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SHORT ARTICLE

The Morphological and Molecular Characterization of Trichoderma spp. in Cocoa Agroforestry System

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Abstract:

Species of the genus Trichoderma have been used as growth inhibitors and regulators of phytopathogens. The aim of this work was to study and molecularly characterize the species of the genus Trichoderma that can be found in the rhizosphere of cocoa agroforestry systems in the state of Tabasco, Mexico. Four composite samples were taken from each of three municipalities (Cardenas, Comacalco and Cunduacan). Thirty homogeneous sub-samples were taken (0-30 cm of depth) from each site (plantation) in a random zig-zag pattern across the middle part of the plantations, avoiding the edges. Thirteen strains of the Trichoderma genus were isolated and morphologically identified, while two species, T. harzianum and T. koningiopsi, were molecularly identified. Of the 13 strains, only five were amplified, and of these five, four corresponded to T. harzianum and one to T. koningiopsi.

Keywords: Phytopathogens, Rhizosphere, Agroforestry, Cocoa, Molecular

Introduction

The species Trichoderma harzianum was first described in 1871 by Harz, who emphasized the importance and the microscopic characteristics of Trichoderma, especially the presence of phialides[1]. The classification taxa are Kingdom: Fungi; Division: Ascomycota; Subdivision: Pezizomycotina; Class: Sordariomycetes; Order: Hypocreales; Family: Hypocreaceae and Genus:

Trichoderma; approximately with around 40 taxa of Trichoderma have been described to date[2], of which more than 20 have been reported to have some agricultural use. The cocoa agroforestry systems in Tabasco, Mexico, isolated and identified, morphologically and molecularly, nine Trichoderma species which include; Trichoderma asperellum, T. brevicompactum, T. harzianum/H. lixii, T. koningiopsis/H. koningiopsis, T. longibrachiatum/H. sagamiensis, T. pleuroticola, T. reesei/H. jecorina, T. spirale and T. virens/H. virens[3]. The genus Trichoderma have been used in biological control programs to control phytopathogens such as Phytophthora spp., Sclerotium cepivorum, Macrophomina phaseolina, Fusarium oxysporum, Rhizoctonia solani, and Pythium sp. [4, 5, 6 ,6, 7, 8, 9]. This development tripled the number of described species of Trichoderma, especially those that can be used for biological control, a true revolution in fungal systematics [10]. The secondary metabolites produced by Trichoderma have antifungal as well as mycoparasitism actions, defense response induction and resistance in host plants which are mechanisms that act synergistically [11]. In this mechanism, extracellular enzymes degrade the cell wall of pathogenic fungi, especially chitinases, lipases, proteases and glucanases [12]. The molecular identification of species of the genus Trichoderma has been done using different molecular biology techniques, including RAPD (Random Amplification of Polymorphic DNA) and UP-PCR (Universal Primer-Polymerase Chain Reaction). Reported the identification of Hypocrea crassa/T. crassum by analyzing the ITS1 sequence (internal transcribed spacer region) and tef1 (elongation factor 1 alpha), which were amplified by PCR using the primers ITS1 and ITS4 for the ITS, and primers EF1-728F and TEF1 for the tef1[13]. The several species at the molecular level using RAPD and rDNA-ITS markers, including T. harzianum, T. koningiopsis, T. atroviride, T. pseudo-koninngii and T. longibrachiatum [14,15]. In Mexico of the cocoa production is in located Chiapas and Tabasco. These states have a combined area of 61 444 ha. The planted area in Tabasco is 41 117 ha, from which are obtained 16 560 t of dry cocoa, sustaining 31 139 families 96% of the cocoa area is distributed in the municipality of Cardenas, Comalcalco, Cunduacan and Huimanguillo and 4% in the Teapa and Tacotalpa [16]. Therefore, the Comalcalco, Cunduacan and Cardenas is the main cocoa producing area in Tabasco, Mexico and where most studies on the cultivation of cocoa have developed. The agroecosystem that constitutes a cacao plantation is the closest thing to a tropical jungle; thus contributing to the mitigation of the effects of climate change, by capturing and filtering water into the aquifers, soil retention, oxygen generation, wildlife refuge, important role in the decomposition microbiology and transformation of matter Organizes, mainly within the soil, the rhizosphere zone between bacteria and fungi, which entails symbiosis, protection of biodiversity and assimilation of various nutrients and contaminants [17,18,19]. Among this interaction is the Trichoderma fungus, in which the diversity of species is unknown. Continuing with the study and molecular characterization of the Trichoderma species that can be found in the rhizosphere of different agricultural systems, including cocoa agroforestry systems.

