

Antibiosis of *Trichoderma* spp strains native to northeastern Mexico against the pathogenic fungus *Macrophomina phaseolina*

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Abstract

Sampling of agricultural soils from the Mexican northeastern region was performed to detect *Trichoderma* spp., genetically characterize it, and assess its potential use as a biologic control agent against *Macrophomina phaseolina*. *M. phaseolina* is a phytopathogen that attacks over 500 species of cultivated plants and causes heavy losses in the regional sorghum crop. Sampling was performed immediately after sorghum or corn harvest in an area that was approximately 170 km from the Mexico-USA border. Sixteen isolates were obtained in total. Using colony morphology and sequencing the internal transcribed spacers (ITS) 1 and 4 of 18S rDNA, 14 strains were identified as *Trichoderma harzianum*, *T. koningiopsis* and *T. virens*. Subsequently, their antagonistic activity against *M. phaseolina* was evaluated *in vitro*, and 11 isolates showed antagonism by competition and stopped *M. phaseolina* growth. In 4 of these isolates, the antibiosis phenomenon was observed through the formation of an intermediate band without growth between colonies. One strain, HTE808, was identified as *Trichoderma koningiopsis* and grew rapidly; when it came into contact with the *M. phaseolina* colony, it continued to grow and sporulated until it covered the entire petri dish. Microscopic examination confirmed that it has a high level of hyperparasitism and is thus considered to have high potential for use in the control of this phytopathogen.

Key words: *Macrophomina phaseolina*, antagonism, hyperparasitism, *Trichoderma*.

Introduction

Trichoderma spp. is a fungus that can be isolated from soil and agricultural waste and can form opportunistic interactions with and cause hyperparasitism in other fungi (Monte, 2001; Samuels, 2006). This fungus is frequently used in the textile industry, and agricultural reports have indicated that it promotes vegetable growth and facilitates the absorption of water, mineral salts, nutrients and the use of carbohydrates. It also produces secondary metabolites such as antibiotics, mycotoxins, and phytotoxins (Lifshitz *et al.*, 1986; Benhamou and Chet, 1996; Mukherjee *et al.*, 2006; Shores and Harman, 2008a, 2008b). These substances are involved in the antagonism phenomenon as a result of either competition or antibiosis or through hyperparasitism using structures called appressoria, which release enzymes

(glucanases, chitobioses and chitinases) or antibiotics (viridin, gliotoxin or peptaibols) (Howell *et al.*, 1993; Mukherjee *et al.*, 2006). These factors encourage the use of *Trichoderma* spp. in controlling agricultural crop phytopathogens (Benitez *et al.*, 2004). The increase in the number of its isolates, its high phenotypic variability, and its similarity with *Hypocrea* and *Gliocladium* complicate the identification of *Trichoderma* spp. through classic taxonomy. With the recent application of nucleic acid-based identification techniques, the number of described *Trichoderma* species has tripled. Various methods have been used in these studies, including the following sequences: (ITS) the 5 introns of the protein gene, which encodes the elongation alpha 1 (*tef1*) translation factor; the gene that codes for actin (gene *act*); calmodulin (gene *cal*);

and one partial exon of the gene *ech42* for chitinase (Bailey and Lumsden, 1998; Castle *et al.*, 1998; Hermosa *et al.*, 2000; Lu *et al.*, 2004; Vera *et al.*, 2005; Druzhinina *et al.*, 2006; Samuels *et al.*, 2006). *Macrophomina phaseolina* (Tassi) Goid is a phytopathogenic filamentous fungus that belongs to the anamorphic Ascomycota *Botryosphaeriaceae* family (Crous *et al.*, 2006; Arora *et al.*, 2012) and produces both sclerotia and pycnidia. *M. phaseolina* is responsible for the plant disease called charcoal rot, which affects both roots and stems. *M. phaseolina* is widely distributed in tropical regions, specifically in areas that are subjected to water stress, where it infects hundreds of different hosts (Songa *et al.*, 1997) and causes severe economic losses (Smith and Carvil, 1997). In Mexico, *M. phaseolina* is found in both the northern region, where the climate is mostly hot and dry, and in the south, where the humidity is high and where the temperatures range from 30 °C in the summer to 5 °C in winter. This microorganism penetrates host tissues through mechanical pressure exerted by the spore germ tube and the sclerotia hyphae and through the dissolution of the cell wall via processes that are mediated by secreted enzymes (Ammon *et al.*, 1974). Therefore, the present study was conducted to isolate native strains of *Trichoderma* spp. that are present in northeastern Mexican agricultural soils and to evaluate their *in vitro* antagonistic capacity against *M. phaseolina*.

