Morpho-molecular characterization of fungi in harvested fruits of *Theobroma cacao* L.

Caracterización morfo-molecular de hongos en frutos cosechados de *Theobroma cacao* L.

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ABSTRACT

In the present research, various isolates of pathogenic, endophytic, and epiphytic fungi were molecularly identified from diseased fruits collected in the harvest phase of *Theobroma cacao* L. coming from two different farms of Norte de Santander and Bolívar departments, Colombia. Standard microbiological procedures were used to phenotypically and molecularly characterize 26 fungi. *Moniliophthora roreri*, *Scopulariopsis* spp., 7 species belonging to the *Fusarium solani* complex, 6 species of *Aspergillus*, *Neocosmospora tonkinensis*, *Penicillium paneum*, *Pestalotiopsis microspora,* and *Geotrichum candidum* were found. Besides this, the identification of *Neofusicoccum parvum*, PQ721376.1, the causal agent of cocoa pod rot in the analyzed samples, is one of the most remarkable results since it is the first time that this recognized destructive fungal pathogen is reported in the country. We concluded that cocoa crops from two geographically different farms located in Colombia harbor a wide variety of fungi, most of them with pathogenic behavior, which could influence their low productivity. This finding supports the need to implement differentiated strategies for disease management depending on the regions where *T. cacao* L. is cultivated and the pathogenicity profile of the fungus found, to improve crop productivity.

Keywords: cocoa, pathogens, fungal infection, agroecology.

RESUMEN

En la presente investigación se identificaron molecularmente varios aislados de hongos patógenos, endófitos y epífitos a partir de frutos enfermos, recolectados en la fase de cosecha de *Theobroma cacao* L. provenientes de dos fincas diferentes ubicadas en los departamentos de Norte de Santander y Bolívar, Colombia. Se emplearon procedimientos microbiológicos y moleculares estándar para caracterizar fenotípica y genotípicamente 26 hongos. Se encontraron *Moniliophthora roreri*, *Scopulariopsis* spp., 7 especies pertenecientes al complejo *Fusarium solani*, 6 especies de *Aspergillus*, *Neocosmospora tonkinensis, Penicillium paneum, Pestalotiopsis microspora* y *Geotrichum candidum*. Además de esto, la identificación de *Neofusicoccum parvum*, PQ721376.1, el agente causal de la pudrición de la mazorca del cacao en las muestras analizadas, es uno de los resultados más notables, debido a que es la primera vez que este reconocido patógeno fúngico destructor es reportado en el país. Se concluyó que los cultivos de cacao de dos fincas geográficamente diferentes ubicadas en Colombia, albergan una gran variedad de hongos, la mayoría de ellos con comportamiento patógeno, lo que podría estar influyendo en su baja productividad. Este hallazgo apoya la necesidad de implementar estrategias diferenciadas para el manejo de enfermedades dependiendo de las regiones donde se encuentre establecido el cultivo de *T. cacao* L. y del perfil de patogenicidad de los hongos encontrados, con el fin de mejorar la productividad de los cultivos.

Palabras clave: cacao, patógenos, infección fúngica, agroecología.

Introduction

Cocoa (*Theobroma cacao* L.) is a plant species native to equatorial tropical moist forests of South America. It is established in warm, humid regions at latitudes spanning 10°N and 10°S of the equator. Its beans are primarily used as raw materials to produce chocolate, and lipids are used in the food, pharmaceutical, and cosmetics industries (Arvelo *et al*., 2016). Worldwide, cocoa production is led by the African continent with 70.3% followed by the American continent with 17.4% and finally Asia and Oceania with 12% (Ministerio de Desarrollo Agrario y Riego, 2023).

