Presence of endophytic fungi in cacao plantations (*Theobroma cacao* L.), in the state of Tabasco, Mexico

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ABSTRACT

Objective: The present work was done with the objective of identifying endophytic fungi associated with *Theobroma cacao* L. in Centro, Cunduacán and Comalcalco, locations in the state of Tabasco, Mexico. The molecular identity used was the region of the Internal Transcribed Spaces (ITS), ITS 1 and ITS 4.

Design/methodology/approach: The study identified 15 fungal strains, grouped into 13 different species, belonging to the *Ascomycota phylum*, distributed in three different classes: *Dothideomycetes*, *Eurotiomicetos* and *Sordariomycetes*. It is important to mention that it is the first record of *Endomelanconiopsis endophytica* and *freycinetiae* found in cacao in Tabasco. In addition, we also identified *Aspergillus foetidus*, *Fischeri*, *Delicatus arcoverdensis*; *Thielaviopsis ethacetica*, *Cophinforma atrovirens*, *Neurospora udagawae*, *Diaporthe miriciae*, *Nodulisporium indicum*, *Cophinforma atrovirens*; *Colletotrichum tainanense* y *hebeiense*.

Findings/conclusions: Many of these endophytic fungi produce secondary metabolites and antioxidants that can be used in the medical industry or for biological control of phytopathogenic diseases, such as *Moniliophthora roreri*.

Keywords: Cocoa, fungi, endophytes.

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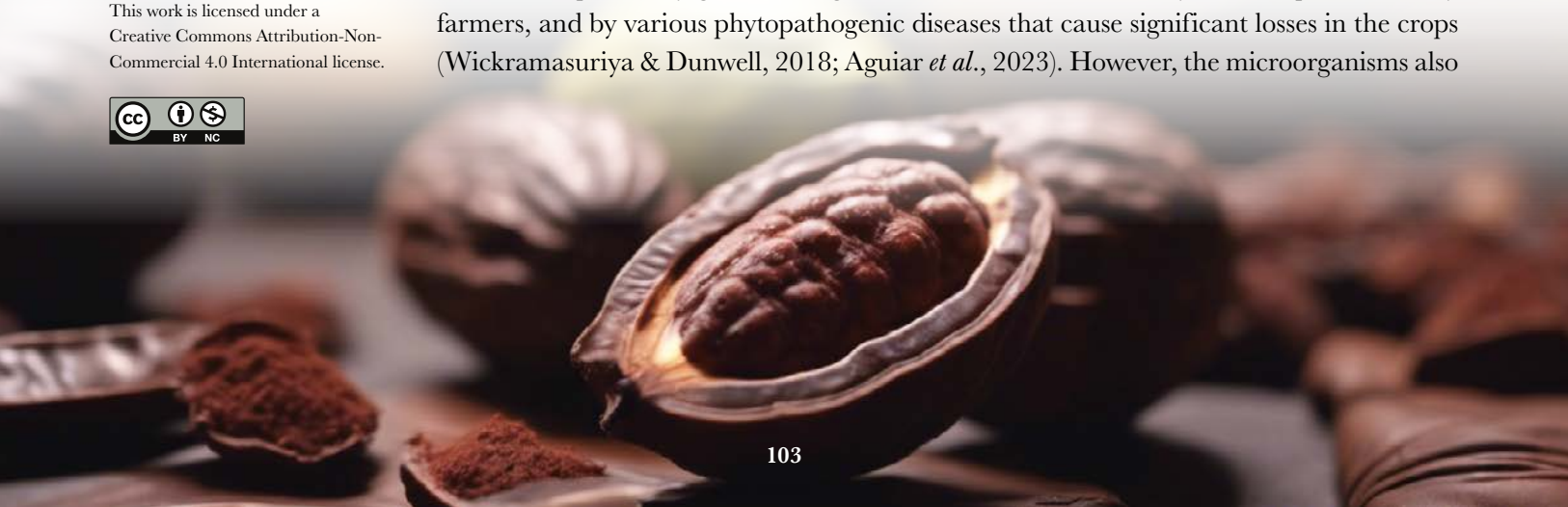
INTRODUCTION