Materials and Methods

Origin of the strains

Forty-two samples were obtained from approximately 2 kg of soil from the top 15 cm of lands recently cultivated with maize. To isolate the fungus, a technique developed in this laboratory was used, which consists of depositing 150 g of soil into 180-mL Styrofoam cups, adding 10 maize seeds (Pioneer 3025) and soaking with sterile deionized water according to their needs. The cups were covered and observed for 15 days, after which they were checked to detect colonies with the typical morphology of *Trichoderma* spp. on the seeds and in the soil (Barnett and Hunter, 1998; Samuels *et al.*, 2006). The isolations were performed on potato dextrose agar PDA (Difco, Sparks, MD), and only the colonies with different morphological characteristics were selected for further analysis. The HMP5 strain of *M. phaseolina* used in this study was isolated from bean plants (*Phaseolus vulgaris* L.) in Cotaxtla, México, and was provided by the Plant Biotechnology Laboratory of the Genomics Biotechnology Center of the IPN.

Antagonism

The fungi were cultivated on PDA (Difco, Sparks, MD) in petri dishes first individually and then in confrontation. The growth rate was measured every 12 h. Antagonism tests were performed according to the methodology described by Acevedo (1995), which involves placing a

7-mm-diameter agar disc with a seven-day-old *M. phaseolina* mycelium and an agar disc with a four-day-old *Trichoderma* mycelium at adjacent points of a PDA (Difco, Sparks, MD) petri dish. Each test was replicated three times, and statistical analysis (Mean comparison, Tukey $p = 0.05$) was performed using the SAS program. Graphs were made using Microsoft Excel XP and GraphPad Prism 4.00.

DNA extraction, ITS amplification and DNA sequencing

Isolates of *Trichoderma* spp. were grown in 500 mL Erlenmeyer flask, with 50 mL of Luria-Bertani broth (Difco, Sparks, MD), incubated at 27 °C with 200 rpm shaking. The 72-h culture was centrifuged, and the biomass obtained was prepared for DNA extraction. The Hoffman and Wriston (1987) method was used, which consists of transferring the biomass to an Eppendorf tube, washing with deionized sterile water and discarding the supernatant. The cells were lysed through chemical and physical treatment and were then centrifuged at 12,000 rpm followed by RNase addition. The obtained DNA was stored in 10:1 TE buffer at -20 °C until used. The quantity of DNA was analyzed using a 1% agarose gel in a horizontal electrophoresis unit (Bio-Rad Laboratories Inc., Hercules, CA) at 100 V for 50 min. The gel was observed in an ultraviolet transilluminator, and the image was captured with the Kodak Digital Science® 1D program (Kodak Company, Rochester, NY). PCR was performed in a final volume of 50 µL using 1 µL of genomic DNA (50 ng), 5 µL of 10X Buffer, 1.5 µL of magnesium (50 mM), 1 µL of mixed dNTPs (10 mM), 1 µL of each primer (100 mM) and 0.4 µL of the enzyme Taq DNA polymerase (5 U/µL), completing the volume with sterile milliQ water. For amplification of the ITS1-18S-ITS4 region of *Trichoderma* genome, the primer pair T/ITS1 TCTGTAGGTGAACCTGCGG and T/ITS4 TCCTCCGCTTATTGATATGC was used. The amplification program consisted of one cycle of 3 min at 94 °C and 35 cycles of 1 min at 94 °C, 1 min at 53 °C and 1 min at 72 °C. There was a final extension step of 1 min at 72 °C. The PCR was performed in a Peltier MJ Research Thermal Cycler® (MJ Research/Bio-Rad, Hercules, CA). We proceeded to visualize the PCR fragments on a 1% agarose gel, adding 0.1 µL SYBR gold (Thermo Fisher Scientific Inc., Waltham, MA) and 0.4 µL Orange loading dye (Thermo Fisher Scientific Inc., Waltham, MA). We loaded 5 µL of each sample in the gel, which was run in a horizontal electrophoresis unit (Bio-Rad Laboratories Inc., Hercules, CA) at 80 V for 1 h. The gels were digitized using the Kodak Digital Science® 1D program (Kodak Company, Rochester, NY). The PCR products at a 50 ng/µL concentration were sequenced in the Applied System Model 3130® column sequencer (Thermo Fisher Scientific Inc., Waltham, MA). For species-level identification, the obtained data

were compared with the reference sequences deposited in the NCBI GenBank and in the Isth *Trico*OKEY databases. The *Trichoderma* species was determined based on concordance with these databases.