Colombian cocoa stands out internationally for its aroma and delicate flavor, characteristics present in nearly 95% of its national production (Fernández Niño *et al*., 2021),

Received for publication: May 21, 2025. Accepted for publication: July 26, 2025. Doi: 10.15446/agron.colomb.v43n2.120512

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providing higher quality and price in the foreign market (Antolinez *et al*., 2020). It is cultivated throughout the national territory, approximately in 30 of the 32 departments of the country. Its production and export increased 28% over the last decade, increasing from 46,730 t in 2013 to 73,000 t in 2024 (Fedecacao, 2025). The sector has had a significant socioeconomic impact on the nation, thus becoming an alternative for more than 65 thousand families (MADR, 2021).

Theobroma cacao crops are established in pantropical regions, characterized by two rainy and two dry annual periods. This causes variables such as high temperatures and relative humidity to facilitate the growth of fungi and contribute to the spread of diseases, affecting the quality of the final product (Delgado-Ospina *et al*., 2021). At a global level, it has been estimated that cocoa infections with pathogenic microorganisms can cause losses exceeding 30% of worldwide productivity (ICCO, 2021). But if the infection occurs during the initial twelve weeks of its development, the losses can exceed 60% (Albores *et al*., 2022). Some of the most impactful disease-causing agents, such as *Phytophthora palmivora,* responsible for root rot, have global distribution.

In contrast, others, such as *Moniliophthora roreri*, the causal agent of moniliasis, are restricted to Latin America and the Caribbean (Marelli *et al*., 2019). It is estimated that approximately 20,000 fungi cause diseases in plants, including those with phytopathogenic and endophytic behavior. The latter promotes the vital functions of the plant. But, under conditions of physiological stress, they can change their behavior to opportunistic pathogens (Jain *et al*., 2019). In the case of *T. cacao*, it is estimated that there are up to 30 species of fungi that significantly affect its production, both globally and in more specific local contexts (Bailey & Meinhardt, 2016).

The aforementioned supports the need to carry out characterization studies of phytopathogenic fungi in the different geographical areas where the cultivation of *T. cacao* is established and to learn more about its agroecology, especially in those regions with low production rates (Amaro *et al*., 2021). To our knowledge, this is the first research carried out on the phenotypic and molecular characterization of fungal species present in different diseased cocoa biological materials, collected in the harvest phase, from the Norte de Santander and Bolívar departments of Colombia, where national production is not higher than 454 and 436 kg ha^{-1} per year, respectively (Fedecacao, 2021). This study aims to identify the primary fungal pathogens that may be affecting the productivity of small producers and to propose mitigation strategies in the future.

Materials and methods

Collection of plant material

Between eight and ten fruits were randomly selected in the harvest phase, which presented morphological alterations such as humps, oily spots, presence of necrotic tissue, superficial white mycelium, etc. These were randomly chosen (Villamizar-Gallardo *et al.,* 2019). We carried out sampling in two cocoa plantations whose characteristics are shown in Table 1. We packed the collected samples independently in plastic bags. We placed them in expanded polystyrene cellars with cooling gels for subsequent transport and processing at the laboratory of the group of research in Nanotechnology and Sustainable Management (NANOSOST) at the University of Pamplona, Norte de Santander, Colombia.

TABLE 1. Characteristics of the sampling sites.

Description/ Sampling sites	S1	S2
Georeferencing	8°16´24" N - 72°26´46" W	8°02´02" N - 74°04´23" W
Farm	Finca Miraflores	Finca los Pinos
Village	Vereda la Floresta	Vereda Juan Pablo II
Town	Cúcuta	Santa Rosa del Sur
Department	Norte de Santander	Bolívar
Elevation	64 m a.s.l.	734 m a.s.l.
Air temperature	21°C - 33°C	22°C - 30°C
Precipitation	878 mm	1000 mm
Pest control	Chemical and cultural	Chemical and cultural
Average annual yield (kg ha^{-1})	377	475
Cocoa accesiones	FSA-13, FTA-2, FEAR-5, CCN-51, ICS-95, SCC-61, and FLE3	ICS-95, CCN-51, and ICS-60

Fungal isolation

We carried out abundant superficial washing with sterile distilled water on each of the fruits to remove impurities. The isolation technique was based on direct cultivation, which consisted of making superficial cuts of the fruit of approximately 5 mm^2 to 10 mm^2 from the observed lesions. The samples were placed in Petri dishes with potato dextrose agar culture medium (PDA-Condolab®) and incubated at 25°C for 5-14 d, due to the heterogeneous growth observed in the crops. Subcultures of the different morphospecies were carried out until axenic cultures were obtained. To avoid redundancy and ensure

representativeness, a single strain was selected for each group of isolates with similar morphological characteristics (Villamizar-Gallardo *et al*., 2019).

