CONFRONTATION OF *Trichoderma asperellum* VSL80 AGAINST *Aspergillus niger* VIA THE EFFECT OF ENZYMATIC PRODUCTION

CONFRONTACIÓN DE *Trichoderma asperellum* VSL80 CONTRA *Aspergillus niger* A TRAVÉS DEL EFECTO DE LA PRODUCCIÓN ENZIMÁTICA

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RESUMEN

El género *Aspergillus* ha sido aislado a nivel mundial de plantaciones de cacao, sin embargo, no hay suficiente información sobre su implicación en la aparición de enfermedades. Las cepas de *Aspergillus niger* se caracterizaron morfológica y molecularmente. Las secuencias de *A. niger* se depositaron en las bases de datos del GenBank. También se evaluó el efecto antagonístico de *Trichoderma asperellum* VSL80 frente a las cepas de *A. niger*, obteniéndose un índice de biocontrol (BCI) de entre 15,36 y 88,71%. Se observó por microscopía electrónica de barrido que las hifas de *Trichoderma* crecieron en paralelo con las hifas de *A. niger* y en algunos casos se encontraron unidas con las hifas del hongo patógeno impidiendo su desarrollo. Por otro lado, la actividad enzimática máxima de xilanasas producida por *A. niger* en cultivo sumergido fue de 1,128 U mL⁻¹ a las 96 h. La caracterización morfológica y molecular de las cepas de *A. niger* confirmó su presencia en México. Además, la información obtenida del BCI puede ser importante para el desarrollo de estrategias para la prevención y control de este hongo. La actividad enzimática de *A. niger* durante el micoparasitismo por *T. asperellum* podría explicar el bajo BCI observado en algunas cepas de *A. niger* confrontadas con *T. asperellum*. Finalmente, la producción de enzimas xilanolíticas por *A. niger* indicó que este hongo podría usarse en la industria para obtener xilanasas.

Palabras clave: Biocontrol, micoparasitismo, *Theobroma cacao*, xilanasas.

ABSTRACT

The genus *Aspergillus* has been isolated from cocoa plantations worldwide. However, there is not enough information about its role in the occurrence of diseases. *Aspergillus niger* strains were characterized by morphological and molecular techniques. The sequences of *A. niger* were deposited in the GenBank databases. In addition, the antagonistic effect of *Trichoderma asperellum* VSL80 against *A. niger* strains was evaluated, obtaining a biocontrol index (BCI) between 15.36 and 88.71%. Scanning electron microscopy showed that the *Trichoderma* hyphae grew in parallel with the hyphae of *A. niger* and, in some cases, they were found together with the hyphae of the pathogenic fungus preventing their development. On the other hand, the maximum enzymatic activity of xylanases produced by *A. niger* in submerged culture was 1.128 U mL⁻¹ at 96 h. The morphological and molecular

characterization of *A. niger* strains confirms its presence in Mexico. In addition, information obtained of the BCI may be important for the development of strategies for the prevention and control of this fungus. The enzymatic activity of *A. niger* during mycoparasitism by *T. asperellum* could explain the low BCI observed in some *A. niger* strains confronted with *T. asperellum*. Finally, the xylanolytic enzyme production by *A. niger* indicated that this fungus could be used in the industry to obtain xylanases.

Key words: Biocontrol, mycoparasitism, *Theobroma cacao*, xylanases.

INTRODUCTION

Cocoa (*Theobroma cacao* L.) is a crop with great commercial value worldwide due to the large number of commercial applications cocoa beans have. Unfortunately, diseases caused by fungal pathogens decrease crop yield. In this sense, *Aspergillus* fungi are considered to be responsible for food spoilage due to mycotoxin production (Soares et al., 2013), and they can also lead to infectious diseases such as pulmonary invasive aspergillosis (Thompson and Patterson, 2008). This fungus is able to contaminate several foodstuffs, particularly cocoa (Mounjouenpou et al., 2012). In Mexico, few studies have focused on the identification of these cocoa fungi (Cuervo-Parra et al., 2011). The morphological and molecular identification of *Aspergillus niger* strains associated with cocoa crop is important for the development of strategies for their prevention and control.

Filamentous fungi secrete a number of enzymes that are capable of degrading cell wall components of plants (Vásquez, 2013). Cellulases have shown their potential application in textile bioclarification (Kuhad et al., 2011) and in the energy industry (Martínez-Anaya et al., 2008). The growing demand for these enzymes has promoted research, study and use of different lignocellulosic microorganisms. In this sense, filamentous fungi are the main source of hydrolases, including cellulase and xylanase, due to their easy handling and reduced costs (Izarra et al., 2010). *Aspergillus versicolor* and *A. niger* are the most widely used species in the production of industrial enzymes, mainly for their high levels of protein secretion, production of extracellular enzymes, easy retrieval and status generally recognized as safe (Gautam et al., 2011).

