] used *Trichoderma longibrachiatum* spore suspensions to control *Leucinodes orbonalis*, one of the major pests of aubergine. This treatment showed similar efficacy to that of the pesticide malathion in the field trials. [10] showed that *Trichoderma harzianum* was a pathogenic agent of *Atta laevigata* workers, based on detection of mycelial growth and conidiogenesis of this fungus on inoculated ant corpses. *Trichoderma* has also been described in natural environments associated with ants, for example as, endophytes of plants which the ants cut and carry into their colonies [34,35]

Even if *Trichoderma* is not directly pathogenic to the ants, it can be used as an additional tool for ant control as it can act as an antagonist of the symbiontic fungus which the ants cultivate and feed on [36]. Notwithstanding the production of metabolites that adversely act on ant colonies.

In a general, when we consider all isolates from the different species we assessed (Table 2), the low fungal recovery rate might indicate that:

a) most of them did not behave as entomopathogenic agents in the infection process and were eliminated during superficial disinfection of cadavers;

b) cadaver disinfection was efficient in eliminating epicuticular fungi, meaning that only the fungi present within the ants were detected;

c) inoculation without addition of surfactants may not have been efficient in breaking the superficial tension to ensure spores adhesion, germination and posterior ant infection.

Beauveria bassiana occurred spontaneously in three ants inoculated with *Phomopsis phyllanthicola* and *Alternaria* sp. It is possible that these ants were carrying these fungi infection since they had been collected in the field, indicating the natural occurrence of this entomopathogen. Cadavers also exhibited other fungal species that had not been inoculated, such as *Aspergillus* sp., *Penicillium* sp. e *Paecilomyces* sp., which have previously been reported as entomopathogenic agents [37,16,10,38]. It is likely that the ants had already been contaminated with these fungi before being collected in the field and used in bioassays.

Table 2. Endophytic and soil-borne fungi from the restinga tested against leaf-cutting ants *Atta sexdens rubropilosa* by inoculation and incidence of re-isolated of these fungi from ant cadavers

Inoculated Fungus	Isolate Codes	Number Recovered Fungi*/Ant
Acremonium sp.	ACR1	1 *Fusarium* sp.
	ACR2	1 *Fusarium* sp.
	ACR3	1 *Fusarium* sp1; 1 *Fusarium* sp2
	ACR5	1 *Fusarium* sp.
	ACR6	1 *Fusarium* sp1; 1 *Fusarium* sp2
	ACR8	1 *Fusarium*; 1 *Penicillium* sp.; 1 non sporulated
	ACR9	1 *Fusarium* sp1; 1 *Fusarium* sp2
	ACR10	1 *Fusarium* sp.
	ACR11	1 *Fusarium* sp.
	ACR12	1 *Fusarium* sp.

Pestalotiopsis sp.	117D3	1 *Pestalotiopsis* sp.
	109D4	1 *Fusarium* sp.
	116D1	1 *Fusarium* sp.
Trichoderma sp.	TR1	1 *Fusarium*; 1 *Trichoderma* sp.
	TR2	1 *Fusarium* sp1; 1 *Fusarium* sp2
	TR3	1 *Trichoderma* sp.; 1 *Fusarium* sp1; 1 *Fusarium* sp2
	TR4	1 *Fusarium* sp1; 1 *Fusarium* sp2; 1 *Fusarium* sp3
	TR5	1 *Trichoderma* sp.
	TR7	1 *Trichoderma* sp.
	TR8	1 *Trichoderma* sp.
	TR9	1 *Paecilomyces* sp.
	TR10	1 *Trichoderma* sp.
	TR11	1 *Trichoderma* sp.; 1 *Fusarium* sp.
Diaporthe perseae	35C1	1 *Fusarium* sp.
Phomopsis phyllanthicola	34C1	1 *Beauveria* sp1; 1 *Beauveria* sp2
Pestalotiopsis protearum	17B3	1 *Penicillium* sp.
Cladosporium perangustum	38C2	1 não esporulado
Lasiodiplodia theobromae	5 A2	1 *Aspergillus* sp; 1 non sporulated
Trichoderma atroviridae	7 A2	1 *Fusarium* sp.
Rhinocladiella similis	8B2	1 *Fusarium* sp.
Alternaria sp.	5B1.1	1 *Fusarium* sp.; 1 *Beauveria* sp.
Alternaria sp.	61 A	1 *Fusarium* sp.
Metarhizium anisopliae	Positive control	1 *M. anisopliae*; 1 *Fusarium* sp1; 1 *Fusarium* sp2
Beauveria bassiana	Positive control	1 *B. bassiana*