Material methods

Location of the study area

The state of Tabasco located in the southeast of Mexico, between 17° 58′ 20″ N; 92° 35′ 20″ O. The prevailing climates Cfa (Humid subtropical climate); in the state are Am (Tropical monsoon climate) and Af (Tropical rainforest), which correspond to humid warm climates, according to the climatic classification of Köppen [20]. It has 191 km of coast (1.58 % of the total coastal length of the country). Its surface covers an area of nearly 25,000 square kilometers. Chontalpa is a subregion of Tabasco; in terms of territory, it is the second in importance, covering 7,482.13 km^2, that is, 31.34 % of the territory of Tabasco; it has 593,668 inhabitants, of which 241,168 live in urban areas and 352,500 in rural areas28. It is made up of three municipalities: Cunduacán, Cárdenas and Comalcalco (Table 1). A sampling site was selected within each municipality, 15 soil samples were taken at a depth of 20 cm, from which a composite sample was homogenized from each study site within the municipalities.

Sampling

Four composite samples were taken at each sampling site. Thirty homogeneous sub-samples were taken (0-30 cm of depth) from each site (plantation) in a random zig-zag pattern across the middle part of the plantations, avoiding their edges. The sub samples were then homogenized into composite samples (Table 1). Four samples were taken in each of the three municipal districts under study (Comalcalco, Cardenas and Cunduacán), for a total of 12 composite soil samples, each formed by 30 random homogeneous horizons (0-30 cm depth) subsamples.

Table 1. Location of the sampling sites in the municipalities of Tabasco under agroforestry systems

Municipalities	Location	Coordinates	Key of the strains
Comalcalco	Chipilín	18° 19' 03.8''N	T1, T2, T3, T4.
Cardenas	Arroyo hondo 2°	18° 01' 37.2''N	T5, T6, T7
	sección	17° 59' 33.1''N	T8
	Poblado C-28		
Cunduacan	Yoloxochilt 1ra sección	18° 06' 16.9''N	T9, T10
	Yoloxochilt 2da	18° 04' 55.7''N	T11
	sección	93° 16' 50.2''W	T12
	Miauhuathal		

Laboratory work

The experimental work was carried out in the Rizosphere Molecular Ecology Laboratory of the Sinaloa Unit of the Interdisciplinary Research Center for Regional Integral Development (CIIDIR); National Polytechnic Institute (IPN).

Isolation of strains

The isolation of Trichoderma strains was done using the plate dilution method following the protocol[21]. Ten grams of soil material were taken from the composite samples from each sampling site. The soil material from each sampling site was suspended separately in 500 mL Erlenmeyer flasks containing 100 mL of sterilized distilled water, and stirred for 5 min. One-mL aliquots were then taken with a graduated micropipette (100-1000 µL) and transferred to test tubes containing 4 mL of sterile distilled water. Serial dilutions (1/10 w/v) were made of each sample, and 0.5 mL aliquots from each dilution were seeded in Petri dishes containing PDA culture medium supplemented with ampicillin and amoxicillin (1 mg mL^{-1} of each), spreading the sample material uniformly on the surface of the culture medium using a glass L. There were three replications per dilution. The petri dishes were incubated at 25 °C ± 1 for eight days. Trichoderma strains were identified by their rapid and extended growth and white to green color, according to the criteria suggested18. Mounts were examined under the microscope to observe the morphological characteristics of conidia and conidiophores. The Trichoderma strains were then re-isolated to obtain pure colonies. After eight days, samples of mycelium were taken from the colonies using a platinum loop and streaked onto PDA culture medium. The petri dishes were incubated at 25 °C ± 1 for eight days and preserved for DNA extraction [22].

Morphological identification

The strains were identified based on the morphological characteristics of conidiophores and conidia, using the taxonomic keys [23, 24]. Sub-samples of each fully sporulated Trichoderma strain culture were mounted on slides, fixed with lactic acid and stained with lactophenol. The slides were examined under a compound microscope (Carl Zeiss) to observe morphological structures.

DNA extraction

The DNA of the Trichoderma species was obtained from the mycelium of the colonies grown in Petri dishes with PDA. The mycelium was homogenized in Eppendorf tubes and genomic DNA was extracted using the DNAzol Genomic DNA Isolation Reagent (cat. No. DN 127, Molecular Research Center, Inc.), following the manufacturer's instructions. This method is based on the use of a guanidine-detergent lysis solution that hydrolyzes RNA and allows the selective precipitation of DNA from a cell lysate. The DNA was quantified using a spectrophotometer (NanoDrop 2000, Thermo Scientific).