Macroscopic characterization

To evaluate the macroscopic characteristics, monosporic cultures were subcultured in APD medium and incubated at 25°C for 5-14 d. The parameters proposed by Muñoz and collaborators were evaluated to determine the color, appearance, and growth rate of the colony (Muñoz *et al*., 2020). The latter was obtained by measuring colony growth daily in APD medium for 10 d. For this purpose, a 7 mm diameter disc was placed in the center of the plate that contained the inoculum of each isolate, and incubated at 25-27°C. Daily photographic records of the growth of each isolate were taken along with a known scale and then processed in ImageJ™ software to obtain growth values. The procedure was done in triplicate for each isolate. The growth rate was obtained with the following formula:

$$GR = \frac{\Delta d}{\Delta t} = \frac{fd - id}{ft - it} \quad (1)$$

where:

GR: Growth rate; *fd*: final diameter; *id*: initial diameter; *ft*: final time; *it*: initial time. To determine the statistical differences between the growth rates of the different fungi, the Kruskal-Wallis test was used with a *P*-value < 0.05. To identify the groups that presented significant differences, Dunn's post hoc test was applied. All analyses were performed using RStudio statistical software, version 4.3.3. The characterization information was contrasted with the classification guide proposed by Garcés de Granada *et al*. (2015).

Microscopic characterization

To evaluate the microscopic characteristics, monosporic cultures were grown in microcultures using APD culture medium. Concentrated suspensions of conidia were prepared, and 2 µl were taken from these to inoculate 1 cm² of medium. They were incubated at 25°C for 5-7 d, stained with lactophenol blue, and observed under the Zeiss Primo Star binocular microscope with 40x and 100x objectives. Different characteristics, such as shape and size of conidia, presence or absence of septate hyphae, and characterization of fruiting bodies, were evaluated as described by De Hoog *et al*. (2020). The size of the conidia was measured using the Zeiss® ocular micrometer, and the respective photographic record was taken for processing in the ImageJ software.

Molecular characterization

DNA extraction

DNA extraction started from cultures in liquid medium (Sabouraud dextrose broth) incubated at 28°C for 3-5 d with constant agitation, to promote the growth of mycelium. For DNA extraction, the commercial Exgene® Plant SV mini kit (GeneAll Biotechnology Co., Korea) was used following the manufacturer's instructions. The quality of the DNA obtained was visualized in a 0.8% agarose gel and was subsequently quantified by spectrophotometry (Agilent BioTek Epoch, CA, USA).

Polymerase chain reaction (PCR)

The ITS region of the ribosomal DNA was used as an amplification target using the oligonucleotides ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') (Luo & Mitchel, 2002). To amplify, a 1X buffer was used: 2 mM $MgCl_2$, 0.25 mM dNTPs, 0.3 µM oligonucleotides, 3.5 U of Taq DNA polymerase, 50 ng of DNA for a total reaction volume of 50 µl. One cycle at 95°C for 3 min was used; 30 cycles of 95°C/30 s, 55°C/30 s, 72°C/30 s and 1 final cycle at 72°C/3 min were also used. This procedure was carried out using the Corbett Research thermal cycler. The amplification products were visualized on a 1.5% agarose gel and subsequently purified using the DNA Clean & Concentrator kit or the Zymoclean® Gel DNA Recovery kit (Zymo Research, USA) for the subsequent sequencing process.

Sequencing

The purified fragments were sent for sequencing to the Molecular Biology Laboratory of the Institute of Cellular Physiology of the UNAM, using the Sanger chain termination method, on an Applied Biosystems 3500 Series Genetic Analyzer 3500 (Thermo Fisher Scientific, USA).