Therefore, the aims of this study were: (i) characterize morphologically and molecularly eleven strains of *A. niger*; (ii) test the antagonistic effect of *T. asperellum* VSL80 strain against *A. niger* strains; and (iii) determine the capacity for xylanase production by *A. niger* strains through solid medium and submerged fermentation culture production.

MATERIALS AND METHODS

Fungal strains

The *Aspergillus* strains used in this study were isolated from diseased tissue of cocoa fruits from commercial plantations located in the municipality of Huimanguillo, Tabasco State, Mexico. Strains were identified as HT-ITV18, HT-ITV21, HT-ITV24, HT-ITV28, HT-ITV39, HT-ITV42, HT-ITV47, HT-ITV49, HT-ITV51, HT-ITV52 and HT-ITV53. *Trichoderma asperellum* VSL80 antagonistic strain was obtained from the culture collection of the Genetic Laboratory of the Technological Institute of Veracruz, Mexico.

Morphological analysis

Morphological descriptions were based on comparisons with other strains of *A. niger* (Silva et al., 2011; Mounjouenpou et al., 2012; Vega et al., 2012). Pure culture strains were incubated in Potato Dextrose Agar (PDA) medium for 7 days and 25°C. Scanning electron microscope (SEM) pictures (JEOL, Model IT300, Boston, United States of America) was taken at Superior School of Apan-Universidad Autonoma del Estado de Hidalgo, Mexico.

Molecular characterization and phylogenetic analysis

DNA extraction from *A. niger* were performed as described by Cuervo-Parra et al. (2014). A region of nuclear DNA of each strain (containing the ITS I/5.8s/ITS II sequence) was amplified by PCR using the primers ITS1 and ITS4 (Kendall and Rygiewicz, 2005). PCR amplifications were performed in a total volume of 50 µl reaction, which contained: 10 µL of 5x Colorless buffer, 200 µM dNTPs, 0.2 µM of each primer, 2.5 units of Taq DNA polymerase, and 10-50 ng of template DNA. PCR reactions were placed in a thermal cycler (Techne PRIME3 Model, Series No. 31309, Staffordshire, United Kingdom) under the following parameters: initial denaturation at 95°C 5 min⁻¹, followed by 30 cycles of denaturation (95°C 1 min⁻¹), annealing (57°C 1 min⁻¹) and extension (72°C 1 min⁻¹), and a final extension period at 72°C 5 min⁻¹. The resulting products were purified with the Geneclean® II kit

(Bio101 Inc., California, United States of America) according to the manufacturer's protocol. DNA sequences from each strain were assembled using the Chromas 1.45 software (School of Health Science, Griffith University, Gold Coast Campus, Southport, Queensland, Australia). Sequences were submitted to a similarity search against sequences entries from the National Center for Biotechnology Information (NCBI) GenBank databases with the BLAST program (Tamura et al., 2013). Phylogenetic analysis of the aligned sequences was done by Neighbor-Joining (NJ) tree generated using Kimura's two-parameter model and supported by bootstrap method using 1000 random replicates with the MEGA v 6.0 software package (Tamura et al., 2013).

Confrontation experiments

The interactions between *A. niger* and *T. asperellum* VSL80 strains were evaluated by using the technique described by Szekeres et al. (2006). In Petri dishes with PDA medium, three-day-old *Trichoderma* mycelia discs of 5 mm in diameter were put at equidistant points and *A. niger* pathogen was left to confront. Petri dishes were incubated at 25°C in darkness. Antagonist activities were recorded after a week of incubation and digital images were taken at a distance of 18 cm with a camera (Cybershot DSC-P72, San Diego, California, United States of America). The percentage growth inhibition of the phytopathogenic fungus *A. niger* was calculated using the biocontrol index (BCI) according to the formula: $BCI = [A/B] \times 100$ (Szekeres et al., 2006), where letter A represents the area occupied by the colony of *T. asperellum*, and letter B represents the total area occupied by the colonies of *T. asperellum* VSL80 and *A. niger*. BCI was calculated using the software ImageJ (<http://rsbweb.nih.gov/>). Observations of the interaction region between *Trichoderma* and *A. niger* were made by SEM pictures according to Cuervo-Parra et al. (2014). Cuts were mounted on a pedestal with graphite conductive paint and coated with gold by evaporation method and sputtering (Bozzola, 2007).

