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Biocontrol of *Leucoagaricus gongylophorus* of leaf-cutting ants with the mycoparasitic agent *Trichoderma koningiopsis*

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Abstract

Leaf-cutting ants are one of the main agricultural and agroforestry pests in the Neotropic region. The essential food source of these ants is *Leucoagaricus gongylophorus*. Therefore one of the main biocontrol agents under study are *Trichoderma* species, because of their biocontrol activity against a diverse range of fungi. Here, *Trichoderma koningiopsis*, isolated from a leaf-cutting ants nest was tested against three *Leucoagaricus gongylophorus* strains from leaf-cutting ants by dual culture technique under laboratory conditions. The molecular analysis of ITS sequence data showed three well-separated main clades in which the isolated *Trichoderma* strain was assembled as a sole subclade among *T. koningiopsis* strains. The tests also showed that *T. koningiopsis* strain inhibited the growth of all *L. gongylophorus* strains tested. The values of radial inhibition of *L. gongylophorus* ranged from 58% to 69% with an average mean value of 65%. This is the first report on a strain of *T. koningiopsis* isolated from a naturally parasitized nest of leaf-cutting ants with biocontrol ability over *L. gongylophorus* tested in dual culturing method.

Key words – biological control – leaf-cutting ants – *Leucoagaricus gongylophorus* – *Trichoderma koningiopsis*.

Introduction

In the New World, leaf-cutting ants are dominant herbivores and major agricultural and agroforestry pests (Folgarait et al. 2011, Napal et al. 2015). Furthermore, the economic damage in the neotropic agricultural industry caused by *Atta* and *Acromyrmex* genera of ants is enormous and these ants are among the most serious agricultural pests of tropical and subtropical America (Silva et al. 2006, Della Lucia et al. 2014).

Ants of the *Attini* tribe live in obligate symbiosis with a basidiomycete fungus of the *Agaricaceae* family which is an essential food source for the larvae and queen (Fig. 1) (Fisher et al. 1994, Currie 2001, Miyashira et al. 2010). The fungal symbiont of the leaf-cutting ants is the basidiomycete *Leucoagaricus gongylophorus* (Fisher et al. 1994, Silva et al. 2006, Miyashira et al. 2010, Napal et al. 2015, Bich et al. 2016).

Despite their low specificity, high toxicity, adverse effects to the environment and development of resistant insect populations, chemical insecticides have been used traditionally for

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Fig. 1- *Leucoagaricus gongylophorus* grown in a fungal garden of leaf-cutting ants

leaf-cutting ants control (Lopez & Orduz 2003). Consequently, there is need for new and alternative control methods that can provide more sustainable and efficient control of leaf-cutting ant colonies (Folgarait et al. 2011, Napal et al. 2015). Alternatives to chemical insecticides are being researched and developed (Lopez & Orduz 2003, Folgarait 2013). Consequently application of chemical insecticides is being replaced or complemented by biocontrol agents because of the emergence of insecticide-resistant populations and public concerns regarding the health and environmental impacts of these chemicals.

During the last few decades, several potential biocontrol organisms have been isolated, characterized and commercialized, and thus, biocontrol of insects in forestry has received more attention (Shali et al. 2010, Folgarait et al. 2011). The genus *Trichoderma* was widely studied due to its rapid growth, capability of utilizing diverse substrates, and resistance to diverse chemicals (Kamala et al. 2015). Furthermore, *Trichoderma* species have been investigated as biological control agents (BCAs) and growth promoter agents of many crops (Hermosa et al. 2000, Howell 2003, Kubicek et al. 2008, Savazzini et al. 2009), but it is only recently that these strains have become commercially available. The competition with pathogens, parasitism and the production of antifungal compounds are the most important mechanisms in biocontrol activity of *Trichoderma* (Savazzini et al. 2009, Yao et al. 2016). Usually *Trichoderma* strains have adaptation capacities in different types of soil and persist at detectable levels for months.