The agrifood sector is one of the most important socioeconomic activities in the world because it provides a large diversity of food products to satisfy human needs. This sector has been impacted by global changes that influence the economy and the production by farmers, and by various phytopathogenic diseases that cause significant losses in the crops (Wickramasuriya & Dunwell, 2018; Aguiar *et al*., 2023). However, the microorganisms also



carry out an important role in the ecosystem; plants could not survive without mutualist microbes, since they improve the immune system, promote growth, and eliminate the diseases transmitted by the soil; the microbiome is considered as a gene reservoir. Cacao (*Theobroma cacao* L.) from the *Malvaceae* family, is one of the most important crops in the world and has faced various problems, primarily from phytopathogenic diseases (Aikpokpodion *et al.*, 2009). It is cultivated in more than 58 countries of Africa, America, Asia and Oceania. The International Cocoa Organization (ICCO) reported a global production of 4,923 thousand tons in 2021/2022 (MIDAGRI, 2022). In 2021, the Ministry of Agriculture and Rural Development (*Secretaría de Agricultura y Desarrollo Rural*, SEDER) reported that Mexico occupies the fourteenth producer at the global level with 28,106 tons of grain and 44,500 to 47,800 hectares of cacao; Tabasco, Chiapas and Guerrero are the main producing regions. Grain production has been affected primarily by phytopathogenic fungi with losses of 30 to 70% (Díaz *et al.*, 2020). Some important phytopathogens are: *Moniliophthora roreri*, *M. perniciosa*, (Bailey *et al.*, 2018); *Phytophthora palmivora*, *P. theobromicola*, and *Nodulosporium* sp., (Decloquement *et al.*, 2021; González *et al.*, 2019). Other fungi reported in the literature are endophytes which inhabit plants without causing apparent symptoms of a disease, in a balanced antagonistic relationship, in which nutrients and residence are provided for the fungus. In addition, the fungus favors the immune system of the host, produces secondary metabolites, and improves the resistance to pathogens (Tiwari & Bae, 2022). The following have been identified as endophytic fungi of plants: *Fusarium graminearum*, *F. equiseti*, *Lasiodiplodia jatrophicola* (Cruz *et al.*, 2022). In *T. cacao*, the following have been isolated: *C. gloeosporioides*, tropicale, theobromicola (Christian *et al.*, 2019); *L. theobromae*, *F. chlamydosporum*, *F. oxysporum*, *Verticillium luteo* (Rubini *et al.*, 2005), to mention a few. Because of this, and due to the great importance that fungi organisms have in plants, specifically in *T. cacao*, the objective of this study was focused in the isolation and the molecular identification of endophytic fungi of three cacao plantations in the state of Tabasco, with the aim of contributing knowledge about the fungal diversity of this important crop for Mexico and the state of Tabasco.

MATERIALS AND METHODS

Sampling sites

Three sites were selected for sampling in the state of Tabasco, Mexico (Figure 1): Centro (17° 58' 39.0" N; 93° 03' 45.0" W -Hacienda Buena Vista); Cunduacán (18° 06' 14.8" N; 93° 18' 26.3" W -Hacienda Río Seco); and Comalcalco (18° 15' 54.2" N; 93° 13' 39.9" W -Hacienda a Luz).

Collecting the plant material

The collection was done in March, 2019, with random sampling by selecting healthy and infected fruits and leaves, the latter with a slight infection; small dark spots with oily appearance or deformations; fruits that presented necrosis or white powder characteristic of a fungal disease were not selected (Aikpokpodion *et al.*, 2009). Five cacao plants per plantation were selected, and two fruits and two leaves were collected from each individual (a healthy one and an infected one), with a total of 60 samples. The leaves selected were

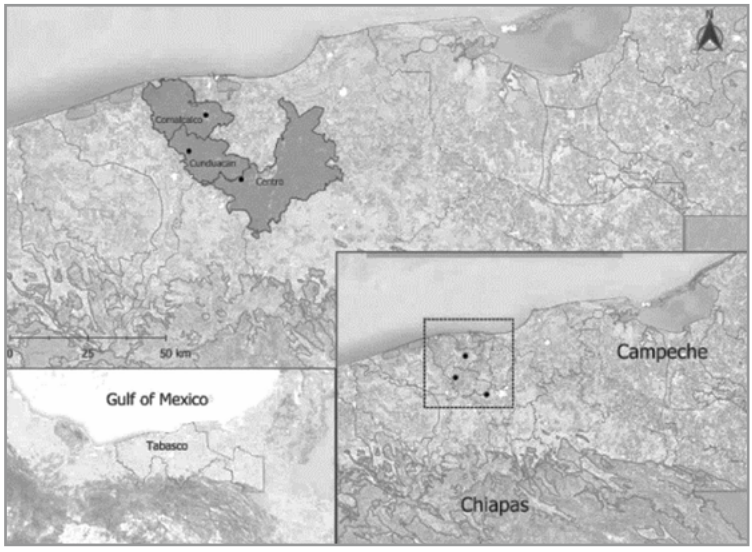


Figure 1. Location of the three collecting sites in the municipalities of Centro, Cunduacán and Comalcalco, in the state of Tabasco, Mexico.

cut with a pole previously disinfected with alcohol at 70%, while a pruning scissor was used for the fruits. The plant material was placed in Kraft paper bags, with the corresponding data, and conserved at a temperature of 4 °C. The isolation of fungi was carried out in the microbiology laboratory, while the molecular analyses were carried out in the genomic laboratory of Universidad Juárez Autónoma de Tabasco in the Academic Biological Sciences Division (*División Académica de Ciencias Biológicas*, UJAT-DACBiol).