Amplification of DNA by PCR

The internal transcribed spacer region (ITS) was amplified by PCR using primers ITS1-5.8S rDNA (TCCGTAGGTGAACCTGCGG 290 pb) and ITS4 (TCCTCCGCTTATTGATATGC 330 pb)[25]. The PCR reaction mixture was prepared in a final volume of 25 µL (1X); it contained: H_2O (17.8 µL), buffer without $MgCl_2$ (2.5 µL), 10 nm dNTPS (0.5 µL), $MgCl_2$ (1 µL), ITS-1 (0.5 µL), ITS-4 (0.5 µL), Taq Polymerase (0.2 µL) and 2 µL of DNA (samples diluted 1:10). The amplification program consisted of 1 cycle of 95 °C for 4 min, 32 cycles of 95 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min, with a final cycle of 72 °C for 5 min. All PCR reactions were carried out in a thermal cycler (Labnet International Inc., MultiGene Gradient, Cat. TC9600-G, 090863 series).

Electrophoresis

The PCR products were analyzed by electrophoresis (Electrophoresis chamber, Galileo bioscience, model 80-0708, 023629 series) in agarose gel at 1% in 0.5X TAE buffer (Tris Acetate-EDTA), stained with ethidium bromide and subjected to electrophoretic separation at 95 V for 30 min (Electrophoresis Power Supply-EPS 301, Amersham Biosciences, T80010972 series), using a 1 kb DNA Ladder marker (Invitrogen, Cat. No. 15615-016). The bands were visualized in a gel documentation system (Bio Rad, Universal Hood II, 765/07032 series).

Purification of amplified DNA

The amplified PCR products were purified using the QIAquick PCR Purification Kit (Cat. No. 28104, QIAGEN) following the manufacturer's instructions. The DNA concentrations of the amplified products were measured with a spectrophotometer (NanoDrop 2000, Thermo Scientific). Subsequently, each purified PCR product with a minimum DNA concentration of 400 ng/µl was dried in a water bath at 45 °C, packaged and sent to the National Genomics Laboratory for Biodiversity (Langebio, CINVESTAV, Mexico; http://langebio.cinvestav.mx/labsergen/ for sequencing.

Phylogenetic inference

The sequences returned by Langebio were assembled and edited using the Software package DNASTAR Lasergene (version 7.0). The edited sequences were analyzed for homologies to known sequences using the BLAST-N program of the NCBI (https://blast.ncbi.nlm.nih.gov). A similarity of ≥ 97% was used as a threshold when comparing the values generated by BLAST-N with the sequences under study. Phylogenetic inference was performed using GenBank sequences. An Aspergillus Flavus sequence (GenBank access number: AF138287) was used as outgroup. Sequences of 16 Trichoderma species were also used: T. Harzianum (EU280079, LN846720, KU896349, AF057593 and AF057600), T. Pleuroticola (JQ040377), T. Virens (JQ040400), T. Spirale (EU280082), T. Brevicompactum (T. JQ040334), T. Reesei (JQ040380), T. Longibrachiatum (JN108926), T. Koningiopsi (JQ040370, FJ884183), T. Asperellum (JQ040317), T. Ovalisporum

(DQ315458), T. Erinaceum (EU280106), T. Hamatum (DQ109530), T. Viridescens (EU280104), T. Atroviride (KC469612), T. Rossicum (EU280089), and T. Crassum (EU280085). The sequences were aligned using the software MEGA 6 (version 6.06), eliminating the gaps between them. A phylogenetic tree was built based on the neighbor joining algorithm, using Maximum Parsimony as optimality criterion, with 1000 bootstrap repeats.

Results and discussion

Morphology

Table 2 shows the results of the present study regarding the isolation and morphological identification of strains of the genus Trichoderma/Hypocrea. A total of 13 strains were isolated and identified in the rhizosphere of cocoa agroforestry systems in the state of Tabasco. four strains were found in Comalcalco and in Cunduacan, while five were found in Cardenas. A study identified six species Trichoderma in the rhizosphere of T. cacao plants from the state of Carabobo, in Venezuela, and found nine species of the genus Trichoderma/Hypocrea in cocoa agroecosystems in the state of Tabasco: T. asperellum, T. longibrachiatum/H. sagamiensis, T. brevicompactum, T. koningiopsis/H. koningiopsis, T. spirale, T. pleuroticola and T. reesei/H. jecorina [25].

Table 2. Isolation and morphological identification of species of the genus Trichoderma . BLAST, NCBI.