Phylogenetic analysis

After sequencing, the ITS1-18S-ITS4 region of *Trichoderma* isolates were compiled SeqMan software (DNASTAR Inc., Madison, WI) and edited using the BioEdit software (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Multiple alignments were performed using the ClustalW software (<http://www.ebi.ac.uk/clustalw/>). Each sequence type was compared by a BLAST search with those available in GenBank (<http://www.ncbi.nlm.nih.gov>) and those in the Isth *Trico*OKEY databases to determine approximate phylogenetic affiliations. Evolutionary distances were calculated using the Kimura two-parameter model. The phylo-

genetic tree was constructed with a neighbor-joining method using MEGA 4.0.

Results

A total of 42 soil samples were obtained and processed, from which 16 strains were isolated based on their similarity of morphology to *Trichoderma* spp. (Figure 1). These isolates were coded HTE801 to HTE816. To identify the species, sequence typing was performed, and the sequences obtained from each isolate were aligned and compared with the sequences of the GenBank database from NCBI and the *Trico*OKEY database from Isth.

Isolate identification by this method showed that the strains coded from HTE801 to HTE807 and from HTE810 to HTE816 correspond to *T. harzianum* and that HTE808 corresponds to *T. koningiopsis* and HTE809 to *T. virens* (Figure 2). These results show that there is no association

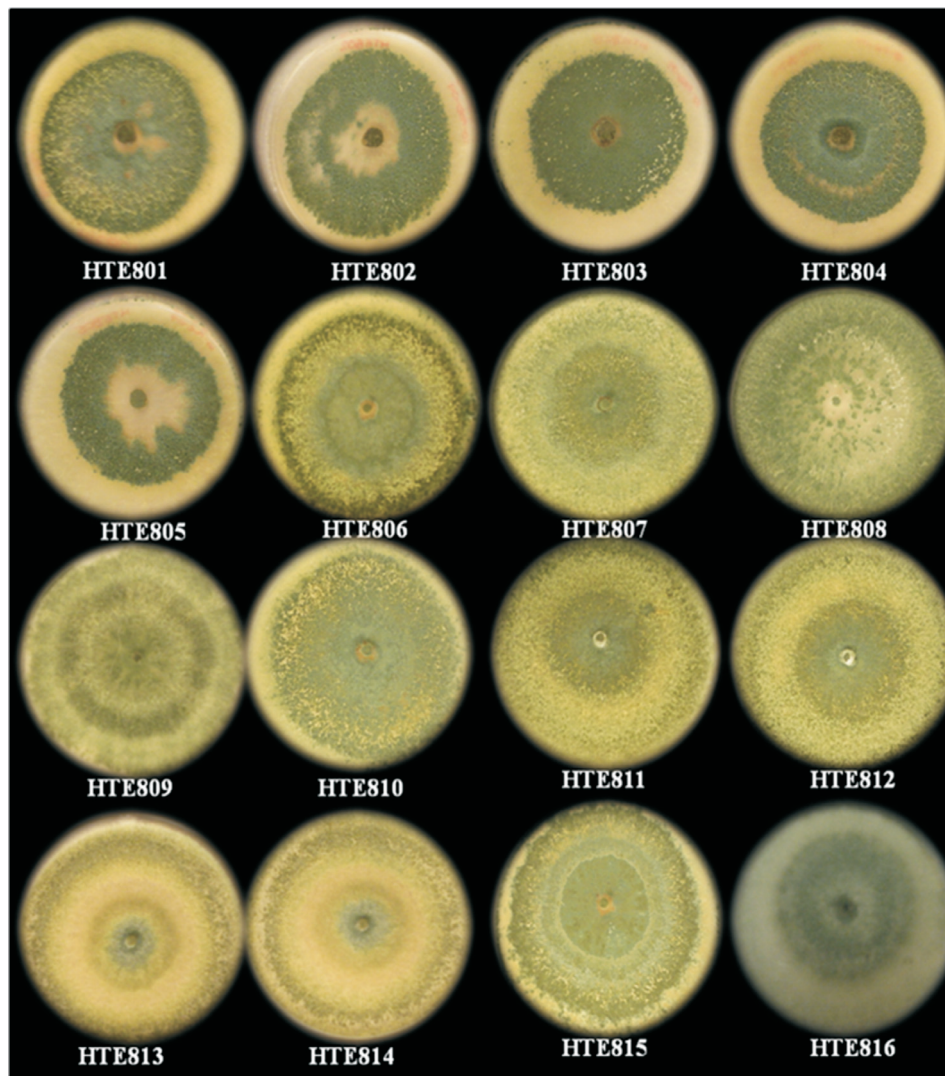


Figure 1 - *Trichoderma* spp. colonies obtained from soils with different crops and agronomic conditions.