** Number and species of fungi recovered from approximately 30 cadavers of ants/inoculated isolate.*

It is important to point out that more than 30 *Fusarium* sp. isolates (Table 2) occurred in ants treated with different inoculated fungal spore suspensions. [30] worked on fungi prospection for leaf-cutting ant biological control and analyzed 88 fungi isolated from *A. sexdens rubropilosa* queens. Among those, 43 isolates were identified as *Fusarium*, making this genus the most represented within these samples.

It is likely that *Fusarium* infections occur in natural conditions in the field, indicating this fungus has a high affinity with leaf-cutting ants. Several *Fusarium* species have exhibited entomopathogenic activities and also been considered as promising organisms for the biological control of insects [39] or the ants symbiotic fungus, since some *Fusarium* species have already been described as potential antagonistic agents of fungi [40].

Thirty ants were inoculated separately with either *B. bassiana* or *M. anisopliae* spores. Only one treated ant for each of the two fungi died and exhibited posterior external colonization. This could be explained by the fact that individuals from different casts are more susceptible than others [41,42]. Besides, there is high genetic variability between entomopathogenic fungal isolates, resulting in different pathogenicity and levels of virulence [43]. Pathogenicity tests of entomopathogenic fungi in insects can vary greatly even in relation to the two most studied species [44,45,46]. [47] studied 72 *B. bassiana* and *M. anisopliae* isolates and reported that the mortality they caused varied greatly in stored grains pests, some were completely inefficient and others caused 100% mortality, hence the importance of studies to select new isolates or promising species for the control of serious pests such as leaf-cutting ants.

3.3 Effect of Ingestion or Contact with *Trichoderma* Extracts and Effects of Volatile Compounds Produced by this Fungus

In the previous bioassay, involving direct contact between ants and fungal cultures, worker survival was only affected by *Trichoderma* after the ants had fed on fungal cultures. Thus, as consequence, only isolates from this genus were considered for the production of extracts to be used in subsequent assays.

Eleven *Trichoderma* isolates were assessed for their toxic effects via ingestion on ant survival. All of them exhibited deleterious effects when compared to the controls (Fig. 1), whose equations are shown in Table 3.