Strain	% Identity	Access number GenBank	Organism Genbank
T1COMALCALCO	100	AF057600	Trichoderma harzianum
T2COMALCALCO	100	KU896349	Trichoderma harzianum
T3COMALCALCO	100	AF057593	Trichoderma harzianum
T5CARDENAS	100	FJ884183	Trichoderma koningiopsis
T10CUNDUACAN	99	LN846720	Trichoderma harzianum

The physical-chemical properties [26] of the soil in the sampling sites from where Trichoderma species were isolated were as follows: moderately acid (5.6) to neutral (6.9) pH, 9.3% of organic matter and bulk density of 1.26 g/cm^3. These soil properties indicate that Trichoderma fungi inhabit acid-neutral soils with a high content of organic matter that form sustainable ecosystems. Twenty-four hours after being seeded, the strains of the genus Trichoderma isolated from these soils grew a white, cottony mycelium of different hues, forming two to seven concentric rings (Figure 1). The colonies changed from light green to intense green at 48 h due to the high production of spores in the mycelium, until acquiring a final red wine color [27].

Regarding the microscopic characteristics (Figure 2) of the Trichoderma colonies, the 13 strains identified in the present study had conidiophores with the following general characteristics: long, hyaline, branched, not whorly, with individual phialides, or phialides in groups of two or three, emerging from small

terminal clusters at 90° from the conidiophore; the conidia are hyaline, single-celled, ovoid, some of them coarse or wrinkled. These characteristics coincide with those described by [28], who reported that several species of the genus Trichoderma stand out by their conidiophores with a tuft-like conidial mass of fertile conidia, and short branches growing at 90° or 100° from the conidiophore; they have one to four phialides, long, not whorly and single-celled, in the shape of a bottle, with small, irregular ramifications that emerge from the center of the vesicle, with conidia at the apex, globose, subglobose or ellipsoidal. Some Trichoderma species could produce chlamydospores in natural substrates; these structures are vital for the survival of Trichoderma species growing under adverse conditions, as demonstrated by the studies [29, 30].