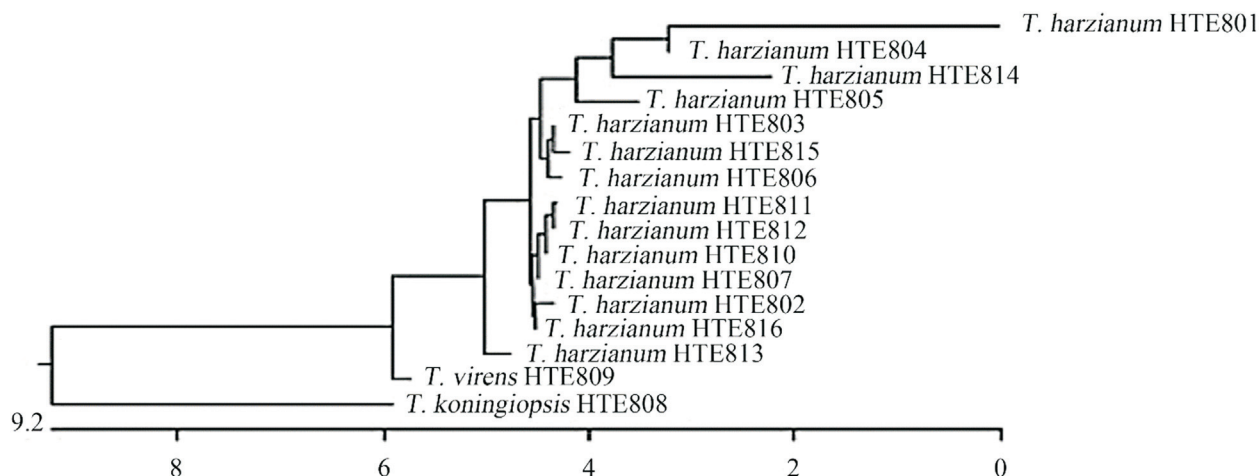


Figure 2 - Neighbor-joining phylogenetic tree of *Trichoderma* spp. isolates based on sequences from the ITS1-18S-ITS4 region.

among the geographical origins of the sample, the cultivated species, growing condition and the isolated fungus.

Growth kinetics of assessed fungi: *Trichoderma* spp.

The data obtained from the tests performed when cultivating each of the *Trichoderma* spp. strains individually shows that generally between 96 and 120 h after seeding, the mycelium completely covers the petri dish surface (Figure 3).

The growth rate curve is exponential up to 96 h and stabilizes thereafter. HTE801, HTE807 and HTE808 are considered fast growing because they cover the petri dish within 96 h after seeding, whereas a lower growth rate is observed for HTE809, with 132 h required post-inoculation for complete coverage. In most instances where the *Trichoderma* spp. is grown in the same petri dish as *M. phaseolina*, the fungus has the same developmental kinetics, except with strains such as HTE810, where a phenomenon that has not yet been reported in assays from the confrontation of *Trichoderma* spp. with other fungi is observed. Therefore, there are several mechanisms involved in *Trichoderma* antagonism, namely antibiosis, whereby the antagonistic fungus produces antibiotics and competes for nutrients. In the case of mycoparasitism, *Trichoderma* directly attacks the plant pathogen by excreting lytic enzymes such as chitinases, β -1, 3 glucanases and proteases (Haran *et al.*, 1996). Because the skeleton of pathogenic fungi cell walls contains chitin, glucans and proteins, enzymes that hydrolyze these components must be present in a successful antagonist to play a significant role in cell wall lysis of the pathogen. Filamentous fungal cell walls also contain lipids and proteins. It therefore was expected that antagonistic fungi synthesize proteases might act on the host cell wall (Lorito *et al.*, 1994).

Growth kinetics of assessed fungi: *M. phaseolina*

M. phaseolina is a fungus that has an exponential kinetic growth in PDA (Difco, Sparks, MD) media and covers the entire dish 120 h post-inoculation. The analysis of variance was performed using a Tukey test ($p = 0.05$), which was performed by comparing the growth data of the *M. phaseolina* strain by itself and in antagonism with the different *Trichoderma* spp. strains, showed that at 60 h post-inoculation, the control and confrontation tests exhibit similar growth, except for strain HTE805, which develops more slowly. At the 72nd h, only nine of the evaluated strains (HTE801, HTE808, HTE813 and HTE816) came into contact with the *Trichoderma* spp. colonies (Figure 4). In all other treatments, contact was observed at the 84th h post-inoculation.