Enzymatic production of *A. niger*

Aspergillus strains were grown on PDA medium at 25°C for 3 days. Then 5-mm mycelium disks from young fungi cultures were placed in 250 mL Erlenmeyer flasks containing 50 mL of potato dextrose (PD) broth according to Cuervo-Parra et al. (2011) at a concentration of 25, 50, 100 and 200 g L⁻¹ glucose, respectively. Erlenmeyer flasks were maintained at 25°C for 7 days under constant agitation at 120 rpm. Biomass was measured by dry weight (Collins et al., 1995).

Biomass quantification as dry matter

A spore suspension (1×10^6 spores mL⁻¹) was prepared in a sterile solution of Tween 20 at a concentration of 2% and collected on a cellophane sheet in 168 Petri dishes containing the culture medium (Arévalo et al., 2005). The amount of biomass was determined at different times for each culture medium concentration (25, 50, 100 and 200 g L⁻¹ glucose). The cellophane sheet containing the mycelium was dried in an oven at 60°C for 24 h, and stored in desiccators for 24 h. Subsequently, samples were weighed on an analytical balance. The sample was obtained by the difference in dry weight and the result was expressed as gram of dry matter (g DM).

Enzyme assays

Extracts were filtered using Whatman paper filter No. 1. Mycelium disc (5 mm) of *A. niger* were inoculated in 250 mL Erlenmeyer flasks containing 50 mL of minimal medium (Cuervo-Parra et al., 2011), and the flasks were incubated for 144 h (250 rpm, 30°C). Xylanase activity was determined according to Rawashdeh et al. (2005). Xylan was used as substrate; one unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar (using xylose as a standard) per mL per min. Protease activity was measured using the method described by Kunitz (1946). The enzyme activity was defined as the amount of enzyme required to release 1 μg of tyrosine per mL per min. Amylase activity was measured using the method described by Sugita et al. (1997). The enzyme activity was defined as the amount of enzyme required to hydrolyze 1 mole of glucose in 1 minute (AU min⁻¹) under constant experimental conditions. Cellulase activity was measured using the method described by Zhang et al. (2006). The enzyme activity was defined as the amount of enzyme present capable of releasing one μg of glucose h⁻¹ (μg h⁻¹, 1 mL⁻¹). Lipase activity was measured using the method described by Nawani et al. (1998). The enzyme activity was defined as the amount of enzyme that produced 1 mg of *p*-nitrophenol per mL per mL. Enzymatic activities were measured every 24 h. Statistical analysis was done as described above.

RESULTS AND DISCUSSION

Morphological analysis

The *Aspergillus* strains grown on PDA medium showed a colony diameter of 66-70 mm, with an initial growth of white color, which with time acquires a black coloration due to the production of spores (Fig. 1a). These observations

Fig. 1. *Aspergillus niger* morphology. (a-b) colonies on PDA; a) top view of the fungus; b) fungus culture viewed from below the Petri dish; (c-f) fungi viewed by SEM c) conidiophores, bar = 100 µm; d) vesicle, bar = 20 µm; e) chain of conidia, bar = 20 µm; f) conidia, bar = 5 µm.

Fig. 1. Morfología de *Aspergillus niger*. (a-b) colonias en PDA; a) hongo visto en la parte superior; b) cultivo de hongos visto por debajo de la placa de Petri; (c-f) hongos vistos por SEM; c) conidióforos, barra = 100 µm; d) vesícula, barra = 20 µm; e) cadena de conidios, barra = 20 µm; f) conidios, bar = 5 µm.

coincide with those reported by other authors for *A. niger* strains grown in Malt Extract Agar (Silva et al., 2011), and Czpeck Yeast Agar (Araujo-Blanco et al., 2016). The reverse side of the colony was colorless to light yellow (Fig. 1b). The conidial heads were dark brown to black, biserial, conidia globose, irregularly rough to finely rough, crests and grooves $2.9-4 \mu\text{m} \pm 0.7$. The conidial heads that present only phialides are characterized as uniseriate, whereas if they have metula and phialides they are biserial (Cruz, 2014). The hyphae are well developed, profusely branched, septate and hyaline near the apex (Vega et al., 2012). As a general rule the cells are multinucleated, with three well-defined zones (Nithiyaa et al., 2012). Of these, the first is the foot cell that joins the fruiting body with the somatic mycelium, which may or may not be septate (Eltem et al., 2004). The second zone is the conidiophore, with cylindrical and elongated shape, which projects the reproductive mycelium out of the somatic mycelium (Samson and Varga, 2007) in aerial form with negative geotropism (Araujo-Blanco et al., 2016). The young mycelium produces conidiophores in abundance that arises solitary from the somatic hyphae (Araujo-Blanco et al., 2016). The hyaline or pigmented conidiophores are long, erect, each ending in a bulbous head called vesicle, subglobally shaped, $254-300 \mu\text{m} (\pm 27)$ in diameter (Fig. 1c).