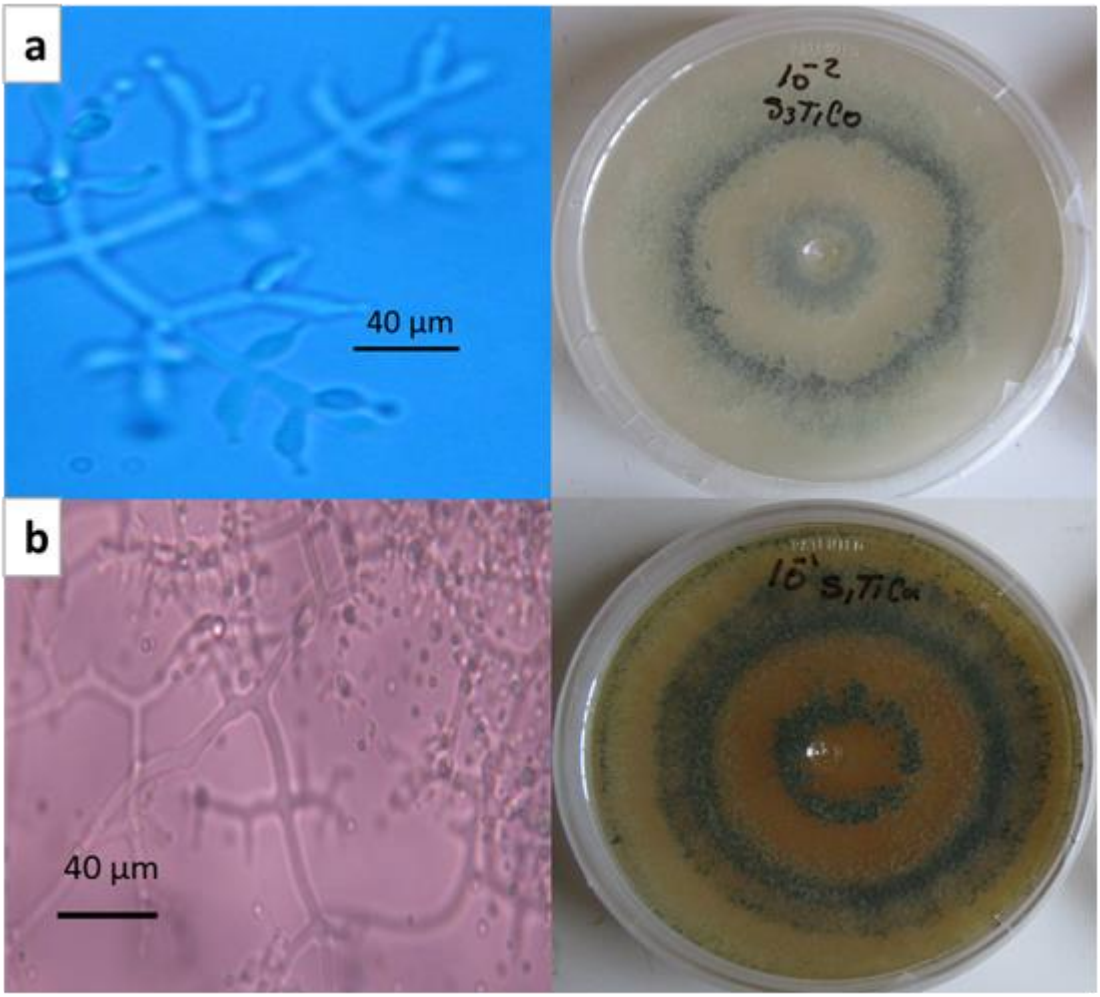

Figure 1. Macroscopic and microscopic characteristics of Trichoderma: a) T1 strain, white mycelium, green concentric rings, conidiophores and phialides; b) T2 strain, wine red mycelium at 48 h of growth, concentric dark green rings, conidiophores and phialides.

Amplification of genomic DNA by PCR

The molecular identification of Trichoderma species was performed using the primers ITS1-ITS431, which are universal primers for the amplification of the ITS region of fungal rDNA. The electrophoresis of amplified genomic DNA

resulted in bands between 500 and 750 bp. However, no bands were observed in most wells containing agarose gel and amplified genomic DNA PCR products. The absence of amplification bands in Trichoderma species may be due to the presence of products that inhibited PCR amplification in the DNA extracted with the DNAzol protocol [32].

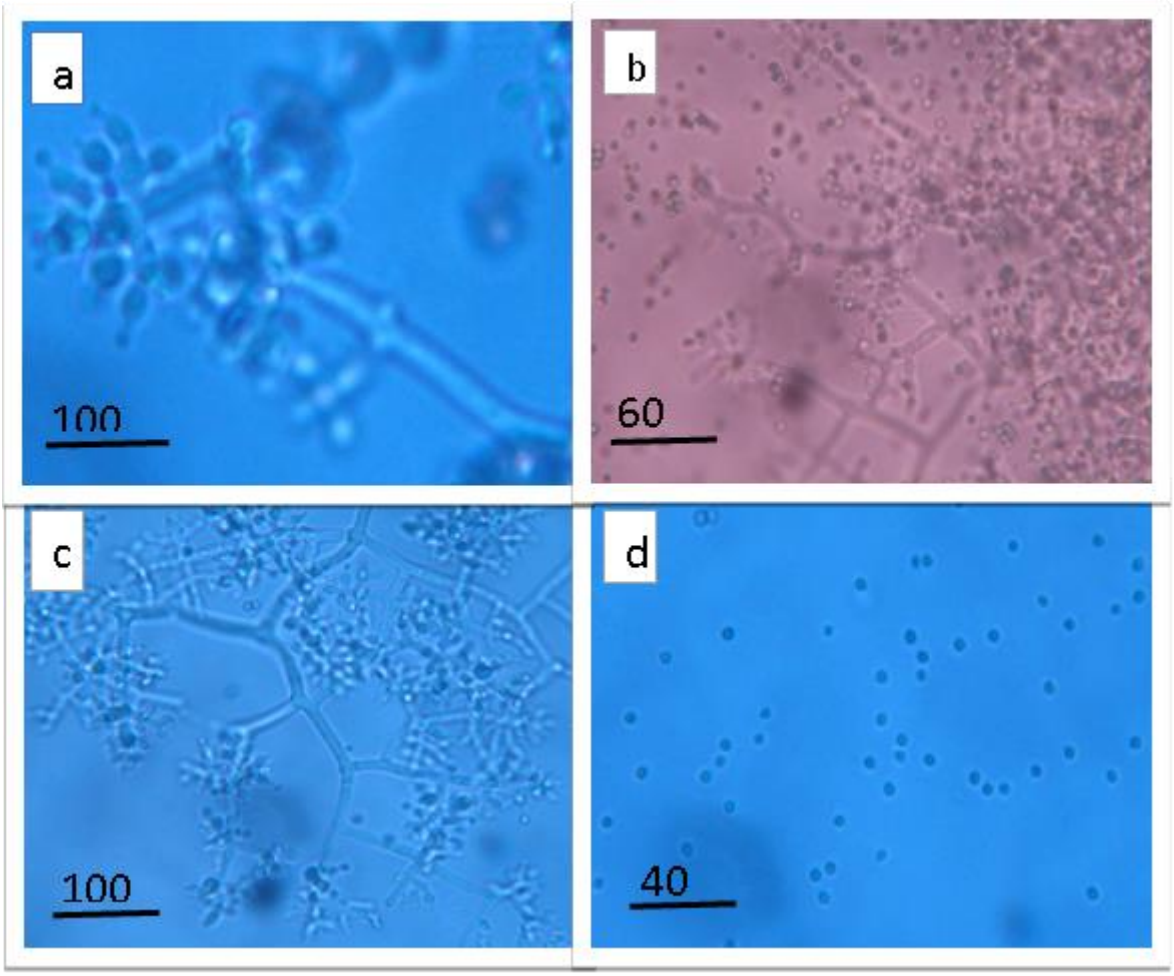

Figure 2. Microscopic characteristics of Trichoderma. T4 strain: a) phialides in groups emerging from small terminal clusters, b) conidiophores and conidia of ovoid shape; T6 strain: c) conidiophores and conidia, d) spores.