A statistical analysis of the growth kinetics between the *M. phaseolina* strain (Figure 4) growing by itself and in confrontation showed that strains HTE801 and HTE803 of *Trichoderma* spp. increased the phytopathogen colony development rate. As observed in Figure 4, for strains HTE805, HTE809 and HTE813, immediately before the contact between the colonies of *M. phaseolina* and *Trichoderma* spp., the detected growth rate of the phytopathogen decreased, which modified the exponential growth line until it stabilized.

Antagonism tests

From the antagonism tests between *Trichoderma* spp. and *M. phaseolina*, the following four phenomena were detected: antagonism through competition, antibiosis, hyperparasitism caused by *Trichoderma* spp. over *M. phaseolina* and the developmental delay of *T. harzianum* HTE810 when grown in competition with *M. phaseolina*. These tests were repeated three times, and the results are statistically significant. Competition is observed when the two fungi grow in the petri dish until their mycelia come into contact,

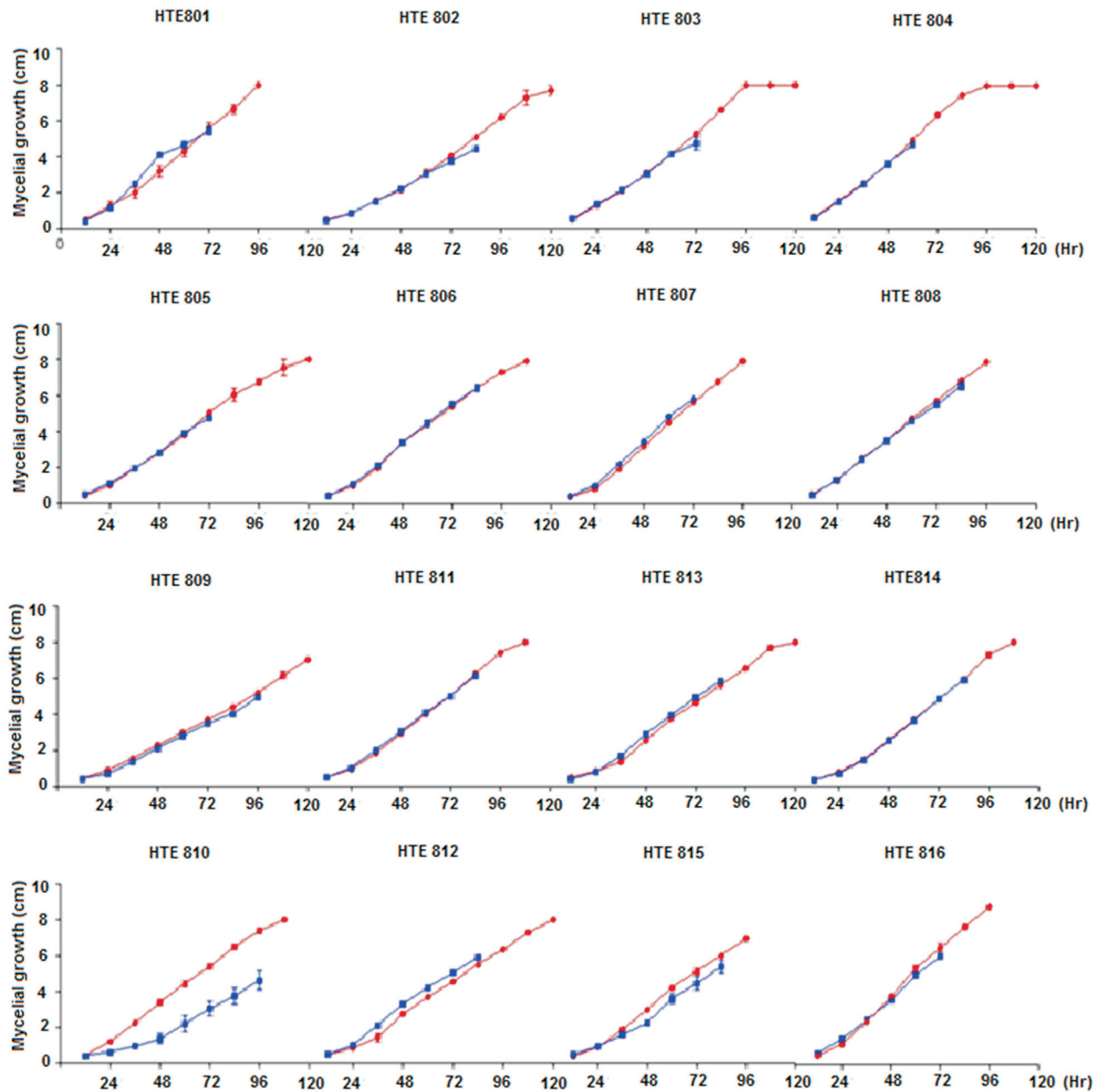


Figure 3 - Mycelial growth (cm) of *Trichoderma* spp. after inoculation growing by itself and in confrontation with *M. phaseolina*. The Percentage Inhibition of Radial Growth of *M. Phaseolina* (PIRGM) was determined as follows: $PIRGM = [(R1-R2)/R1] \times 100$ where, R1 = radial growth of the pathogen and R2 = radial growth of the pathogen against *Trichoderma* spp; The Percentage of Radial Growth Acceleration of *Trichoderma* (PRGAT) was determined as follows: $PRGAT = [(R2-R1)/R2] \times 100$ where, R1 = Radial growth of *Trichoderma* spp. and R2 = radial growth of *Trichoderma* spp. against the pathogen. Red lines indicate the growth of *Trichoderma* spp. and blue lines indicate the growth of *Trichoderma* spp. against *M. phaseolina*.

and the *Trichoderma* spp. initiates the formation of a barrier, which prevents the growth of *M. phaseolina*. Over time, the barrier consolidates, thereby preventing the advance of the phytopathogen (Figure 5).

Antagonism through competition

All of the *T. harzianum* and *T. virens* strains that were studied underwent antagonism by competition. Both *Trichoderma* species stopped the growth of the fungus *M.*

phaseolina at the site of contact by forming a barrier that prevents *M. phaseolina* development.

Antibiosis

From the confrontation between the *Trichoderma* spp HTE815 strain and *M. phaseolina*, it is observed that at 48 h and without contact between mycelia, a zone of growth inhibition exists and the culture medium changes color, which could be due to secondary metabolite excretion. As