However, this structure can measure up to

2500 microns (Gautam and Bhadauria, 2012). The third zone is the vesicle, which develops at the apical end with a variable shape ranging from spherical, hemispherical, globular, subglobose or ellipsoidal (Abarca, 2000). A large number of conidiogenic cells are produced on the surface of the multinucleated vesicle as it develops, and are thus seen as radiated conidial heads. The vesicles of the conidiophore showed a thick, rounded, globose to subglobose wall (Colin et al., 2013), between $30-112 \mu\text{m}$ (Fig. 1d). Metula present between $10-20 \times 3-6 \mu\text{m}$. Conidiogenic cells, either primary or secondary, are typical phialides, ranging from $7-10 \times 3-4 \mu\text{m}$; as they reach maturity, these produce conidia one after another, in chains (Fig. 1e). Conidia are globular, $2.9-4 \mu\text{m}$ in diameter, ornate, brown, with rough walls, which form at the end of the tubular phialides (Fig. 1f). These asexual spores produce rosaries or chains of conidia (Cruz, 2014). By means of these structures, these fungi propagate clonally if environmental and nutritional conditions are favorable for their growth (Araujo-Blanco et al., 2016). As conidiophores and conidia are produced abundantly, their color is predominant in pigmentation of the colony, being black or brown. These observations allowed the identification of isolated strains within the *A. niger* species and are in agreement with Abarca (2000) and Vega et al. (2012).

Molecular characterization and phylogenetic analysis

Amplification of ITS I/5.8s/ITS II region of the ribosomal DNA (rDNA) isolated from each strain was carried out with primers ITS1 and ITS4 (Samson et al., 2014). In this study, 11 sequences of fungi were amplified; these regions had a variable size ranging from 530 to 609 base pairs (bp). Sequences were aligned using the Basic Local Alignment Search Tool (Morgulis et al., 2008) with other sequences of *Aspergillus* genus from GenBank, reaching a sequence identity in the range of 98 and 99%. Finally, a phylogenetic tree was constructed for the characterized strains and the phylogenetically related fungi.

The results obtained with the BLASTN program confirm that strains HT-ITV5, HT-ITV24, HT-ITV53, HT-ITV42, HT-ITV21, HT-ITV47, HT-ITV28, HT-ITV52, HT-ITV39, HT-ITV49, and HT-ITV18, deposited in the NCBI GenBank databases with the accession number KP963950, KF010173, K096352, KP963947, KF010172, KP963948, KF010174, KP963951, KP963946, KP963949 and KF010171, correspond to new strains of *A. niger* for Mexico.

The sequences examined in this study for the ITS I/5.8s/ITS II region, for the cocoa *A. niger* strains and other related sequences from NCBI GenBank databases had a length ranging from 413 to 1452 bp, and a length of 1758 bp of consensus region for the alignment sequences. Between ITS I and ITS II regions, the ITS I region was the most conserved *Aspergillus* sequence used in this study. For the analysis of 5.8S subunit, it was observed that all species of *Aspergillus* were highly conserved with only a few base changes (insertions, deletions and substitutions) in the sequences of *A. glaucus*, *A. niger*, *A. nidulans*, *A. ochraceoroseus*, *A. cremeus*, *A. flavipes*, *A. flavus* and *A. versicolor* strains. On the other hand, 18S and 28S subunits showed higher variations but limited to point mutations, insertions or deletions, predominantly among isolates of *A. fumigatus*, *A. sparsus*, *A. niger*, *A. candidus* and *A. flavus*. Deletions, insertions and base substitutions varied greatly within the ITS regions, especially in the ITS II, although many conserved regions were present in the ITS region I and ITS II. In general, the length of the sequences of the ITS I and ITS II regions was highly conserved in all species of *Aspergillus*, with limited variations to the few base change.

