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Original article

Morphological and molecular characterization of a new autochthonous *Trichoderma* sp. isolate and its biocontrol efficacy against *Alternaria* sp.

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ABSTRACT

The development of agriculture requires the use of microorganisms in the management of phytopathogens as a way to compensate for the use of chemical pesticides, in order to produce healthy crops. The objective of this study was to characterize a new isolate of *Trichoderma* sp. based on morphological and molecular features, and its potential ability to control the pathogen *Alternaria* sp. The antagonistic isolate was isolated from soil samples of potato fields in Guasave Sinaloa, Mexico, whereas the pathogen was collected from infected apple leaves in the orchard "La Escondida" in Guerrero County, Chihuahua, Mexico. For morphological characterization both fungi were grown on solid PDA medium. DNA of *Trichoderma* sp. was isolated using the CTAB method and PCR analyses were done using ITS1, ITS4 primers resulting in amplified products of 600 bp. These were sequenced, submitted to Genbank (acc. no. MN950427) and used for further phylogenetic analysis through Bayesian inference approach. Five clades were identified and the polytome topography recovered from clade 4 indicates a high genetic similarity with *T. asperellum*. A BLAST examination of the resulting sequence in GenBank showed 98.11% similarity with *T. asperellum*. This result together with the morphological and the phylogenetic analyses indicates that the isolate belongs to *Trichoderma asperellum* Samuels, Lieckfeldt & Nirenberg. Biocontrol tests of this isolate showed inhibition of *Alternaria* sp. between 50% and 93%. These results are essential for biodiversity research and give some new possibilities for pest management.
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1. Introduction

The genus *Malus* Mill. gathers 25 to 33 species (Ma et al., 2017) and includes one of the most economically important fruit trees worldwide: the apple (*Malus domestica* Borkh.) (Posadas-Herrera et al., 2018). Apple production is constantly affected by various biotic stresses such as *Alternaria alternata* (Fries) Keissler, the fungus predominantly associated with the moldy heart of the apple fruit (Reuveni et al., 2006).

The disease caused by *A. alternata* is considered the most important of Red Delicious cultivars in northern Mexico and is characterized by the growth of mycelium within the locules, with or without penetration into the mesoderm of the fruit (Reuveni et al., 2003). The external symptoms of diseased fruits are difficult to perceive, although some may acquire color and fall prematurely (Spotts, 1990). Chemical control is the most widely used method, with efficient results, but with residual effects, which causes chemicals to accumulate in water bodies, soil, plants and animals. This is one of the main reasons for the application of ecological strategies such as microorganisms (Michel-Aceves et al., 2009).

The use of antagonistic microorganisms that are naturally present in the soil offers options to reduce environmental contamination by use of chemical pesticides. Biological control implies the

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use of natural enemies for the regulation of pest populations. These enemies include bacteria, viruses, nematodes and fungi. The genus *Trichoderma* is one of the most studied fungus and is widely used as biocontrol since it includes species which are versatile, adaptable to various environments and are easy to manipulate (Duarte-Leal et al., 2017).

The mechanisms used by *Trichoderma* sp. to control plant-pathogens include antibiosis, mycoparasitism and competition for space and nutrients (Harman et al., 2004; Bailey et al., 2008). In addition, when it interacts with the roots, *Trichoderma* promotes growth and increased resistance, producing positive effects on the development of the plant, due to the presence of growth-regulating hormones and formation of iron chelating siderophores, stimulating the primary meristematic tissues in young parts (Candelero et al., 2015). Because the plasticity of morphological traits in *Trichoderma* species is not enough to make a precise taxonomic diagnosis, it is essential to characterize them with accurate molecular tools (Hermosa et al., 2000).