Isolation of the fungal material

The processing of the samples was carried out under controlled sterile conditions in a laminar flow bell. The methodology for fungi isolation was the one proposed by Cañedo and Ames (2004), and Azuddin *et al.* (2021). Small fragments of 3 to 5 mm were cut from the borders with lesions and healthy tissue, using a sterile scalpel blade. Consecutively, the plant tissue was disinfected with hypochlorite at 1% and alcohol at 75%, each during one minute, and washed with sterile tri-distilled water (30 seconds). Four to five fragments were transferred to moisture chambers and some cuts were placed on a potato dextrose agar plate (PDA-Bioxon®). They were incubated at 27 °C for 3 to 5 days. The purification was done by transferring hyphae growth to PDA plates, to obtain new monosporic growth.

DNA extraction, PCR amplification, and DNA sequencing

The isolates obtained were transferred to 40 mL of potato dextrose broth (PDB) in Erlenmeyer flasks of 250 mL, incubating at room temperature for 3 to 7 days to obtain mycelium growth. The resulting mycelium was filtered with Miracloth paper (20-25 μm) washed twice in sterile tri-distilled water. Later, the mycelium is pulverized with liquid nitrogen (N_2) with the help of a porcelain pestle and mortar. The total genomic DNA was extracted from the mycelium of each of the individual isolates according to the protocol proposed by Stirling (2003). The quality of the DNA was analyzed with a spectrophotometer

at a wavelength of A260 nm and the purity based on the A260/280 rate. Electrophoresis in agarose gel at 1.5% dyed with ethidium bromide was used to verify the integrity of the DNA (0.5 μg/mL).

Amplification by Polymerase Chain Reaction (PCR)

The region of the Internal Transcribed Spaces (ITS), between ribosomal (rADN) 18S-5.8S and 5.8S-28S were amplified by PCR in each sample. The first were ITS1 (5´-TCCGTAGGTGAACCTGCGC-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´), and the amplification protocol was the one proposed by White *et al.* (1990). The PCR amplification per sample consisted in: 15 μL of ultrapure water free of nucleases, 10 μL of 5X green: 1 μL of the following reagents: bovine serum albumin (BSA), 0.2mM dNTPs, MgCl at 1.5 mM, 10 μM of each starter, 1.25u of GoTaq® DNA polymerase and DNA at 100 ng. The amplifications obtained were verified by electrophoresis in Ultrapure™ Agarose 1000 at 2.5% w/v (1XTAE buffer), dyed with ethidium bromide (0.5 μg/mL), and visualized under UV light in a Bio-Print (Vilber®) transilluminator. The fragments were determined by comparison with a marker of 1-Kb (Invitrogen®). The sequencing was carried out with the Genetic 3500xl Analyzer (Applied Biosystems, Foster City, CA) at the Instituto Potosino de Investigación Científica y Tecnológica A.C. (IPICYT), in both directions ITS1 and ITS4. The ITS sequences were edited and assembled manually using the Bioedit 7.2.5 software (Hall, 1999). The sequences were aligned using the ClustalX 2.1 software (Thompson *et al.*, 1997), with the predetermined configuration. The set of sequences aligned built a phylogenetic tree with the sequences of endophytic fungi using the Molecular Evolutionary Genetics Analysis (MEGA) XI software (Tamura *et al.*, 2021). The ITS sequences were analyzed with searches in the Basic Local Alignment Search Tool (BLAST) system of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nl-m.nih.gov).

RESULTS AND DISCUSSION

In total, 15 isolates from cacao (*T. cacao*) plantations were obtained, in the municipalities of Centro, Cunduacán and Comalcalco in Villahermosa, Tabasco, Mexico. Amplicons of 450 to 650 pb corresponding to the identification gene (ITS1 and ITS4) were obtained. The Blast analysis revealed that the fungi belong to the *Ascomycota phylum*, grouped into three classes: *Dothideomycetes*, *Eurotiomicetos* and *Sordariomicetos*. Table 1 shows the results obtained from the 15 fungi strains grouped into 13 different species; and the percentage of identity, total score, and number of access provided by the database from National Center for Biotechnology Information (NCBI) were also observed.