Phylogenetic analysis of *A. niger* cocoa sequences and 49 other representative *Aspergillus* sequences from GenBank were used to generate a tree by the Neighbour-Joining (NJ) method, forming three major clades (I, II, and III) based on sequencing of the ITS regions of rDNA sequences (Fig. 2). To be able to infer phylogenetic

relationships, the NJ tree was rooted with *Bacillus subtilis* (AB474001, KM659218) and *Lactobacillus fermentum* (AF382391) GenBank sequences as outgroup. The optimal tree with the sum of branch length = 2.04833715 is shown. The percentage of times in which the associated taxa were grouped together in the bootstrap test (1000 replications) is shown next to the branches in the phylogenetic tree. The three main clades represented by different sequences of species of *Aspergillus* genus were supported by high bootstrap values. Clade I could be subdivided into four subclades (I, II, III, and IV).

At the top of the subclade I, it can be observed that the Mexican cocoa strains were phylogenetically related to the NCBI GenBank sequences of *A. niger* from India (KY657577), Mozambique (AM745113), Portugal (KX897144), and Spain (AY656630). Considering the high bootstrap value obtained during the construction of the phylogenetic tree, these strains were easily distinguishable from the other *Aspergillus* sequences branches in other clades.

The subclade II includes sequences belonging to the Sections *Sparsi* and *Wentii* isolated from USA (EF661184, EF661183), France (FR670319), Ecuador (KX712427) and Brazil (KP691043). Subclade III comprises a large group, belonging to the Sections *Aspergillus*, *Ornati*, *Cervini*, *Clavati*, *Fumigati*, *Candidi* and *Restricti*, which include the species *A. glaucus*, *A. ornatus*, *A. cervinus*, *A. clavatus*, *A. fumigatus*, *A. candidus*, and *A. restrictus* isolated from different hosts and geographic regions (KX394538, KF298065, KM232504, EF669704, EF669705, HQ608146, AJ874118, JQ316526, KF669481, HQ844676, GU566242, JX421732, KP724998, JQ781823, LT626947, JQ724415, NR_135333, JX156352). On the other hand, subclade IV includes sequences for species of the Sections *Terrei* (KR610362, KF364668), *Flavipedes* (GU566209, JF817254), *Circumdati* (HQ843030, DQ336712), *Cremeri* (EF652148, NR_137455) and *Flavi* (AM745114, FJ878656, JQ316529), supported with high bootstrap values, ≥ 84 (Fig. 2).

The other two clades of the phylogenetic tree comprise species of the Sections *Ochraceoreosei* (NR_135381, JN217239), for clade II and species of the Sections *Nidulantes*, *Versicolor* and *Usti* for clade III, isolated from different host and geographic regions of the American continent (KR012899), Europe (KM063206, EU982032, FJ878645, KX690140, HE962600), Oceania (KF020313) and Asia (HQ116387, JF817276).

The clades generated in the phylogenetic analysis reveal the formation of groups corresponding to their morphologic characteristics, allowing for the identification of

Fig. 2. Neighbour-Joining tree derived from fungal ITS sequence data. Numbers on nodes are bootstrap values.

Fig. 2. Árbol filogenético generado por el método Neighbour-Joining derivado de datos de secuencias de ITS de hongos. Los números en los nodos son valores de bootstrap.

the species within each of the Sections. Similarly, the use of the ITS regions of the rDNA genes allowed the comparison of isolated cocoa strain sequences with other sequences related from other GenBank specimens. This result confirms that the strains identified as *A. niger* may belong to different specimens of these species, based on bootstrap values that support these alignments. The morphological differences constitute an important tool for the characterization of new species, even in members of the same clade (Silva et al., 2011). On the other hand, mutation and recombination are a major source of genetic variation in fungal pathogens (Zhang et al., 2013). Recombination, specifically, allows horizontal gene transfer or lateral gene transfer in fungi species (Fitzpatrick, 2012). Furthermore, within a species, genes flow between populations; propagules which diffuse from one epidemiological area to another and from one population of closely related interbreeding organisms to the next add to this process. This would explain the observed differences between the cocoa *A. niger* sequences isolated in this study and the GenBank sequences of the same species compared.

Confrontation experiments

The antagonistic ability of *T. asperellum* VSL80 strain was tested against *A. niger* cocoa strains by confrontation experiments in dual culture. The digital images taken on 7 days of incubation (Fig. 3) were used to calculate the BCIs values.

A characteristic that gives an advantage to an antagonistic microorganism over phytopathogens is its ability to grow rapidly and compete for

space and nutrients with pathogens (Benítez et al., 2004). In this sense, after 7 days of incubation, *T. asperellum* VSL80 grew rapidly on the culture medium in most of the Petri dishes. However, the BCI values of *T. asperellum* VSL80 against the *A. niger* strains studied ranged from 15.36 ± 0.150 and 88.71 ± 0.015 , respectively (Table 1).