Previous research used molecular techniques to characterize 16 biological control strains formerly known as *T. harzianum* and one strain identified as *T. viride*. Hybridizations using a mitochondrial DNA probe, showed a certain degree of polymorphism and sequence analysis of internal transcribed spacers 1 and 2 (ITS1 and ITS2) revealed three different ITS lengths and four different sequence forms (Hermosa et al., 2000). García-Núñez et al. (2017) characterized by morphology and molecularly 10 native strains of *Trichoderma* (TL2, TL4, TL5, TL6, TX7, TX8, TT6, TF8, TF10 and TJ6) and their phylogenetic relationship, as well as their biocontrol capacity against *Phytophthora infestans.* This study found that six of the analyzed strains corresponded to *T. asperellum* (TF8, TT6, TX7, TX8, TL2 and TL4) and four to *T. harzianum* (TJ6, TF10, TL5 and TL6). In addition, the phylogenetic analysis showed a close correlation among the strains of these two groups.

According to the data above, it is inferred that the use of antagonistic microorganisms such as *Trichoderma* sp. could be a viable option for the management of fungal diseases for different crops, and by increasing the availability with autochthonous strains, integrated control strategies would become more efficient. The morphological and molecular studies of isolates around the world may reveal novel species or strains. This may constitute a framework for future taxonomic, phylogenetic and biological investigations. Therefore, the objective of the present research was to characterize a novel isolate of *Trichoderma* sp. based on morphological and molecular approaches, as well as to evaluate its potential biocontrol against *Alternaria* sp., the causing agent of moldy heart of the apple fruit.

2. Materials and methods

2.1. Purification of isolates

A native isolate of *Trichoderma* sp., was obtained from 15 soil samples from a potato field in Guasave, Sinaloa located at 108° 19′ 8.5″ West and 25° 32′ 25″ North, at an altitude of 21 m above sea level (masl). These samples were taken from a surface of 1 ha. Soils were taken at a depth of 20 cm and close to the roots. The samples (≈500 g) were stored in plastic bags and taken to the laboratory under refrigeration at approximately 18 °C until processing (Sadeghian, 2018).

Each sample was added to sterile distilled water (1 g 100 mL^{-1}), from which 1 mL was diluted to 10^{-3}, afterwards, 100 μl were placed in Petri dishes with PDA dextrose agar medium (Potato Dextrose Agar medium). After spreading the suspension, the plates were incubated at 26 °C for 7 days until the *Trichoderma* sp. colonies developed. Afterwards they were purified and transferred to Petri dishes with PDA medium (Girard, 1964; Maniscalco and Dorta, 2015).

The pathogen (*Alternaria* sp.) was isolated from infected apple leaves from the orchard "La Escondida" which is located in the Mesa of Miñaca, Guerrero County, Chihuahua, Mexico located at 107° 27′ 02.6″ West and 28° 28′ 11.7″ North, at an altitude of 21.50 masl. The collected leaves were disinfected with 1% sodium hypochlorite and washed three times with sterile distilled water to proceed to the direct plating of leaf portions (half healthy and half diseased) in PDA medium (BD Bioxon) at pH 5.0–5.5. The Petri dishes were incubated in the dark at 25 ± 2 °C during 7 days.

2.2. Morphological characterization

2.2.1. Macroscopic identification

The morphological characterization was done after obtaining a pure isolate of *Trichoderma* sp. The isolate was identified following the method of Barnett and Hunter (1972), considering concentric rings, conidia development and pigmentation, and mycelium texture.

2.2.2. Microscopic identification

The microscopic identification was implemented through the recognition of structures observed in culture, such as, phialides hyphae, shape of conidia and number of conidiophores. Once of the fungal structures were visualized, microcultures were done on slides with agar water at 2%, and they were incubated at 25 °C. Subsequently at 48–72 h, microcultures were observed with an optical microscope (VELAB). The *Trichoderma* sp. isolate was identified using the codes and descriptions of Barnett and Hunter (1972), for fungi genera and species. The *Alternaria* sp. isolate was characterized using the guide of Morales-Mora et al. (2020).