Identity by homology

From the sequences obtained, bioinformatic analyses were performed using the BLAST (Basic Local Alignment Search Tool) of the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/) and the MycoBank database (https://www.mirri.org/upcp_product/mycobank/). The objective of this procedure was to determine the percentage of identity (%I) by comparing it with curated sequences deposited in these databases, specifically for the ITS region, which is widely recognized as a reliable molecular marker for the identification of fungal species (Tekpinar & Kalmer, 2019). The selection

criteria considered included an E-value equal to 0.0 and an identity percentage between 99% and 100%, as reported by Suárez-Contreras and Peñaranda-Figueredo (2022) (Tab. 2).

Representation of the phylogenetic relationship

For the analysis of the phylogenetic relationship, the "Molecular Evolutionary Genetic Analysis" software (MEGA 11.v) was used to understand the evolutionary relationships between the molecularly characterized organisms. The sequences obtained were aligned with a sequence used as an "Outgroup", corresponding to the gene that encodes the ITS region of the basidiomycete fungus *Amanita muscaria*, selected as an external reference point not related to the "Ingroup". The alignment of the DNA sequences was performed using the MUSCLE algorithm. The sequences were aligned using the MUSCLE algorithm for DNA alignment. Subsequently, a phylogram was constructed using the Neighbor-Joining statistical method, with a bootstrap analysis based on 1000 replications (Suárez & Peñaranda, 2022).

Results

It was possible to identify 26 axenic isolates from the samples taken of which 15 (H1 to H15), that is, 58% of the total, were obtained from Norte de Santander department (S1) (Fig. 1). In comparison, 11 isolates (H16 to H26), corresponding to 42% were obtained from Bolívar department (S2) (Tab. 2). From S1 isolates, we found that H2-H6, H8, H11 and H13 presented white and beige colonies. In the majority, the center of the colonies presented a denser growth of light brown color (except in isolates H11 and H13). The texture of most colonies was cottony on the periphery and more compact in the center. Isolate H7 showed a greenish-yellow color, with a powdery appearance. Isolates H10, H12, and H14 presented an olive-green color, with irregular