Strains of *Trichoderma* spp. can vary in their biocontrol activity from pathogen to pathogen, so each *Trichoderma* strain must be assayed against the target fungal species (Rojan et al. 2010). The knowledge concerning the behavior of these fungi as antagonists is essential for their effective usage in agronomy and forestry since they act against target organisms in different ways. The commercial usage of *Trichoderma* as BCAs must be then preceded by precise identification, adequate formulation and studies about the synergistic effects of the biocontrol mechanisms (Hermosa et al. 2000). Accurate species identification of *Trichoderma* based on morphology is very difficult and even ambiguous because of similarity of morphological characters and increasing numbers of morphologically cryptic species (Druzhinina et al. 2005, Samuels et al. 2010). The increase in the number of isolates, high phenotypic variability and similarity with *Hypocrea* and *Gliocladium* can complicate the identification of *Trichoderma* spp. through classic taxonomy (Hernández et al. 2015). These features have already led to misidentifications (Kubicek et al. 2008).

There is strong evidence that molecular methods and identification tools based on sequence analysis of genes enable the identification of every *Trichoderma* isolate (Druzhinina et al. 2005,

Kubicek et al. 2008). To date there is limited knowledge about molecular identification and evaluation as BCA of newly isolated *Trichoderma* strains from Argentina. There are few studies of dual culture methods of *Trichoderma* against *Leucoagaricus gongylophorus* strains from leaf-cutting ants.

The development of new biocontrol products against plant pests requires screening of candidate antagonists which have to fulfill many requirements to be commercially successful (Kohl et al. 2011). In the present study, molecular identification and potential use of a newly isolated *Trichoderma koningiopsis* as BCA was tested *in-vitro* for the biocontrol of *Leucoagaricus gongylophorus* strains from leaf-cutting ant.

Materials & Methods

Microorganisms used

Leaf-cutting ant nests were sampled from Misiones Province (Argentina). The nests were mechanically opened and a portion of the inner fungal garden was placed in a sterile plastic plate and taken to the laboratory. A small portion of a fungal garden naturally parasitized by *Trichoderma* was subcultured in 3.9% Potato Dextrose Agar (PDA) (Britania). The agar plates were incubated for 3 to 5 days in dark at 28 ±1 °C to enable the development of *Trichoderma*. Only one *Trichoderma* strain was isolated from the inner fungal garden. The fungus was first isolated by subculturing in PDA medium and then stored at 4 °C in the biotechnology strain culture collection of the Misiones Biotechnology Institute (INBIOMIS – Misiones National University). Conidiophore structures and morphology were examined using a trinocular microscope (Barnett & Hunter, 1998, Samuels et al. 2006). Three strains of *Leucoagaricus gongylophorus*, previously isolated by our group were tested; two of these strains (HEP4 and HEP12) were isolated from *Acromyrmex pubescens* nests and one strain (HEP20) was isolated from *Atta laevigata* nest. From each leaf-cutting ant nest small portions of fungal garden were subcultured in 3.9% PDA (Britania). The agar plates were incubated as cited previously. The HEP4, HEP12 and HEP20 *L. gongylophorus* strains were also stored at 4 °C in the biotechnology strain culture collection of the Misiones Biotechnology Institute.