2.3. Molecular characterization

The DNA extraction of the isolate was carried out from the mycelium using the method developed by Rajendrakumar et al. (2006). The extracted DNA was observed in agarose gel at 1%. To amplify the internal transcribed spacer region (600–1400 bp), the oligonucleotide pair ITS-1 (TCCGTAGGTGAACCTGCGG) and ITS-4 (TCCTCCGCTTATTGATATGC) (White et al., 1990) were used. The PCR (Polymerase Chain Reaction) was done in a thermocycler (BioRad, CA, EU.), with 25 μl of a reaction combination including 0.2 mM dNTP, 2 mM $MgCl_2$, 0.5 μM of each oligonucleotide and 1.25 μl of recombinant Taq DNA polymerase (Invitrogen). The amplification program was one cycle at 95 °C for 4 min, 30 cycles at 95 °C for 1 min, 60 °C for 60 min and 72 °C by 2 min, with a final cycle at 72 °C for 5 min. The amplification was determined on 1% agarose gel (electrophoresis).

The PCR product was purified using the Wizard SV Gel Kit and PCR Clean-Up System (Promega) and sequenced in the Chemistry DNA laboratory of the CINVESTAV Irapuato, using the Dye Terminator Cycle Sequencing Ready Reaction kit, and the ABI PRISM 377 PERKIN-ELMER Sequencer (Cetus, Norwalk, CT). Forward and reverse sequences were reviewed and aligned in BioEdit 7.0.9 (Hall, 1999) and Clustal W (Thompson et al., 1994) to obtain a consensus sequence for further analysis.

The ITS sequence obtained (GenBank acc. no. MN950427), was compared with sequences available in the GenBank Database (Benson et al., 2018) such as *Trichoderma asperellum* AY380912, *T. longibrachatium* EU401556, *T. brunneoviride* EU518659, *T. hamatum* MK765015, *T. atrobrunneum* MH459162, *T. tawa* KC847184, *T. piluliferum* KF985185, *T. lixii* KT588249, *T. virens* KT588282 and *T. guizhouense* MN258612, using ITS sequences from *Beauveria bassiana* MK246940 and *Metarhizium rileyi* MG637450 as external group. The sequences were aligned with Clustal W and the final

matrix containing 600 bp was used for distance analysis performed in PAUP* 4.0–10 (Swofford, 1998), using the neighbor union method (NJ) (Saitou and Nei, 1987) and Bootstrap (Felsenstein, 1985) with 1000 repetitions, with 100 cycles of random addition each to evaluate the internal branch support and finally the tree was edited in FigTree 1.4.3 (Rambaut, 2016).

2.4. In vitro antagonistic test

The antagonistic test was evaluated in Petri dishes with PDA medium containing a disc (5 mm in diameter) with mycelium of *Trichoderma asperellum* placed at one end, and at the opposite end, another disc of the same size containing the pathogen *Alternaria* sp. A control was included which consisted in a Petri dish inoculated with only the disc with mycelium of *Alternaria* sp. The inoculum of the pathogen was prepared 72 h before the test in PDA medium. The antagonistic test was carried out for 7 days. Petri dishes were incubated at 25 ± 2 °C in the dark. The diameter of each fungal colony was measured daily, until one of these (either the antagonist or the pathogen) completed its growth in the Petri dish.

The inhibition rate of their radial growth was determined using the formula of Samaniego et al. (1989), PIRG = [(R1-R2)/R1 × 100] where R1 is the radial growth of the control and R2 the radial growth of the pathogen confronted against *T. asperellum* in the dual culture.

A randomized experiment was performed with three repetitions and the potential of the isolate as a biocontrol agent of *Alternaria* sp. at the different times was done by analysis of variance and multiple media comparison (Tukey, $p < 0.05$), using the SAS (Statistical Analysis System) software version 9.4.