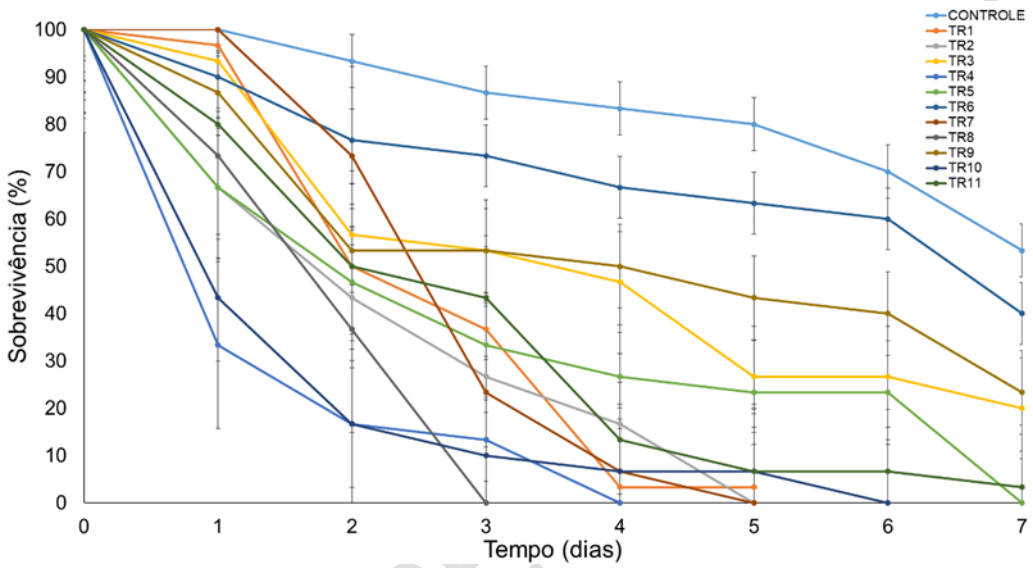

Fig. 1. Survival curves of *Atta sexdens rubropilosa* ants fed with fungal extracts from 11 *Trichoderma* (TR) isolates. Control: sucrose solution.

Only four of the isolates were selected due to their high levels of toxicity, causing 93 to 100% mortality of workers during the first four days of the assays.

Based on these results, extracts of isolates TR1, TR4, TR7 and TR10 were used in new tests to assess toxicity via ingestion, contact and exposure to volatile compounds.

Ingestion of extracts accelerated ant death in comparison to controls. The estimated 100% lethal time was 02 days for isolates TR1, TR2 and TR10; 04 days for isolate TR7; and 10 days for control treatment. Variation in extract toxicity could be related to their chemical composition, since *Trichoderma* is capable of producing several compounds such as: dehydroacetic acid, anhydromevalonolactone, 2-phenylethane, polyketides, peptaibols, terpenoids/steroids, harzianic acid, alamethicins, anthraquinones, azafilones, daucanes, harzialactones, bisorbicillinoides, butenolides, tricholine, glisoprenins, heptelidic acid, gliovirin, pyrons, trichothecenes, isocyanates, trichosetin, viridine among other compounds [48,49,50,51].

TABLE 3. Regression analysis for mortality following ingestion of *Trichoderma* extracts by *Atta sexdens rubropilosa* showing equations and determination coefficients

Treatment	Polynomial equation	Determination coefficient (R^2)
Controle	y = - 0,754x2 - 0,9127x + 99,722	0,9712
TR1	y = 1,25x2 - 28,44x + 107,98	0,9436
TR2	y = 2,4405x2 - 31,25x + 97,976	0,9935
TR3	y = 1,25x2 - 20,536x + 102,92	0,9578
TR4	y = 8,5714x2 - 56,286x + 93,81	0,9418
TR5	y = 1,6667x2 - 23,492x + 93,056	0,9416
TR6	y = 0,0595x2 - 7,7579x + 97,361	0,9439
TR7	y = 0,119x2 - 24,31x + 110,24	0,9257
TR8	y = - 2,5x2 - 26,167x + 100,5	0,9991
TR9	y = 1,0913x2 - 17,202x + 97,361	0,9165
TR10	y = 4,6429x2 - 41,548x + 90,476	0,9358
TR11	y = 2,123x2 - 29,187x + 102,92	0,9814

Several reports describe the use of *Trichoderma* bioactive compounds for control of phytopathogenic fungi such as *Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, *Colletotrichum gloesporioides*, *Verticillium dahliae*, *Fusarium oxysporum* and *Cylindrocladium* sp. [52]; against the *Toxoplasma gondii* parasite [53]; allelopathic effects on cultivated plants and weeds [50]; and antagonistic effects on bacteria such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Rhodotorula* sp. and *Candida* spp. [54]. However, despite the importance and capacity of *Trichoderma* as a bioactive compound producer, literature is scarce when it comes to use of these compounds for insect-pest control.