The results obtained in this study showed a variable effect for the antagonistic interaction observed between *T. asperellum* VSL80 strain and *A. niger* strains confronted. This behavior has been observed in other *T. asperellum* strains. El_Komy et al. (2015) observed that 30 strains of *T. asperellum* confronted with four *Fusarium oxysporum* strains, which represent a major limiting factor of tomato plantations, showed moderate inhibition of the pathogen growth of 61 to 65%, whereas only 6 of these strains caused a significant reduction of *Fusarium* growth (68-71%). Other authors reported a variable antagonistic effect of *Trichoderma* spp. against *A. niger* strains derived from jack bean (*Canavalia ensiformis*) (Amorim and Pascholati, 2011). On the other hand, a commercial strain of *T. asperellum* (Qualiti WG®, Nova city, Brazil) presented a variation in their antagonistic effect of 66.6% against *A. niger* strain isolated from jack beans (Lima et al., 2016). The results obtained between *T. asperellum* and *A. niger* interaction coincide with these studies and with numerous previous studies (Tondje et al., 2007; Marcello et al., 2010; de los Santos-Villalobos et al., 2013). In this study, the interaction of *T. asperellum* VSL80 strain with *A. niger* isolates resulted in significantly different amounts of inhibition of the pathogen confronted. The most susceptible *A. niger* isolates

Fig. 3. Digital images taken at 7 days of confrontation of *T. asperellum* VSL80 against *T. cacao* phytopathogenic fungi (*A. niger*) on PDA medium.

Fig. 3. Imágenes digitales tomadas a los 7 días de confrontación de *T. asperellum* VSL80 contra hongos fitopatógenos de *T. cacao* (*A. niger*) en medio PDA.

Table 1. Biocontrol index (BCI) of *T. asperellum* VSL80 against *A. niger* strains.
Tabla 1. Índice de biocontrol (BCI) de *T. asperellum* VSL80 contra cepas de *A. niger*.

Phytopathogenic fungi	Strain number	BCI ± SD* (%)
<i>A. niger</i>	HT-ITV42	81.04 ± 0.014
<i>A. niger</i>	HT-ITV53	82.41 ± 0.015
<i>A. niger</i>	HT-ITV21	75.51 ± 0.147
<i>A. niger</i>	HT-ITV47	62.29 ± 0.345
<i>A. niger</i>	HT-ITV18	48.94 ± 0.356
<i>A. niger</i>	HT-ITV51	15.36 ± 0.150
<i>A. niger</i>	HT-ITV28	70.21 ± 0.014
<i>A. niger</i>	HT-ITV49	88.71 ± 0.015
<i>A. niger</i>	HT-ITV24	27.49 ± 0.225
<i>A. niger</i>	HT-ITV39	34.96 ± 0.159
<i>A. niger</i>	HT-ITV52	66.74 ± 0.159

* Standard deviation.

(over > 70% growth inhibition) were the strains HT-ITV49, HT-ITV53, HT-ITV42, HT-ITV21 and HT-ITV28, while strains HT-ITV51 and HT-ITV24 were the least inhibited (Table 1). These results are consistent with those obtained in a study conducted by Markovich and Kononova (2003), who reported that *Trichoderma* mycoparasite capacity varies between isolates and species tested. In this sense, some pathogenic strains of fungi of the genus *Fusarium* have been reported to have deleterious effects on *Trichoderma* spp., by producing mycotoxins that would have a direct antagonistic activity that can affect *Trichoderma* genes related to the process of mycoparasitism (El_Komy et al., 2015). Other authors reported the presence of protein in the cell wall of some pathogenic fungi, which would make these strains more resistant to the mycoparasitism exerted by *Trichoderma* (Sivan and Chet, 1989).

Subsequently, SEM photographs of the interaction zone between *Trichoderma* and *A. niger* were taken at 31 days of incubation to evaluate the mycoparasitism exerted by *T. asperellum* VSL80 on the *A. niger* strains. An initial rapid increase of the *Trichoderma* VSL80 colonies was observed in most cases at the first 7 days, while the areas did not change considerably during the following periods in the interaction with the strains HT-ITV51, HT-ITV52 and HT-ITV53 of *A. niger*. On the other hand, a progressive inhibition zone produced by *T. asperellum* VSL280 against *A. niger* HT-ITV42, HT-ITV47, HT-ITV18 and HT-ITV49 strains was observed. In addition, a clear mycoparasitism effect with the formation of pustules in the strains HT-ITV21, HT-ITV28 and HT-ITV39 of *A. niger* was detected. In these isolates, an overgrowth and sporulation of *T. asperellum* VSL80 were observed on the colonies of the pathogen (Fig. 4). Meanwhile, the