3. Results

3.1. Morphological characterization

3.1.1. Macroscopic identification of Trichoderma sp.

A pure culture was obtained and the isolate presented white mycelium of spongy consistency that spread throughout the plate, two to three concentric rings, with yellow-green color (Fig. 1).

García-Núñez et al. (2017) described several *Trichoderma* isolates that developed profuse fluffy mycelium and two to three fine defined concentric mycelium (white) and conidia (green) rings. Other characteristics that define the genus *Trichoderma* are a fast growth in culture medium and development of conidia with green-yellow color (Chaverri et al., 2015). These characteristics are similar to several *Trichoderma* species, so it was difficult to identify the isolate based only on morphological data (Gupta et al., 2013).

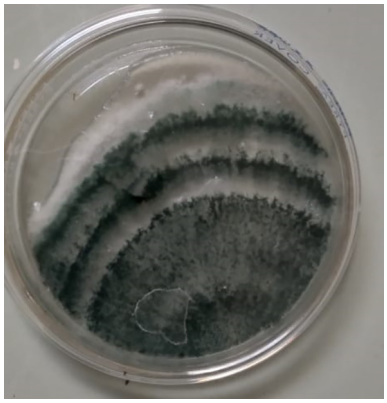

Fig. 1. Colony of the native strain of *Trichoderma* sp. in PDA medium.

3.1.2. Microscopic morphological characterization of Trichoderma sp. and Alternaria sp.

Results from the microscopic observation of *Trichoderma* sp. isolate showed dense conidia, branched conidiophores, ampuliform phialides, slightly globose conidia with yellow-green pigmentation (Fig. 2).

Regarding the *Alternaria* sp. isolate, it showed septated hyaline hyphae, and its conidia are oval to oblong-shaped, transversally septated with three to five divisions, with dark color when mature (Fig. 3).

3.2. Molecular characterization

A fragment of 600 bp was visualized after DNA extraction and PCR amplification. A consensus sequence was obtained for this region and deposited in GenBank with accession number MN95047. The PCR amplification done with primers ITS-1 and ITS-4 is showed in Fig. 4.

The phylogenetic analysis showed that this isolate matched with *Trichoderma* sp. (GenBank acc. no. MN950427), which clustered by 98.11% identity the species *Trichoderma asperellum* (accession number AY380912) from Indonesia, thus confirming by molecular and bioinformatics means that the isolate corresponds to *T. asperellum* (Fig. 5).

Using the ITS sequence information, four clades have been identified, and the polytomous topography suggests a high genetic similarity among *T. asperellum* (clade 4).

These molecular results support the morphological description of the isolate and confirm that the analysis used lead to a precise and reliable taxonomic diagnosis.

3.3. In vitro confrontation

The beginning of the antagonistic activity in the dual culture of *T. asperellum* and *Alternaria* sp. isolates was observed with a marked difference in growth speed in favor of the antagonist, which grew at an average speed of = 0.79 mm h^{-1}. Contact between the colonies began at 48 h, which was most noticeable at 96 h (Fig. 6).

The maturation of the spores of *T. asperellum* and the total coating of the pathogenic colony by them was observed on the 5th and 6th day of the confrontation.

The PIRG varied between 50% and 93% in the evaluated time points with significant differences (Table 1), which indicate an increase of mycelial growth of *T. asperellum* on the pathogenic fungus.

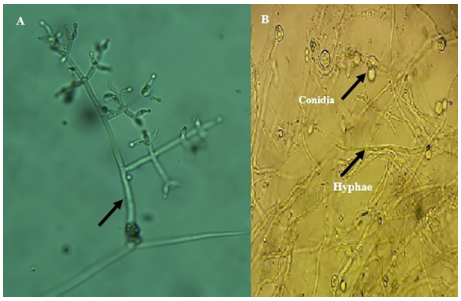

Fig. 2. Microscopic structures of *Trichoderma* sp. (a) Conidiogenic cells (x40); (b) Hyphae and conidia (x100). Scale bars: 10 μm.