[55] studied two strains of *Trichoderma harzianum* that produced a chitinase and basic proteinase which may play a key role in entomopathogenicity action against *Tenebrio molitor* larvae or when applied to the cuticle together with a serine protease. These results suggested that the virulence factors involved in *T. molitor* biocontrol are the same as those for insect pathogenicity. This may affect the use of *Trichoderma* spp. for biocontrol as there may be effects on non-target insect species.

[56] showed that *Trichoderma asperellum* could be used against *Anopheles mosquitoes*, vectors of malaria. They investigated the efficacy of crude methanolic extracts and diferent methanolic fractions of the fungal extracts against anopheline larvae e concluded that the crude methanolic extract has new applications for the control of *Anopheles* spp.

When extracts were pulverized onto the ants, the survival rate was reduced in comparison to controls (Fig. 2B). Average survival was 1.5 days for ants treated with extracts from isolate TR1; 02 days for extracts from isolates TR7 and TR10; 05 days for TR4. Control mean survival rates were 7 days. These lethal time results can be compared to those reported by [57] pointed to a lethal time (LT_{50}) of 2 days for topical application of crude cashew (*Anacardium occidentale* L.) nut oil against leaf-cutting ants. Thus, it suggests that the results for *Trichoderma* extracts are promising for the prospection of natural compounds for biological control.

When using *Trichoderma* metabolites as baits, the ideal procedure would be firstly to test a fungal extract displaying slower mortality effects [58], as it is desirable that the active ingredient can be picked up and spread to other ants in the colony, including the queen. Consequently, individual assessment of the extract effects is essential for determining their best application.

When assessing the volatile compound effects on ants, extracts TR1, TR7 and TR10 displayed statistically different results from control treatments. However, isolate TR4 was similar to controls (Fig. 2C). Average survival was 4 days for ants submitted to volatile compounds from extracts TR1 and TR7; 5 days for TR10; and 6.5 and 7 days, respectively, for control treatment and extract TR4.

[59] considered some *Trichoderma* isolates as possible candidates for leaf-cutting ant control when he detected the production of volatile organic compounds and their negative effects on ants when interacting with the mutualistic fungus.

According to [60], *Trichoderma* produces a wide variety of volatile compounds such as ethylene, ketones and aldehydes. Studies of these compounds, besides exhibiting antifungal characteristics, may also display insecticide action, yet this is an area which has been little explored. One of these studies has pointed out that *Trichoderma* produced a wide range of volatile compounds [59] and the effects of such compounds could be variable when confronting *Trichoderma* and the ants mutualistic fungus, without considering the direct action of the volatiles on the insects which has not been verified.

Where leaf-cutting ants are concerned, most studies that describe *Trichoderma* as being active against these insects are based either on spores suspension inoculation or action against the fungal symbiont. Even if no fungal direct effect on ants can be determined, indirect effects may occur, such as from chitin-degrading enzymes production [61] or the

production of compounds that would negatively interfere with ants or the normal development of the colony.