The amplified products of genomic DNA of the strains T1, T2, T3, T5 and T10, diluted 1:10, generated bands with molecular weight of approximately 500 to 620 bp, which was expected given the size of the partial sequences of the ITS1 and ITS4 regions of rDNA (522-620 bp) [35]. The bands show a faint marking, which may be a result of the genomic DNA extraction protocol used (DNAzol Genomic DNA Isolation Reagent). Three different protocols for the amplification of genomic DNA: 1) CTAB (Hexadecyltrimethylammonium bromide); 2) SDS (sodium Dodecyl Sulfate) + phenol: chloroform: isoamyl alcohol; 3) CTAB + PVP (Polyvinylpyrrolidone). Protocols 1 and 3 yielded faint degraded bands in electrophoresis, while protocol 2 yielded intense bands; however, protocol 2 takes more time and requires the use of highly toxic and expensive compounds. In contrast, the DNAzol method is less toxic, faster and relatively cheap [33].

Phylogenetic inference

Figure 3 shows the results of the alignment of the genomic DNA sequences of the strains T1Comalcalco, T2Comalcalco, T3Comalcalco, T5Cardenas and T10Cunduacan, using the BLAST tool of NCBI. The identity percentage was approximately 100% with the species T. harzianum and T. koningiopsis. The isolated, identified, morphologically and molecularly, nine Trichoderma species in soils of cocoa agroforestry systems in Tabasco, Mexico, including T. harzianum and T. koningipsis [34, 35]. Several works have studied its morphology, its molecular and genomic characteristics and the diversity of its pathogenesis, as well as its function as a biocontrol agent of plant diseases [36,37]. It has been found that Trichoderma strains are more resistant than most fungi to toxic compounds such as chemical fungicides and to the antimicrobial compounds produced by soil and plant microorganisms[38].

Figure 3 shows a phylogenetic tree for 16 species with sequences corresponding to the genus Trichoderma, including the GenBank access number of each of them. The outgroup is the species Aspergillus flavus. The five isolates from cocoa agroforestry systems are grouped in the branches corresponding to the species T. harzianum and T. koningiopsi, with support greater than 70%. Phylogeny was inferred using the neighbor-joining method based on the Tamura model (T92+G); the evolutionary distance was estimated using the distance-proportion method. The numbers in the nodes represent the bootstrap percentage with 1,000 repetitions. The scale bar = 0.2 substitutions per site. The choice of the outgroup and of the Trichoderma species was based on the results of the comparison of the nucleotides of the studied sequences, using the information obtained from the genbank database of the National Center for Biotechnology Information. In the present study, the most abundant species was T. harzianum (34%), which was present in 2 of the 12 sites sampled, followed by T. koningiopsi (8%), which was present in only 1 site.

Figure 4 shows the phylogenetic relationship of five Trichoderma isolates, with two species and Aspergillus flavus as outgroup, inferred by analysis of rDNA sequences (ITS1, 5.8S and ITS4). The grouping of the two species with T. harzianum and T. koningiopsi is reaffirmed as a basis to molecularly identify the five strains of fungi isolated and purified from cocoa agroforestry systems. The results of the BLAST queries showed that the sequences of the ITS gene of the T1COMALCALCO, T2COMALCALCO, T3COMALCALCO, T5CARDENAS and T10CUNDUACAN strains were 94-100% similar to sequences of species identified correctly as T. harzianum and T. koningiopsi. The sequences obtained were visualized, aligned, and edited using the software MEGA 6, which was also used to generate the phylogenetic trees (Figure 3 and 4). The obtained sequences were deposited in GenBank under access numbers KY490998-KY491002.