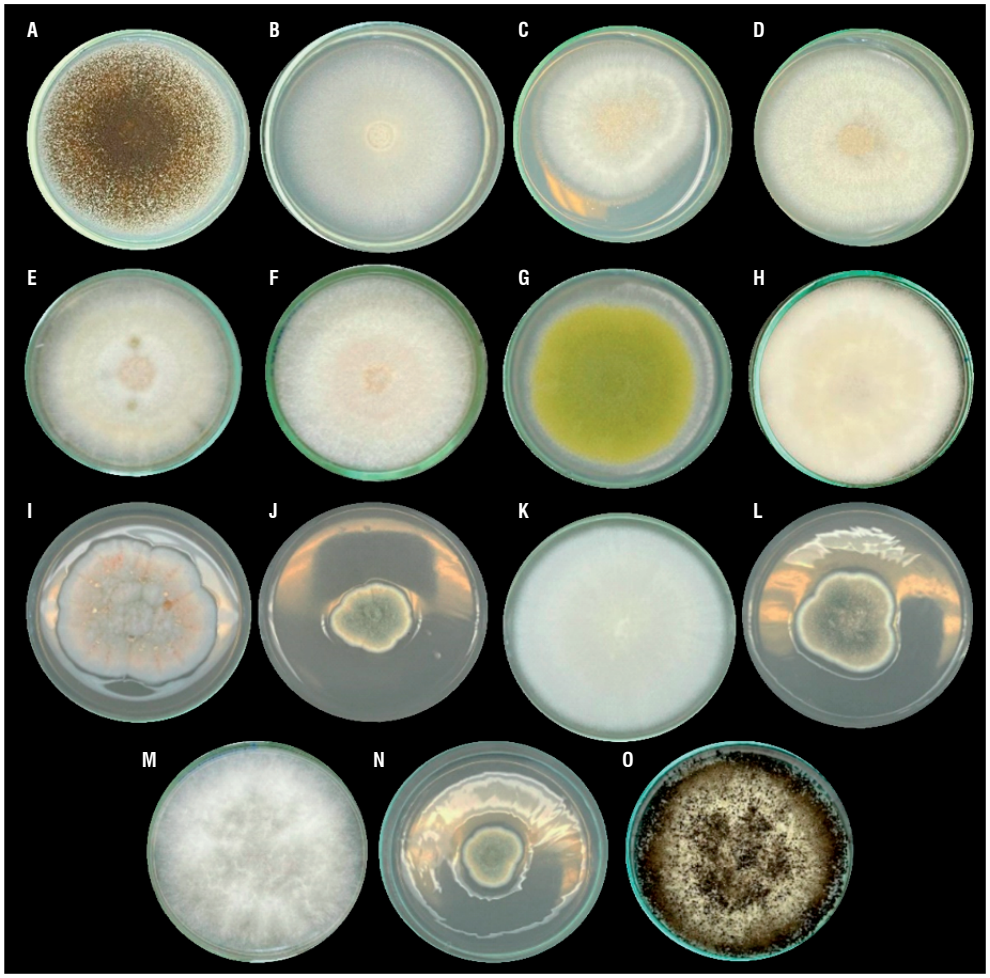

FIGURE 1. Macroscopic characterization of strains isolated from S1. The strains are labeled as follows: (A) H1, (B) H2, (C) H3, (D) H4, (E) H5, (F) H6, (G) H7, (H) H8, (I) H9, (J) H10, (K) H11, (L) H12, (M) H13, (N) H14, and (O) H15. All strains were photographed after 5-14 d at 25°C on PDA medium.

white edges and a velvety appearance. Isolate H9 showed orange pigmentation on the surface of the white mycelium. Finally, isolates H1 and H15 presented light and dark brown colonies, respectively, with a powdery texture.

Eleven isolates were obtained from the Bolívar region (S2) (Fig. 2) of which H16, H18-H20, H22, H24-H26 showed white to beige colonies with a cottony texture and regular edges. Isolates H18 and H19 showed irregular and rhizoid borders. Isolate H17 presented a colony with variable shades from yellow to light brown with asymmetric concentric rings and a cottony texture. Isolate H21 showed a colony with yellow tones on the edge and orange to reddish in the center, including a cottony texture. Finally, isolate H23 presented a green colony with white edges and a yellow center, with a powdery texture.

Regarding the growth rate, Figure 3 showed the significant differences in the 26 isolates. When applying the Kruskal-Wallis statistical test, a *P*-value of 5.212 e^{-07} was obtained, which was much lower than the established level of significance, making it possible to identify statistically significant differences in at least one of the fungi evaluated. Dunn's post hoc test indicated that isolates H17 and H23 were significantly different from the others, having the highest and lowest growth rate, with values of 22.48 mm d^{-1} and 3.28 mm d^{-1}, respectively.