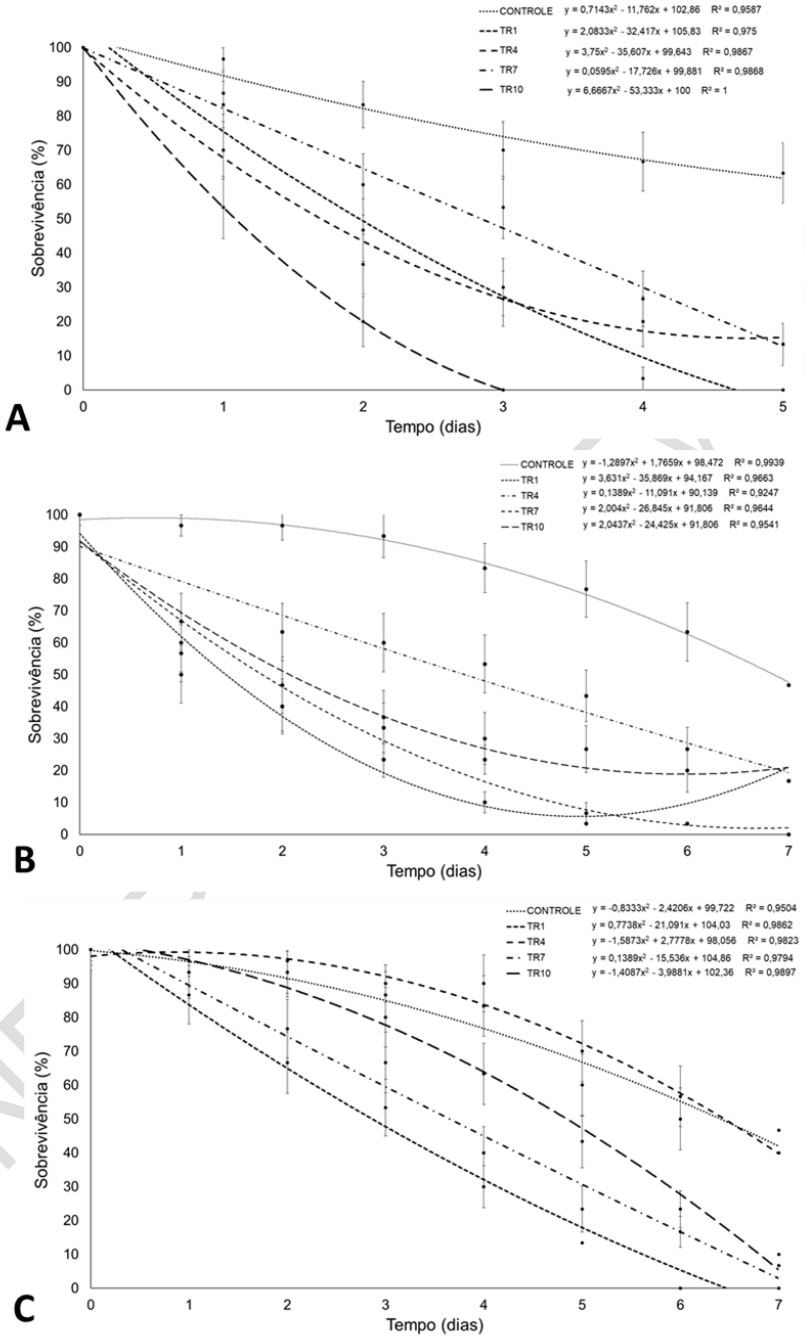

Fig. 2. *Atta sexdens rubropilosa* workers survival curves following ingestion (A), contact (B) and exposure to volatile compounds (C) effects of extracts of four *Trichoderma* (TR) fungal isolates. *P*-value for the Log-rank test was <0.001.

4. CONCLUSION

Extracts from *Trichoderma* fungal isolates (TR1, TR4, TR7 and TR10) caused high levels of mortality of leaf-cutting ant workers and could be used in further research aiming to gain a greater understanding of promising active ingredients. Despite the fact that this fungus does not share any co-evolutionary scenario with leaf-cutting ants, it did exert deleterious action against workers.

Further research will be conducted on these *Trichoderma* isolates to compare their antagonism by means of direct action and to verify whether the metabolites affect *L. gongylophorus* as well as *Atta* mini-colonies, as these environments allow workers to interact more naturally with their colony nest-mates and with the symbiotic fungus, enabling the observation of natural environmental responses of the ant-fungus interactions. *Trichoderma* is likely to be useful in the future as a new tool for leaf-cutting ants control, maybe through entomopathogenic fungi synergism, or maybe through its own potential action on the symbiotic fungus. Such interaction might become an advantage to help us overcome the complex defense system of leaf-cutting ants: a system for which a single biological control agent alone, with a narrow action range, would have less of success.

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