interaction between strains HT-ITV51 and HT-ITV24 of *A. niger* with *T. asperellum* VSL80 strain produced less inhibition. The mycoparasitism effect of *T. asperellum* VSL80 on morphological deformations and disorganization in the structure of *A. niger* abnormal hyphal morphology and lysis of mycelia was observed on the interaction areas (Fig. 5). During the confrontation, there is secretion of hydrolytic enzymes such as chitinases and β -1, 3-glucanases, which hydrolyze the major structural components of the cell walls of pathogenic fungi (Qualhato et al., 2013). The relationship between the antagonistic capacity and the production of hydrolytic enzymes by each *Trichoderma* isolate is fundamental during mycoparasitism (El_Komy et al., 2015).

Biomass and enzyme production

In general, *A. niger* strains rapidly reached their maximum growth at 168 h in the tested conditions. According to Meijer et al. (2011), all *A. niger* strains have similar growth profiles on monosaccharide substrates, but they grow best on starch and pectin. Therefore, the growth of *A. niger* strains in a defined media can be used as a first step in the identification and isolation, since these fungi do not differentiate among strains of the same isolated species.

Biomass determination in dry weight

Biomass quantity is an essential parameter in kinetic studies and for the characterization of the optimum growth of different fungi. The content of the different cell components can change markedly in fungi depending on fungal species, growth conditions, culture age and chemical composition in the media (VanderMolen et al., 2013). In this study, the microbial biomass is a fundamental variable expressed as dry weight,

Fig. 4. Morphology of *T. asperellum* VSL80 strain at 31 days of interaction with *A. niger* HT-ITV21 strain (a); production of green and yellow pustules on the colonies of the *A. niger* HT-ITV39 (b) and HT-ITV21 strains (c) on PDA medium.

Fig. 4. Morfología de la cepa VSL80 de *T. asperellum* a los 31 días de interacción con la cepa *A. niger* HT-ITV21 (a); producción de pústulas verdes y amarillas en las colonias de las cepas *A. niger* HT-ITV39 (b) y HT-ITV21 (c) en medio PDA.

Fig. 5. Scanning electron microscopy micrographs (a, b, c, d and e) showing the mycoparasitic effect by *T. asperellum* VSL80 on PDA medium: (a) Mycelia of *A. niger* parasitized by *T. asperellum* colonies, bar = 100 µm; (b) Mycoparasitism by apresorios structures on conidia of *A. niger* by *T. asperellum*, bar = 5 µm; (c) Mycoparasitism by envelopment of *A. niger* conidiophore by *T. asperellum* hyphae, bar = 20 µm; (d-e) Structural damage caused in conidiophores of *A. niger* by *T. asperellum* enzymes, bar = 20 µm.

Fig. 5. Micrografías de microscopía electrónica de barrido (a, b, c, d y e) que muestran el efecto micoparasitario de *T. asperellum* VSL80 en medio PDA: (a) Micelios de *A. niger* parasitados por colonias de *T. asperellum*, barra = 100 µm; (b) Micoparasitismo por estructuras apresorios en conidios de *A. niger* por *T. asperellum*, barra = 5 µm; (c) Micoparasitismo por envolvimiento del conidióforo de *A. niger* por hifas de *T. asperellum*, barra = 20 µm; (d-e) Daño estructural causado en conidióforos de *A. niger* por enzimas de *T. asperellum*, barra = 20 µm.

which is important for the determination of growth kinetics. In this sense, a direct relationship was found between the dextrose concentration and dry biomass obtained (Fig. 6). In addition, it has been widely reported that *A. niger* is equipped for utilizing sugar as the sole source of carbon. The growth and allocation of *A. niger* response to saccharides generally includes increasing entire colonies, biomass, and reducing the level of carbohydrates in the surrounding environment (Gupta and Neha, 2012).