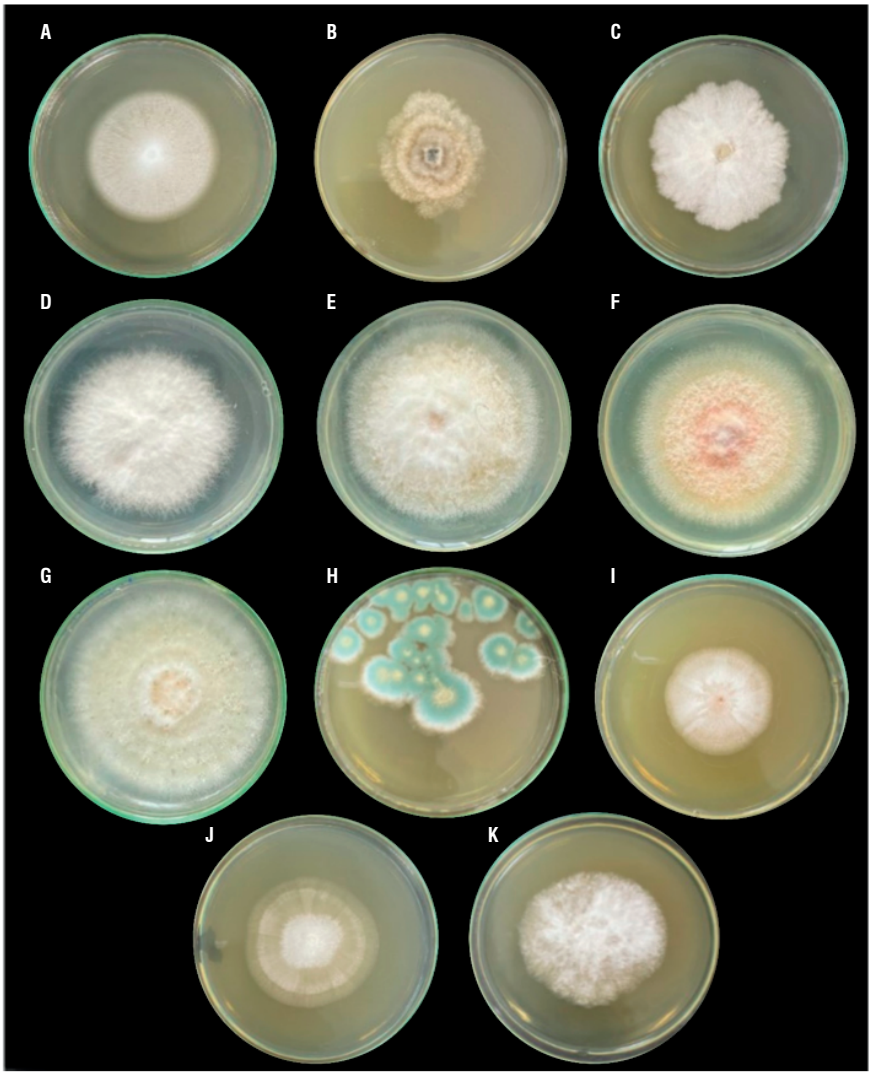

FIGURE 2. Macroscopic characterization of strains isolated from S2. The strains are labeled as follows: (A) H16, (B) H17, (C) H18, (D) H19, (E) H20, (F) H21, (G) H22, (H) H23, (I) H24, (J) H25, (K) H26. All strains were photographed after 5-14 d at 25°C on PDA medium.