Figure 2 shows the phylogenetic analysis that was generated by the UPGMA method with a branch length of 3.3978, and the evolutionary distances were calculated using the method of Maximum Likelihood using 572 positions in the set of final data.

In the dendrogram developed from the sequences obtained, two evolutionary groups can be seen, the first of which is subdivided into two groups; the first group includes the families *Botryosphaeriaceae*, *Aspergillaceae*, *Glomerellaceae*, *Ceratocistidáceas*, *Hipoxiláceas* and *Sordariaceae*; the second group includes species from the *Aspergillaceae*, *Diaporthaceae* and

Table 1. Identification of isolate from the *Ascomycota phylum*, based on data obtained from the ITS rDNA sequences (https://www.ncbi.nlm.nih.gov/).

Isolation	GenBank number	Sample	GenBank ID	Size (pb)	Query Score (%)	Identity (%)	Place		
							Cunduacán	Centro	Comalcalco
H174	156272.1	Healthy leaf	CBS 120397 *Endomelanconiopsis endophytica*	526	100	99.81		X	
H171	158434.1	Healthy leaf	MFLUCC 17-0547 *Endomelanconiopsis freycinetiae*	479	98	98.76		X	
H177	156272.1	Healthy fruit	CBS 120397 *Endomelanconiopsis endophytica*	524	100	99.80		X	
H02	163668.1	Infected fruit	CBS 121.28 *Aspergillus foetidus*	365	88	98.46		X	
H53	137479.1	Infected fruit	NRRL 181 *Aspergillus fischeri*	441	72	77.61		X	
H100	155899.1	Infected fruit	IMI 50560 *Thielaviopsis ethacetica*	448	95	97.2	X		
H05	164291.1	Infected fruit	CBS 124934 *Cophinforma atrovirens*	430	98	99.53	X		
H148	103582.1	Infected fruit	CBS 309.91 *Neurospora udagawae*	358	93	95.85	X		
H130	147535.1	Healthy fruit	BRIP 54736 *Diaporthe miriciae*	437	97	97.18	X		
H76	160206.1	Infected fruit	CBS 101754 *Aspergillus delicatus*	262	85	94.25	X		
H60	166005.1	Healthy fruit	CBS 124.83 *Nodulisporium indicum*	461	99	95.81			X
H68	151816.1	Healthy leaf	JCM 19878 *Aspergillus arcoverdensis*	334	100	96			X
H301	164291.1	Healthy fruit	CBS 124934 *Cophinforma atrovirens*	337	78	93.54			X
H01	171185.1	Healthy fruit	CPC 30245 *Colletotrichum tainanense*	487	94	99.78			X
H120	160815.1	Healthy fruit	MFLUCC 13-0726 *Colletotrichum hebeiense*	480	91	98.86			X

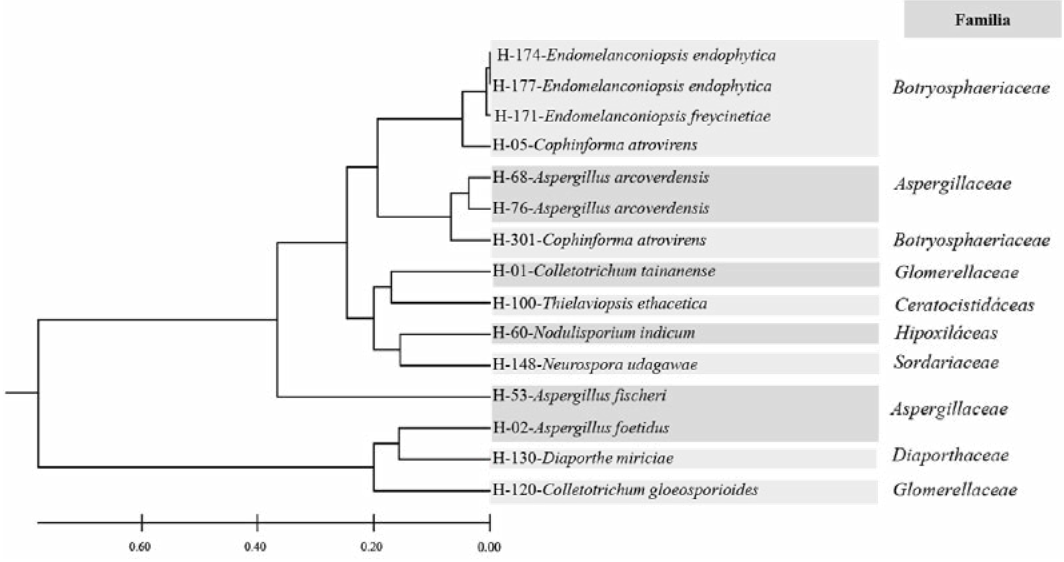


Figure 2. Representation of the phylogenetic proximity based on sequences of the ITS rDNA region of the species identified isolated in cacao trees of the state of Tabasco, using the UPGMA method; the evolutionary distances were calculated using the Maximum Likelihood method.