Fungal molecular identification

Mycelia for DNA extraction were grown in liquid cultures at 28 ±1 C in malt extract broth (MEB) for 3-5 days in the dark. Hyphae were collected on filter paper in a Buchner funnel, washed with distilled water and the extraction of genomic DNA was performed with minor modification as described by Hermosa et al. (2000). DNA was resuspended in 30 µl of sterile distilled free of DNAse water (Biopak®). DNA extracted was further examined by electrophoresis in 1 % w/v agarose gels (InBio, Argentina) and stained with Gel Red Solution (Biotium, 10000X). The final DNA elution was then quantified spectrophotometrically. Primers ITS1 and ITS4 were used to amplify a fragment of rDNA including ITS1 and ITS2 regions and the 5.8S and partial 18S and 28S rDNA genes (White et al. 1990). PCR amplifications were performed in a total volume of 20 µl with approximately 10 µg of genomic DNA using 1 unit of Taq DNA polymerase (InBio, Argentina) per reaction. Each 20-µ1 reaction consisted of 1 X buffer (provided by the manufacturer), 2.5 mM $MgCl_2$ (supplied with the enzyme), 10 pmol of each of the two primer solutions, 200 µM of nucleotide mix (InBio, Argentina) and free of DNAse water to complete de volume. Reactions were amplified through an initial denaturation at 94ºC for 3 min, followed by 35 cycles at the following parameters: 40 seconds at 94°C, 40 seconds at 52°C, and 40 seconds at 72°C, followed by a final extension step at 72°C for ten minutes. A negative control with all the reaction mixtures except the DNA template was included with each set of the PCR amplification reactions. Amplifications were confirmed by standard gel electrophoresis, using 2% w/v agarose gels (InBio, Argentina) in 0.5X TBE Buffer and stained with Gel Red Solution (Biotium, 10000X). After amplification the amplicons were sent to Macrogen Korea to sequence in both directions to corroborate the PCR product.

The ITS1-5.8S-ITS2 sequence generated in this study was deposited in GenBank (National Center for Biotechnology Information) under accession number KU712257. The ITS sequence of the fungal strain was compared with sequences in GenBank and TrichoKey (Druzhinina et al. 2005). Moreover, to identify the isolated *Trichoderma*, 21 accessions of the ITS1-5.8S-ITS2 sequences were selected and retrieved from the GenBank data base representing species within the *Trichoderma* genus. Nucleotide sequence retrieved in this study consisted of about 600 bp which correspond to the ITS1-5.8S-ITS2 and partial 18S and 28S regions. A sequence from the Ascomycote *Beauveria bassiana* (KJ409587) was used as an outgroup to demonstrate the situation of the root. DNA sequences were aligned using the Clustal W program. The phylogenetic methods were based on a distance-based method (Neighbor joining - NJ) and a cladistic method (Maximum Likelihood - ML). Support for specific clades represented in the tree was estimated by bootstrap analysis of 5,000 replications. Nucleotide divergences were estimated using Kimura's two-parameter method. The MEGA 6.0 package was used for the analyses. Phylogenetic data have been submitted to TreeBase with submission number S18932.

Biocontrol in-vitro antagonism technique

In-vitro antifungal activity of *T. koningiopsis* against symbiont *L. gongylophorus* strains was tested on a modified dual culturing method (Hermosa et al. 2000, Rojan et al. 2010, Hernández et al. 2015). Agar plugs (5 mm in diameter) cut from the growing edge of ant fungal symbiont strains (one *L. gongylophorus* at a time) and from *Trichoderma* were placed 3 cm from each other and incubated at 28 ±1 °C. Because *L. gongylophorus* strains have a very slow radial growth (about 0.5 cm/week) related to *Trichoderma*, plates with *L. gongylophorus* strains were previously incubated producing a fungal colony of approximately 1 cm in diameter. The radial growth of all fungi was measured. Growth parameters in all confrontation cultures were daily assessed during ten days after inoculation. Morphological characteristics of the sporulation on the symbiont colony were recorded. Inhibition of radial growth of fungi and *Trichoderma* growth over fungal symbiont were measured and compared to controls. Percent inhibition of mycelial growth of *Leucoagaricus* over control was determined following the formula given by Aquino et al. (2008) where the percentage inhibition of radial growth (PIRG) = (a-b)/a * 100, where a = the radius of the *L. gongylophorus* colony without the biocontrol agent and b = radius of *L. gongylophorus* colony in the presence of *T. koningiopsis*.