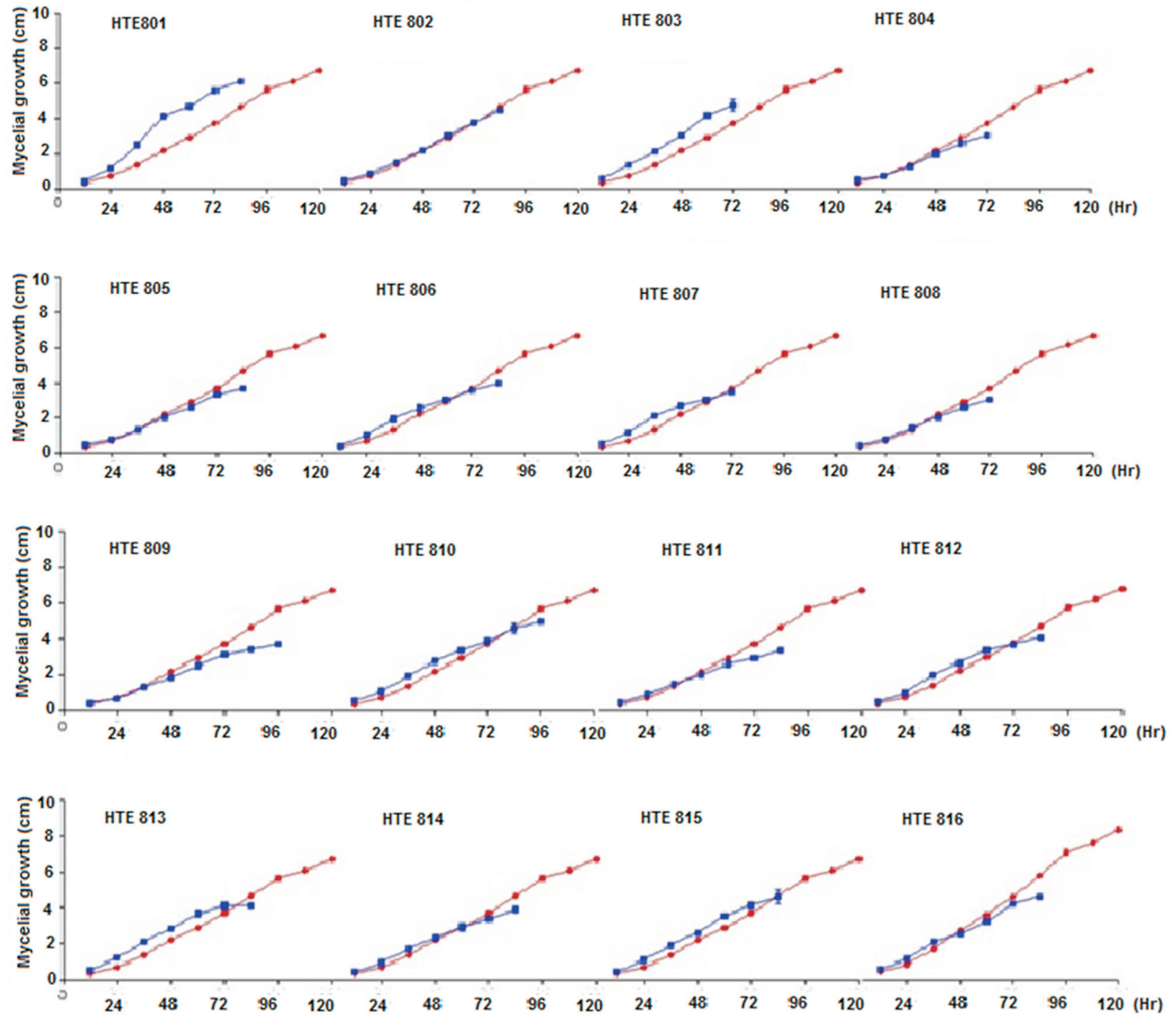


Figure 4 - Mycelial growth (cm) of *M. phaseolina* growing by itself and in confrontation with different *Trichoderma* spp strains. The Percentage Inhibition of Radial Growth of *M. Phaseolina* (PIRGM) was determined as follows: $PIRGM = [(R1-R2)/R1] \times 100$ where, R1 = radial growth of the pathogen and R2 = radial growth of the pathogen against *Trichoderma* spp; The Percentage of Radial Growth Acceleration of *Trichoderma* (PRGAT) was determined as follows: $PRGAT = [(R2-R1)/R2] \times 100$ where, R1 = Radial growth of *Trichoderma* spp. and R2 = radial growth of *Trichoderma* spp. against the pathogen. Red lines indicate the exponential growth of *M. phaseolina* and blue lines indicate the growth of *M. phaseolina* against *Trichoderma* spp.

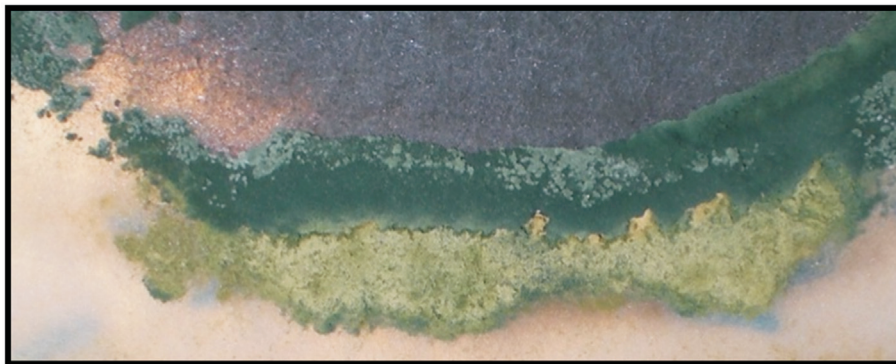


Figure 5 - Details of the growth of *Trichoderma* spp. (bottom green colony), showing the beginning (left) of the formation of a barrier at the contact site with the mycelium of *M. phaseolina* (upper dark part).

shown in Figure 6, a narrow band without growth can form between the fungi, even when they are far from physical contact.

Hyperparasitism

Mycoparasitism involves morphological changes, such as coiling and formation of appressorium-like structures, which serve to penetrate the host. Differential antagonistic activity has been observed for various *Trichoderma* spp., which demonstrates semi-specificity in the interaction of *Trichoderma* with *M. phaseolina*. Our results revealed that of the 16 strains tested, only *T. koningiopsis* (HTE808) shows antagonism by hyperparasitism. Microscopically, *T. koningiopsis* hyphae are rolled into phytopathogenic *M. phaseolina* hyphae. This is a desirable trait for agricultural purposes because this strain (HTE808) has potential for use in phytopathogen control.