Glomerellaceae families, without geographic correlation. It is important to mention that no previous records of *Endomelanconiopsis endophytica*, *E. freycinetiae*, *Cophinforma atrovirens*, *Diaporthe miriciae*, *Neurospora udagawae* and *Colletotrichum tainanense* in Mexican cacao were found. These species have been isolated from tissues of plants from tropical and subtropical regions, similar to the climate in Tabasco (Macabeo *et al.*, 2020). The species *E. endophytica* was reported for the first time by Rojas *et al.* (2008), in the Republic of Panama, in healthy cacao leaves and in other plants of commercial interest such as *Ficus hirta* and tropical palm (Douanla & Scharnhorst, 2021; Sun *et al.*, 2016). *E. freycinetiae* has been identified in the *Pandanaceae* family, closely related with *E. endophytica* (Tibpromma *et al.*, 2018). They reside harmoniously in the plants and are considered antagonistic fungi, capable of inhibiting phytopathogens such as *Colletotrichum truncatum* and *F. oxysporum* (Azuddin *et al.*, 2021); in addition, they can segregate secondary metabolites with cytotoxic activity in cell lines and with antioxidant properties (Sun *et al.*, 2016). This study identified *N. indicum* from the Xylariales order (Bitzer *et al.*, 2008) in the database from the European Bioinformatics Institute (EMBL-EBI); until today it has been recorded in India and Vietnam. In Mexico, *Nodulosporium* sp. was isolated from infected cacao pods, recorded by González *et al.* (2019), causing symptoms of pod rotting and leaf dehydration. Another species isolated was *D. miriciae*, which has been described by Thompson *et al.* (2015) in plants of *Glycine max* and *Vigna radiata* in Australia. Regarding *N. udagawae* from the genus *Sordariaceae*, it is considered an endophytic fungus that colonizes soils, trees and dead shrubs (Fujimoto, 2018; Macabeo *et al.*, 2020). This study reports four species from the *Aspergillaceae* family; *Aspergillus foetidus*, *arcoverdensis*, *fischeri* and *delicatus*, from the *Aspergillus* genus, common in cacao plants and seeds (González *et al.*, 2019). On the other hand, *T. ethacetica* was identified, generalist pathogen with a wide variety of hosts, such as sugarcane, cacao and coconut. Its origin could probably be anthropogenic and it has been recorded in many countries in the five continents (Borges *et al.*, 2019; Mbenoun *et al.*, 2015). Two species from the *Colletotrichum* genus (*C. tainanense* and *hebeiense*) were isolated, frequently found in cacao, and this genus one of the most economically important (Landero *et al.*, 2017). Recent studies in Indonesia, Taiwan and India report *C. tainanense* as a pathogen in *Capsicum annuum* and *Punica granatum* L. (De Silva *et al.*, 2019; Manjunatha *et al.*, 2022), causing a disease called anthracnosis.

Finally, species such as *E. endophytica*, *A. arcoverdensis*, *D. miriciae* and *N. indicum* could be used for the biological control of plant diseases, primarily for cacao, since the literature expresses that they could present a series of chemical substances with antioxidant and anti-inflammatory activity, and of great interest in the pharmaceutical and cosmetic industry, and for biological control (Fujimoto, 2018; Reyes *et al.*, 2021). These results suggest that the agroforestry system sustains a large diversity of fungal species, and many of them could be used as biological control or for development in the pharmaceutical industry (Reyes *et al.*, 2021). It should be highlighted that it is necessary to perform more studies with the fungi identified, such as pathogenicity tests, metabolite detection, or to apply genomic methods based on molecular markers that allow identifying the possible allele variants associated with pathogenicity and aggressiveness of these species (Douanla-Meli & Scharnhorst, 2021).

CONCLUSIONS

This study reports 13 species of different endophytic fungi isolated from cacao trees in the state of Mexico. Non-pathogenic endophytic species are reported until now (*E. endophytica*, *E. freycinetiae*, *A. arcoverdensis*, *D. miriciae*), which with molecular and pathogenicity studies could be used as organisms for biological control of phytopathogens in trees of economic interest, among them cacao.

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