Statistical analyses

All antagonisms experiments were conducted in three replicates and data presented were average of replicates along with the standard deviation. Database was subjected to an analysis of variance (ANOVA) (STATGRAPHICS Centurion XV, StatPoint, Inc., USA) and results which have $p < 0.05$ were considered as significant. Differences among the means were evaluated using Fisher's least significant difference (LSD) procedure.

Results

Isolation and morphological characterization

Fifteen fungal gardens of leaf-cutting ant nests from Misiones (Argentina) were sampled. One *Trichoderma koningiopsis* strain was isolated from the inner fungal garden from an *Acromyrmex pubescens* nest. The colony grown on PDA in darkness produced dense white mycelium with some green areas. There was also aerial mycelium with dispersed conidia. No distinctive pigment diffused to the agar was observed. Microscopically, the phialides were straight (7 μm × 2.6 μm) with ellipsoidal conidia (4 μm × 3.0 μm). The morphology of the colony and conidiophore structures allowed us to classify the fungal isolate as belonging to the *Trichoderma* genus.

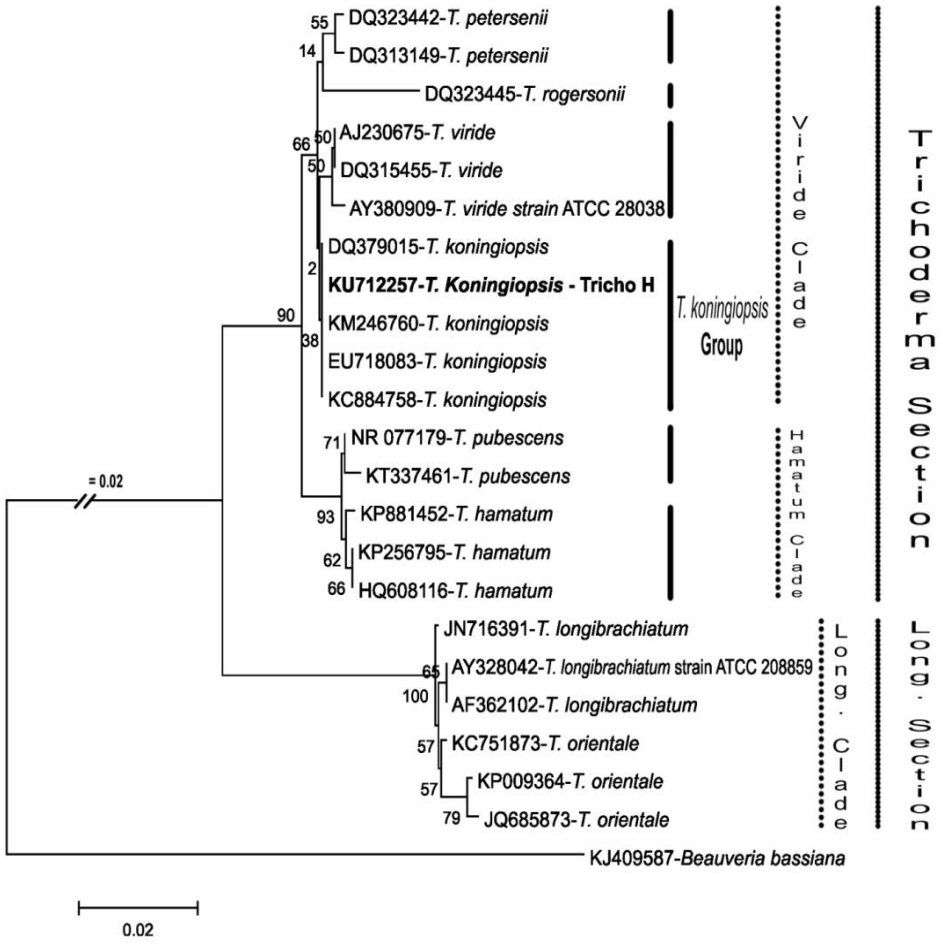

Fig. 2 – Phylogenetic relationships among *Trichoderma* isolates inferred from nucleotide sequences of the internal transcribed spacers and 18S, 5.8S and 28S RNA genes. The sequence of the *T. koningiopsis* isolated from leaf-cutting ant nest is in bold. A sequence of *B. bassiana* was used as outgroup. The numbers next to the branches indicate the percentage in which a given branch was supported in 5,000 bootstrap replications. Long. = abbreviation of Longibrachiatum.