Fig. 3. Microscopic structures of *Alternaria* sp. (A) Conidia (x40); (B) Septate hyphae; (C) Young conidia, and (D) Mature conidia (x100). Scale bars: 10 μm.

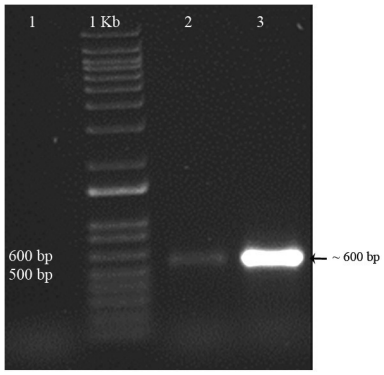

Fig. 4. PCR products amplification of the fungal isolate PCR: (1): negative; (1 kb): molecular weight marker; (2): positive and (3): isolate fragment.

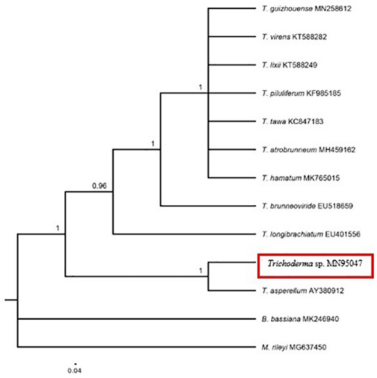

Fig. 5. Neighboring tree of *Trichoderma* sp. MN950427 accessions, based on ITS sequences. The new isolate is marked with a red box. The numbers on the branches indicate Bootstrap compatibility (96%) and the scale bar indicates nucleotide substitutions per site.

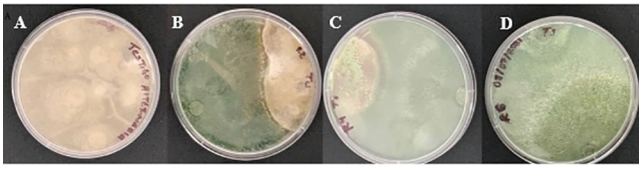

Fig. 6. Interaction between *Trichoderma asperellum* and *Alternaria* sp. isolates where: A. *Alternaria* sp.; B. Dual culture at 48 h; C. Dual culture at 96 h; D. Dual culture at day six.

Table 1
Analysis of the mycelial growth of *Trichoderma asperellum* on the pathogen.

Evaluation Time	R1 (mm h^{-1})	R2 (mm h^{-1})	PIRG (%)
48 h	0.4^{b}	0.2^{a}	50^{c}
96 h	1.4^{a}	0.1^{a}	92^{b}
6th day	1.2^{a}	0.1^{a}	93^{c}

4. Discussion

The previous results demonstrated the importance of the morphological and molecular studies for species identification. Regarding the *Alternaria* sp. isolate the microscopic characteristics are in agreement with those reported by Morales-Mora et al. (2020).

In contrast, the macroscopic features described for the novel *Trichoderma* sp. isolate agree with the taxonomic identification criteria of Barnett and Hunter (1972). These include profuse fluffy mycelium and two to three well defined concentric white (mycelium) and green (conidia) rings. It also was observed fast growth in culture medium and development of conidia with green-yellow color (Chaverri et al., 2015; García-Núñez et al., 2017). Likewise, the microscopic characteristics of the isolate were similar to those reported for the species *T. asperellum* (Barnett and Hunter, 1972). These included the presence of ampulliform phialides, oval-round conidia with greenish-yellow pigmentation and branched conidiophores (Samuels et al., 1999). These features agree with the observations done with the isolate from the present study, as well as with the T2 colony reported by Gamboa-Villa et al. (2020).

Due to the abundant homoplasia of phenetic characters in *Trichoderma* species (Gupta et al., 2013), it is necessary to make additional molecular analyses besides morphology in order to determine the species accurately (Schuster and Schmoll, 2010; García-Núñez et al., 2017).