Discussion

The isolates obtained showed the formation of concentric rings that are typical of *Trichoderma* spp. colonies, where the green color of the conidia is interleaved with the white of the mycelium, which is consistent with the characteristics previously described for this fungus (Barnett and Hunter, 1998; Druzhinina *et al.*, 2006; Samuels, 2006). However, although the colony morphology serves to identify fungi of this genus, it is insufficient to distinguish the species, which makes it necessary to confirm the species through molecular methods (Ospina *et al.*, 1999; Druzhinina *et al.*, 2005, 2006). The identification of the isolates in this study yielded three species, of which *T. harzianum* was the most frequently sampled. The presence of *T. harzianum* had already been reported in this region of the country (Larralde-Corona *et al.*, 2008), and it is the species with the

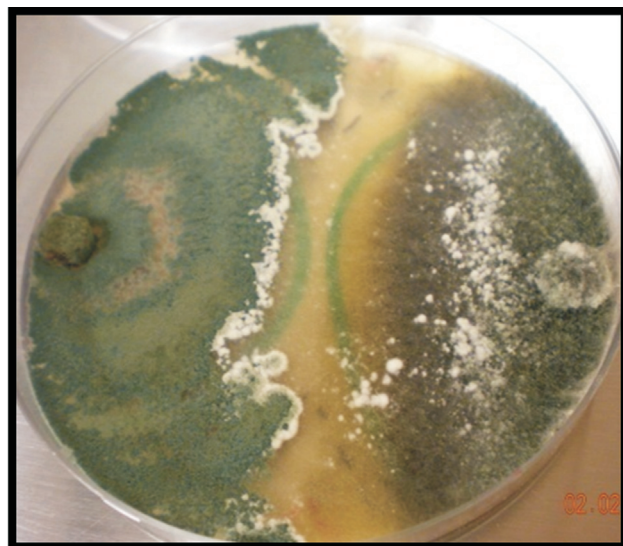


Figure 6 - Growth and sporulation of *Trichoderma* spp. on *M. phaseolina* and the intersection point between them.

widest distribution (Hermosa *et al.*, 2000; Harman *et al.*, 2004). Additionally, the *TichoOKEY* database could only be used to compare the sequences of some *T. harzianum*, *T. virens* and *T. koningiopsis* strains because, for sequencing the strains HTE801, HTE804, HTE805, HTE810 and HTE814, it was necessary to draw upon the NCBI GenBank database, where the identification was corroborated. The antagonism phenomenon observed in these isolates, where the strains of *Trichoderma* spp. did not allow the growth of the *M. phaseolina* colonies, confirm the competition, antibiosis and hyperparasitism phenomena that have been reported for *Trichoderma* spp. (Hjeljord and Tronsmo, 1998; Hermosa *et al.*, 2000; Benitez *et al.*, 2004; Harman, 2006; Hoitink *et al.*, 2006). Two phenomena that have not been previously reported were also observed; the first involves a strong decrease in the growth of the *T. harzianum* colony strain HTE810 when cultivated in confrontation tests with *M. phaseolina* (Figure 3). The other phenomenon detected is counter to the previous model of interaction, namely the induction rather than repression, of *M. phaseolina* colony growth in the presence of the *T. harzianum* strain HTE801 and, to a lesser extent, HTE803. In both cases, the acceleration is statistically significant (Tukey, $p = 0.05$; Figure 3).

Antagonism through antibiosis was detected in strains HTE807, HTE810, HTE815 and HTE809 of *Trichoderma* spp., which show growth-free spaces between both fungi, as well as a change in coloring in the culture medium. These strains correspond to *T. harzianum* and *T. virens*, respectively. The growth-free zone is possibly due to *Trichoderma* spp. producing metabolites such as viridin and its derivatives, which function as antimicrobials (Benitez *et al.*, 2004; Harman, 2006; Hoitink *et al.*, 2006; Vinale *et al.*, 2008). Finally, it was observed in *T. harzianum* strain HTE810 that the *M. phaseolina* strain HMP5 has a statistically significantly decreased growth rate, which was the initial impetus of these trials. We have not found reports of this response against *T. harzianum*.

Conclusions

This sampling shows that *Trichoderma* is a fungus that can be isolated from soil and that there is no relationship between the species and the conditions under which agriculture is carried out in this region. Of the detected fungi, *T. harzianum* is the most frequent species, with *T. koningiopsis* and *T. virens* following in frequency. From the antagonism test results, *T. harzianum* and *T. virens* show antagonistic activity through space competition, while *T. koningiopsis* showed a high level of hyperparasitism on *M. phaseolina*, demonstrating a strong potential for use as a control agent. The *M. phaseolina* HMP5 strain decreased the *T. harzianum* HTE810 growth when cultivated in a confrontation test.

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