Molecular characterization and phylogenetic analysis

For molecular identification the genomic DNA was extracted and amplified using the ITS 1 and ITS 4 primers of the rDNA region. The ITS sequence obtained had 591 bp after sequencing and contig construction. The rDNA sequence was deposited in GenBank under accession number KU712257.

The phylogenetic tree obtained by sequence analysis of ITS region of the *Trichoderma* strains selected is represented in Fig. 2. NJ and ML analyses of the selected ITS sequences of *Trichoderma* strains demonstrated a total of three distinct clades and all the clades were phylogenetically distinct from each other. Clade 1 comprised *T. koningiopsis*, *T. viride*, *T. rogersonii* and *T. petersenii* strains representing the Viride Clade which was supported by a bootstrap value of 66%. Furthermore, based on the ITS region, the Viride Clade included one *T. koningiopsis* group which clustered all *T. koningiopsis* sequences (including our *Trichoderma* strain isolated from leaf-cutting ant nest). Clade 2 (Hamatum Clade) clustered strains of *T. pubescens* along with *T. hamatum* strains were supported by a bootstrap value of 93%, whereas Clade 3 (Longibrachiatum) comprised *T. longibrachiatum* and *T. orientale* strains with a bootstrap value of 100%. In addition, Viride and Hamatum Clades clearly showed a separation of the Longibrachiatum Clade as they belong to different Section in *Trichoderma* Phylogeny (Trichoderma and Longibrachiatum Sections respectively).