Previous studies have shown that ITS primers are valuable to effectively identify *Trichoderma* isolates (Torres-De la Cruz et al., 2015). In the present study, molecular analyses showed a PCR product of ≈600 bp of the amplified ITS region of the *Trichoderma* isolate. This result agrees with findings of several other studies. Ten isolates of *Trichoderma* spp., were analyzed with the same primers ITS1 and ITS4, obtaining amplified DNA fragment sizes of 600 bp (García-Núñez et al., 2017). Another study obtained a single PCR product of approximately 560–600 bp from 17 biocontrol isolates of *Trichoderma* spp. using primers ITS1 and ITS4 (Hermosa et al., 2000). The results of the present study are similar to those obtained by Hermosa et al., (2000).

The phylogenetic analysis of this work supports the data on morphological characteristics found in this investigation and allowed the identification of the isolate as *T. asperellum*. The use of ITS sequences and the phylogenetic analysis enabled to identify as new records several species of *Trichoderma* (*T. asperellum, T. brevicompactum, T. koningiopsis/H. koningiopsis, T. pleuroticola, T. reesei/H. jecorina* and *T. spirale*) in the cacao agrosystem in Tabasco, Mexico (Torres-De la Cruz et al., 2015). Similar results were reported by García-Núñez et al. (2017) using a BLAST analysis of the amplified ITS sequences, where 6 out of 10 isolates tested were highly identical (99%) to *T. asperellum*.

Considering the PIRG results obtained in the present study (antagonistic efficacy between 50% and 93%), it is very possible that *T. asperellum* be an effective control against *Alternaria* sp. Results found by Camacho-Luna et al. (2021) reported the growth inhibition of *A. porri* in 56% using two isolates of *T. asperellum;* while *T. atroviridae* showed only 20% inhibition of the mycelial growth of the pathogen. At 48 h after the confrontation, the value was classified as class III according to the scale proposed by Bell et al. (1982)

and from 96 h on, the value was classified as class I, meaning that *T. asperellum* showed a high antagonistic capacity.

Pandey (2010) found that *T. harzianum* inhibited *A. alternata* at 67% after 10 days of incubation, while *T. viride* inhibited the pathogen at 66.7% and the biocontrol process started at the 5th day of incubation. Other authors found different percentages of *Alternaria* sp. growth inhibition by several species of *Trichoderma.* Examples include 52.9% PIRG of *A. alternata* by *T. harzianum* (Zehra et al., 2017), 53.8–82.8% growth inhibition of *A. alternata* by *T. harzianum* (Kayım et al., 2018), 43.6 ± 6.7% PIRG of *A. niger* by *T. harzianum* (Morales-Mora et al., 2020), and *T. aggressivum* f. europaeum showed high antagonistic activity (≥80%) for different phytopathogens (Sánchez-Montesinos et al., 2021). In the present study, the dual culture of *T. asperellum* colonized half of the medium at 48 h and at the sixth day, this antagonist completely covered the whole medium surface with 93% inhibition of the pathogen radial growth. Hence, this isolate showed a higher biocontrol capacity than all the studies reported with several *Trichoderma* species.

5. Conclusions

This study identified a new autochthonous *Trichoderma* sp. isolate as *Trichoderma asperellum* through morphological and molecular analyses, and it was registered at the National Center for Biotechnology Information (NCBI) database with the accession No. MN95047. The *in vitro* confrontation of *T. asperellum* versus *Alternaria* sp. showed a prominent antagonist capacity (50–93%), indicating that this isolate can be a good candidate to control this phytopathogen in apple fields. Therefore, it is important to carry out research with this system under controlled conditions (greenhouse) and in the field, using various application methods.

This research reports for the first time a *Trichoderma asperellum* isolate from potato fields in Guasave, Sinaloa, Mexico. This fungus could be used as a biocontrol agent against some phytopathogenic fungi for the benefit of several types of crops and the environment.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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