The molecular identification of the species can be done according to four different proposed concepts: 1) the Morphological Species Concept (MSC); 2) the Biological Species Concept (BSC); 3) the Phylogenetic Species Concept (PSC); and 4) the Evolutionary Species Concept (ESC). Argue that ESC is a purely theoretical concept and that the other three try to recognize evolutionary species3. The fungi species should be defined based on the PSC through the analysis of many polymorphic loci29. The identification of the Hypocrea/Trichoderma species has increased considerably with the analysis of sequences of different genes or of neutral sequences such as ITS29,15. The number of genetic markers that can be used to identify Trichoderma species has

increased in recent years; they include the transcription elongation factor (tef1), calmodulin (cal), actin (act) and RNA polymerase subunit II (rpb2).

The PCR protocol for the amplification of genomic ribosomal DNA for the identification of species14. This protocol was used in the present study to identify species of the genus Trichoderma isolated from samples taken from cocoa agroforestry systems. rDNA-ITS sequences were amplified with ITS1, ITS4 and 5.8S rDNA-based primers, and the obtained sequences were compared with sequences deposited in international databases such as EMBL-EBI (European Bioinformatics Institute) and NCBI.

As part of the molecular characterization of anamorphic Trichoderma strains, random molecular markers were used to obtain useful polymorphisms to monitor the colonization and survival of Trichoderma strains used in biological control assays. The most commonly used techniques for obtaining this type of molecular markers for biological control agents (BCAs) are: RAPD (Random Amplified Polymorphic DNA) by Abbasi et al. (1999), UP-PCR (Universal Primed PCR)6, T-RFLP (Terminal Restriction Fragment Length Polymorphism)7, and AFLP (Amplification Fragment Length Polymorphism).