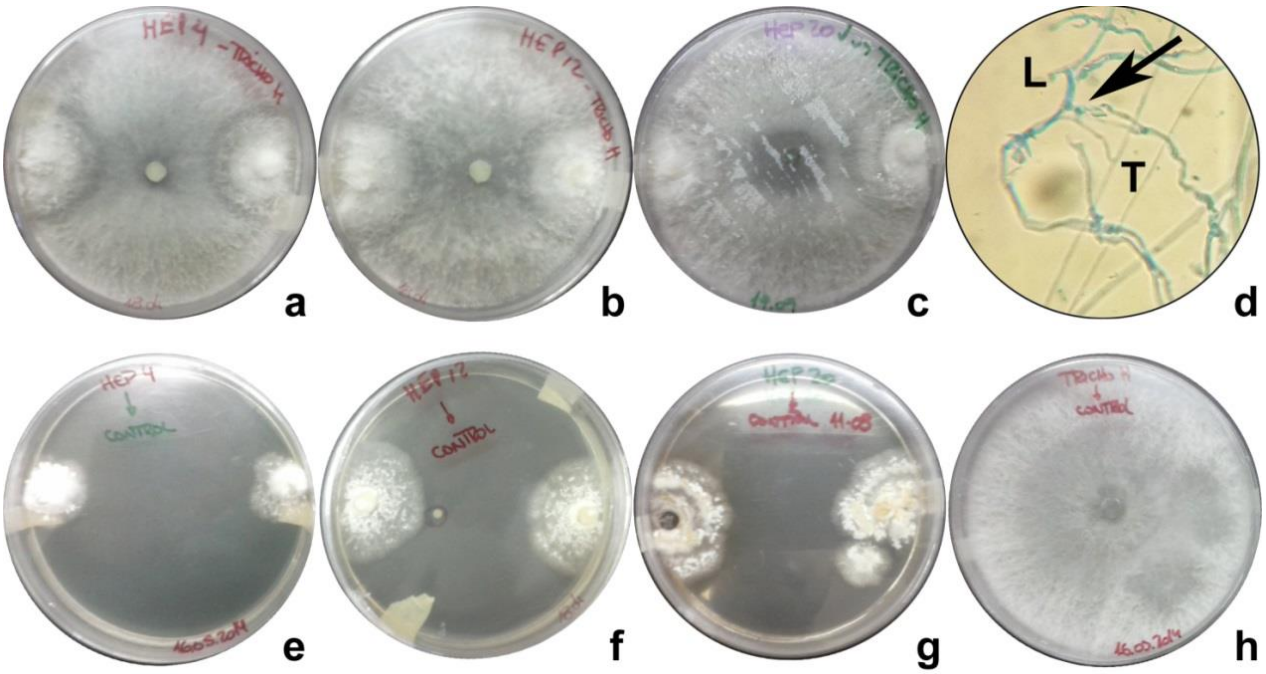

Fig. 3 – Plate assay for antagonistic activity of *T. koningiopsis* against *L. gongylophorus* strains. **a**. to **c**: *Trichoderma* growth and inhibition of *Leucoagaricus* growth after 10 days. **d**. Microscopic photograph of *Trichoderma* (T) and *Leucoagaricus* (L) strains contact zone (arrow marks attack zone) (400x). **e–g**. *Leucoagaricus* control growth. **h**. *Trichoderma* control growth.

Table 1 PIRG rate of *Leucoagaricus gongylophorus* by *Trichoderma koningiopsis*.

L. gongylophorus		PIRG % ± SD (mean)
HEP 4		67.37[a] ± 8.92
HEP 12		69.78[a] ± 3.08
HEP 20		58.03[a] ± 10.01
	Mean	65.06%

Values with same letter denote that the values are not significantly different.

Biocontrol assay

The fungal isolate identified as *T. koningiopsis* was evaluated against three *L. gongylophorus* strains from leaf-cutting ant nests. The parameters with reference to inhibition growth were recorded and evaluated. All *L. gongylophorus* strains had a slower growth rate in agar plates than *T. koningiopsis*. It was observed that contact between fungal colonies occurred after 72 hours post inoculation in all treatments.

The *T. koningiopsis* isolate grew almost completely over all *L. gongylophorus* colonies after the observation period (10 days) (Fig. 3). No further growths of *L. gongylophorus* were observed in *Trichoderma* control plates. *L. gongylophorus* HEP12 was found to be the most susceptible to *T. koningiopsis* presence and revealed the highest percent of inhibition of mycelia growth of more than 69%. In contrast, HEP20 was the most resistant and revealed the lowest percent inhibition of mycelial growth (58%), although there were not significant differences among treatments (Table.1). The mean PIRG value of *T. koningiopsis* against all the tested *L. gongylophorus* was 65.06% when compared to control plates. On the basis of dual culture experiment the antagonist effect of *T. koningiopsis* was significant against all the tested *L. gongylophorus* strains. All *three L. gongylophorus* grew fully on control plates after prolonged incubation.

Discussion

Howell (2003) suggested that the best method for obtaining a potential biocontrol agent might be one where the candidate *Trichoderma* species is isolated from areas of soil where it is expected to function in biocontrol, and where this *Trichoderma* is growing under similar conditions of temperature, moisture, and nutrient availability to those found in nature. So the isolation of the *Trichoderma* strain obtained from the inner fungal garden of leaf-cutting ant can favor its biocontrol efficacy.

The morphological characters used to identified the *Trichoderma koningiopsis* strain are in agreement with Samuels et al. (2006) who described the morphological characters of the sexual and anamorph state of this genus and particularly this species (but we did not observed the development of chlamydospores in the *T. koningiopsis* isolate evaluated).