Xylanase production in liquid fermentation

The highest xylanase production by *A. niger* strains was observed at 96 h (1.128 U mL⁻¹ protein). The β -1,4-xylans are heteropolysaccharides consisting of β -1,4-xylopyranosyl residues, whose groups (such as D-glucuronic acid, L-arabinose, *p*-coumaric acid and ferulic acid) are attacked by enzymes capable of degrading xylan (Seidl et al., 2006). The xylanases production has been studied using different substrates: glucose, rice bran (1.0%, 0.5%), rice straw (1.0%, 0.5%), coarse sugarcane bagasse (1.0%, 0.5%), fine sugarcane bagasse (1.0%, 0.5%), corncob (1.0%, 0.5%), wheat bran (1.0%, 0.5%), xylan (oat spelt; 1.0%, 0.5%), xylan (birchwood; 1.0%, 0.5%), corncob (0.5%) + fine sugarcane bagasse (0.5%), wheat bran (0.5%) + fine sugarcane bagasse (0.5%), wheat bran (0.5%) + corncob (0.5%; De Alencar-Guimaraes et al., 2013) and birchwood xylan (Peixoto-Nogueira et al., 2009).

In addition, there are many studies on xylanase production using several *Aspergillus* species, for example, *Aspergillus terreus*, *A. niger*, *A. terricola*, *A. foetidus*, *A. niveus*, *A. ochraceus* and *A. ficuum*. De Alencar-Guimaraes et al. (2013) observed that 1% wheat bran was the best carbon source for xylanase production by *A. niger* (12.76 U mg⁻¹ of protein), followed by fine sugarcane bagasse 0.5% (10.64 U mg⁻¹ of protein) and corncob 1% (10.00 U mg⁻¹ of protein). For *A. flavus*, the xylanase production was better induced by wheat bran at a concentration of 1% and 0.5% (8.03 and 8.70 U mg⁻¹ of protein, respectively), followed by fine sugarcane bagasse of 1% (7.68 U mg⁻¹ of protein).

The interactions that occur between two microorganisms during mycoparasitism result in the degradation of structures of the cell wall of the pathogenic fungus by the antagonistic microorganism of the genus *Trichoderma* (Peberdy, 1990). From Table 2, it can be observed that all strains of *A. niger* grown in broth PD with sucrose as source of carbon, produced several hydrolytic enzymes in the culture medium. This showed their maximum enzymatic activity at different times. This result could explain the low BCI observed in some *A. niger* strains confronted with *T. asperellum*.

CONCLUSIONS

The morphological and molecular characterization of the strains studied in this

Fig. 6. Dry biomass of *A. niger* cultivation at different dextrose concentration (♦25 g ■50 g ▲100 g and ×200 g).

Fig. 6. Biomasa seca del cultivo de *A. niger* a diferentes concentraciones de dextrosa (♦25 g ■50 g ▲100 g y ×200 g).

Table 2. Enzymatic activities of *A. niger* strains growth in PD broth as carbon source.**Tabla 2. Actividades enzimáticas de cepas de *A. niger* cultivadas en caldo PD como fuente de carbono.**

Hours	Protein mg mL ⁻¹	Enzymatic activities ± SD* (U mL ⁻¹)				
		Xylanases	Proteases	Cellulases	Amylases	Lipases
0	0	0.00	0	0	0	0
24	44.69 ± 1.7	0.383 ± 0.012	21.48 ± 0.2	11.79 ± 0.8	7.29 ± 0.1	0.21 ± 0.01
48	152.47 ± 2.1	0.785 ± 0.007	45.23 ± 0.3	5.86 ± 0.1	11.19 ± 0.05	0.55 ± 0.006
72	214.14 ± 6.1	0.882 ± 0.021	39.08 ± 1.7	5.15 ± 0.2	9.69 ± 0.1	0.89 ± 0.006
96	301.36 ± 8.8	1.128 ± 0.006	28.52 ± 0.5	2.73 ± 0.1	7.72 ± 0.1	1.15 ± 0.002
120	359.97 ± 6.6	0.768 ± 0.061	15.72 ± 0.2	1.62 ± 0.04	3.89 ± 0.2	0.74 ± 0.01
144	425.25 ± 3.6	0.256 ± 0.007	7.32 ± 0.1	0.60 ± 0.02	1.66 ± 0.7	0.27 ± 0.17

* Standard deviation.

work confirm the presence of the *Aspergillus niger* fungi in cocoa plants. It represents the first report of this fungus as a cocoa pathogen. In addition, information obtained on the BCI may be important for the development of strategies for the prevention and control of this fungus. *Trichoderma* isolates from cocoa showed antagonistic activity. The enzymatic activity of *A. niger* during mycoparasitism by *T. asperellum* VSL80 could explain the low BCI observed in some *A. niger* strains confronted with *T. asperellum*. Finally, the results obtained in this study for the xylanolytic enzyme production by *A. niger* cocoa strains through solid medium and submerged fermentation culture production indicate that this fungus could be used in the industry to obtain xylanases.

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