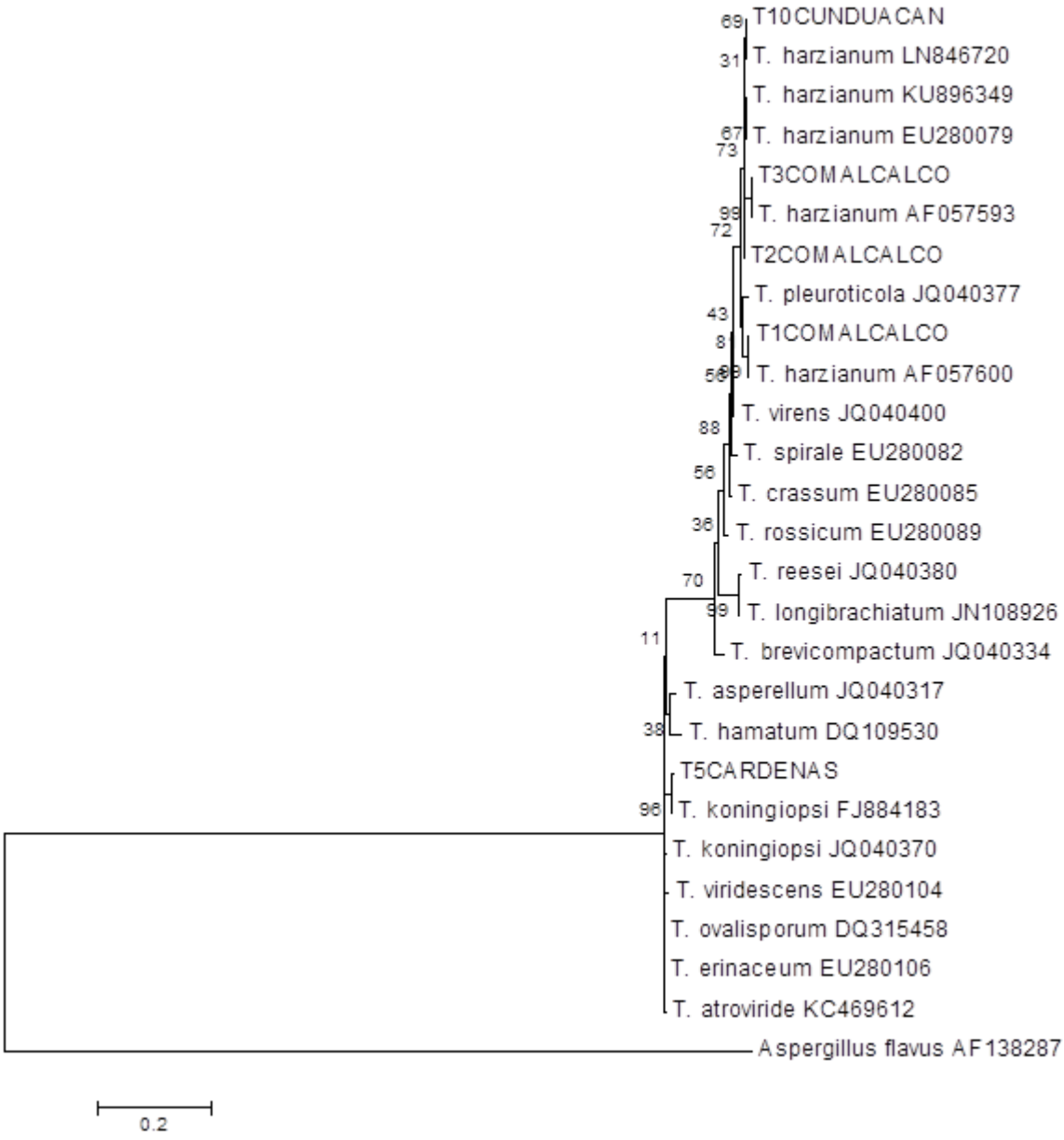

Figure 3. Phylogenetic relationship of five Trichoderma isolates, with 16 species and Aspergillus flavus as outgroup, inferred by analysis of rDNA sequences (ITS1, 5.8S and ITS4). The phylogeny was inferred by the neighbor-joining method based on the Tamura model (T92+G), and the evolutionary distance was estimated by the distance-proportion method. The numbers in the nodes represent the bootstrap percentage with 1,000 repetitions. The scale bar = 0.2 substitutions per site.

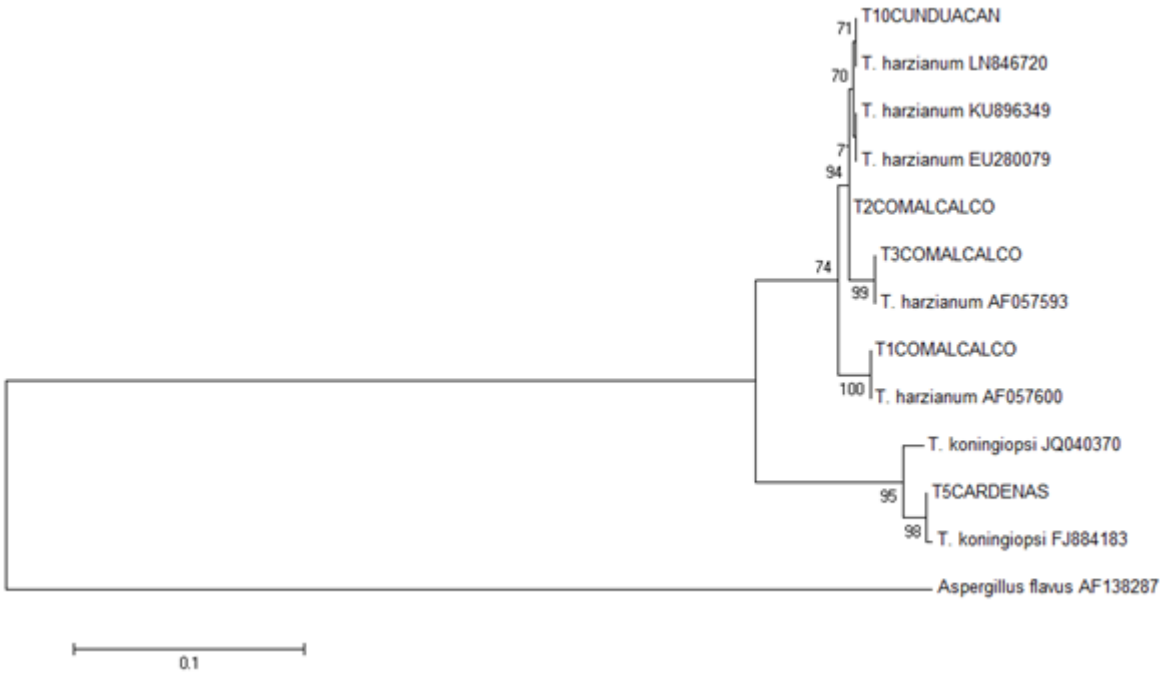

Figure 4. Phylogenetic relationship of five Trichoderma isolates, with two species and Aspergillus flavus as outgroup, inferred by analysis of rDNA sequences (ITS1, 5.8S and ITS4). The phylogeny was inferred by the neighbor-joining method based on the Tamura model (T92+G), and the evolutionary distance was estimated by the distance-proportion method. The numbers in the nodes represent the bootstrap percentage with 1,000 repetitions. The scale bar = 0.1 substitutions per site.

Conclusion

Twelve strains of the genus Trichoderma were morphologically identified in isolates from the rhizosphere of cocoa agroforestry systems in the state of Tabasco. Two Trichoderma species, T. harzianum and T. koningiopsi, were molecularly identified. Of the 13 strains, only five were amplified, and of these five four corresponded to T. harzianum and one to T. koningiopsi. These species of Trichoderma are currently some the most used agents for biological control programs against plant pathogens, and for promoting plant growth.

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