Related to the phylogenetic tree obtained by sequence analysis of ITS region of the *Trichoderma* strains selected, our results were in agreement with previous investigations on topology of *Trichoderma* Phylogeny (Hermosa et al. 2000). Furthermore Kuhls et al. (1997) employing ITS sequences for Phylogenetic analyses discriminated clades of Trichoderma section from clades of Longibrachiatum section.

In the biocontrol assays the *T. koningiopsis* strain showed percentage inhibition of radial growth between 58% and 69%. Silva et al. (2006) found 75% of inhibition in their dual plate growth evaluation of one strain of *T. harzianum* against one strain of *L. gongylophorus* isolated from *Atta sexdens* nest. Lopez & Orduz (2003) in their study of entomopathogenic and mycoparasitic fungi (*Metarhizium anisopliae* and *T. viride*, respectively) evaluated the biological control of *A. cephalotes* nests under laboratory and field conditions. These authors reported that these fungi were more effective in controlling the nests than chemical insecticide, providing evidence that the biological control of leaf-cutting ants using fungi is a viable control alternative.

On the basis of dual culture experiment the antagonist effect of *T. koningiopsis* was significant against all the tested *L. gongylophorus*. All *three L. gongylophorus* grew fully on control plates after prolonged incubation. In a similar study Susanto et al. (2005) also documented that among other genera (*Trichoderma* spp., *Gliocladium* sp., *Bacillus* spp. *Pseudomonas* spp.) a strain of *T. harzianum* gave the highest inhibition capacity in dual culture analysis against the Basidiomycete *Ganoderma boninense*.

As seen in microscopic photographs hyphae of *Trichoderma koningiopsis* presented mycoparasitic phenomena like competition for space and growing along and coiling around the *L. gongylophorus* hyphae (Fig. 3.d). Howell (2003) and Steyaert et al. (2003) cited that one of the most outstanding characteristic of members of the genus *Trichoderma* is their ability to parasitize other fungi through mechanisms including coiling around pathogen hyphae, penetration, and subsequent dissolution of the host cytoplasm. Hernández et al. (2015) also found high levels of hyperparasitism with *T. koningiopsis* hyphae rolled into *Macrophomina phaseolina* hyphae, demonstrating a strong potential for use as a control agent.

Antibiotic production, mycoparasitism, production of cell wall-degrading enzymes and competition for nutrients or space (or a combination of these antagonistic activities) are considered as the main actions involved in biocontrol of pathogens (Zeilinger & Omann 2007, Vinale et al. 2008, Saba et al. 2012, Yao et al. 2016). During direct contact, lectins in the host's cell wall can induce coiling of the *Trichoderma* around the host hyphae and mycoparasite can produce appressorium-like structures to destroy the pathogen (Zeilinger & Omann 2007).

In terms of sporulation and aggressiveness of the BCAs, Hermosa et al. (2000) suggested that these two mechanisms depend on the kind of fungal target. Consequently the study of a native *Trichoderma* strain against native *L. gongylophorus* strains was required in the search of effective BCA against leaf-cutting ants. However it is always difficult to extrapolate the biocontrol activity of a given strain from the laboratory to natural environments.

Due to the widely use of dual culture method in antagonistic studies, the present investigation was based on this technique to test one *T. koningiopsis* strain against three fungal

strains used as food source for leaf cutting ants. Treatments with *T. koningiopsis* presented reduced mycelial growth of fungal symbiont of leaf-cutting ants.

According to Della Lucía et al. (2014) fungus contamination is an interesting control possibility, particularly in small and shallow ant colonies. Based upon these data, biocontrol of leaf-cutting ants with *Trichoderma* may reduce the use of chemical pesticides. To prove the antagonistic action of the selected strain of *Trichoderma* against the tested *L. gongylophorus* these promising results could be tested in field bioassays as a biocontrol agent of leaf-cuttings ants.

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