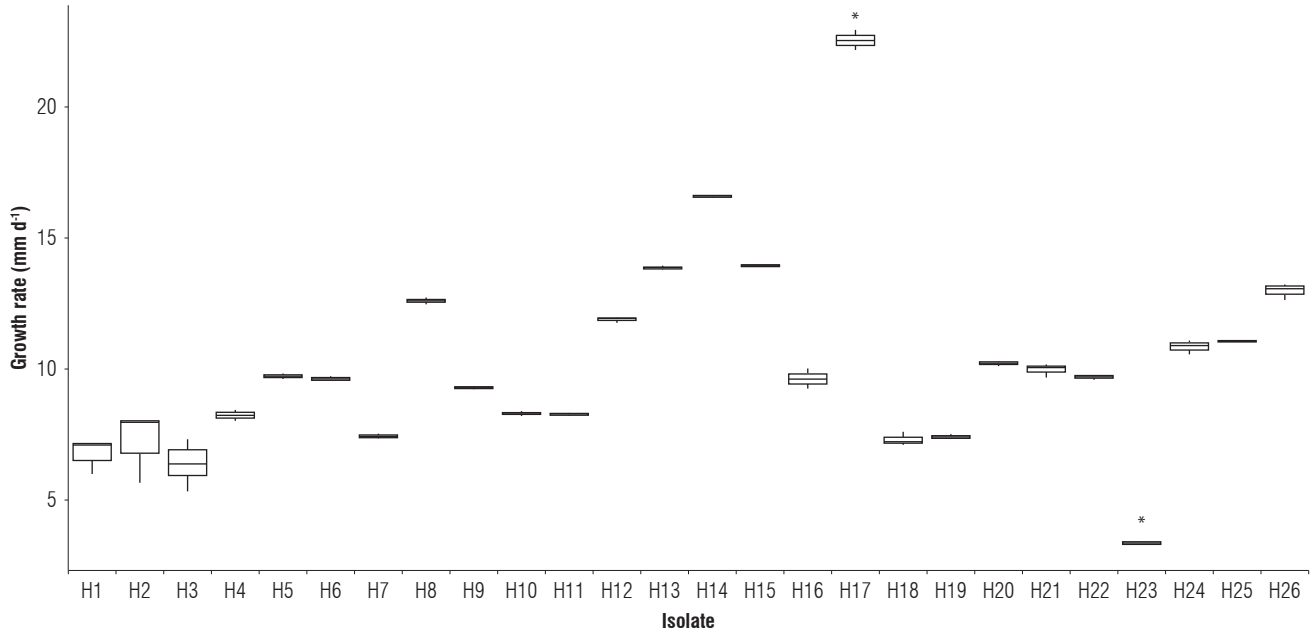

FIGURE 3. Differences between fungal growth rates. *: This represents statistically significant differences according to the Kruskal–Wallis test ($P = 5.21\ e^{-07}$).

TABLE 2. Identification of the isolates obtained in S1 and S2 with their respective E-value from the Mycobank database.

Isolates from Norte de Santander (S1)		
Code	**Species**	**Percentage of identity**
H1, H15	*Aspergillus niger*	100
H2, H11	*Geotrichum candidum*	100
H3, H4, H6	*Fusarium solani* complex (FSCS)	100
H5	*Neocosmospora tonkinensis*	100
H7	*Aspergillus flavus*	100
H8	*Moniliopthora* spp.	NR*
H9	*Scopulariopsis* spp.	NR*
H10-H12	*Aspergillus versicolor*	100
H13	*Sterile mycelium*	NR*
H14	*Aspergillus amoenus*	100
Isolates from Bolivar (S2)		
H16, H25	*Geotrichum candidum*	100
H17	*Neofusicoccum parvum*	100
H18	*Clonostachys rosea*	100
H19	*Bionectria wenpingii*	100
H20, H21, H22, H24	*Fusarium solani* complex (FSCS)	100
H23	*Penicillium paneum*	100
H26	*Pestalotiopsis microspora*	100

NR*: Not recovered.

Regarding the microscopic characterization, we observed that H1 and H15, isolated from S1 (Fig. 4), showed wide conidiophores with thick light brown walls, with conidia of 3 to 5 µm, which arise from a conidial apparatus consisting of globose vesicles and radial phialides. Isolates H2 and H11 exhibited thick, septate hyphae that formed hyaline, rectangular arthroconidia, with rounded ends, with a size of 10 to 20 µm. Isolates H3, H4, H5, and H6 showed septate hyphae, forming monophialides, with ovoid microconidia of 3 to 6 µm and fusiform, septate macroconidia of variable length (between 6 and 30 µm), in addition to the presence of chlamydoconidia.

Isolate H7 exhibited long conidiophores, with a conidial apparatus consisting of a biseriate spherical vesicle and globose conidia of 3 to 5 µm. At the same time, H8 showed globose, ellipsoid, and catenulate conidia of 8 to 20 µm, forming a chain, as well as septate hyphae. H9 was characterized by having thin, long, septate hyphae that stained a light blue tone. The conidiophores were short and branched, from which spherical conidia emerged, arranged in a chain, with a diameter of 4 to 6 µm. Isolates H10 and H12 presented septate and hyaline hyphae. Hyaline conidiophores emerge from these hyphae, ending in vesicles with radially arranged phialides, with spherical conidia of 4 to 6 µm. Finally, in H13, only sterile mycelium was observed.

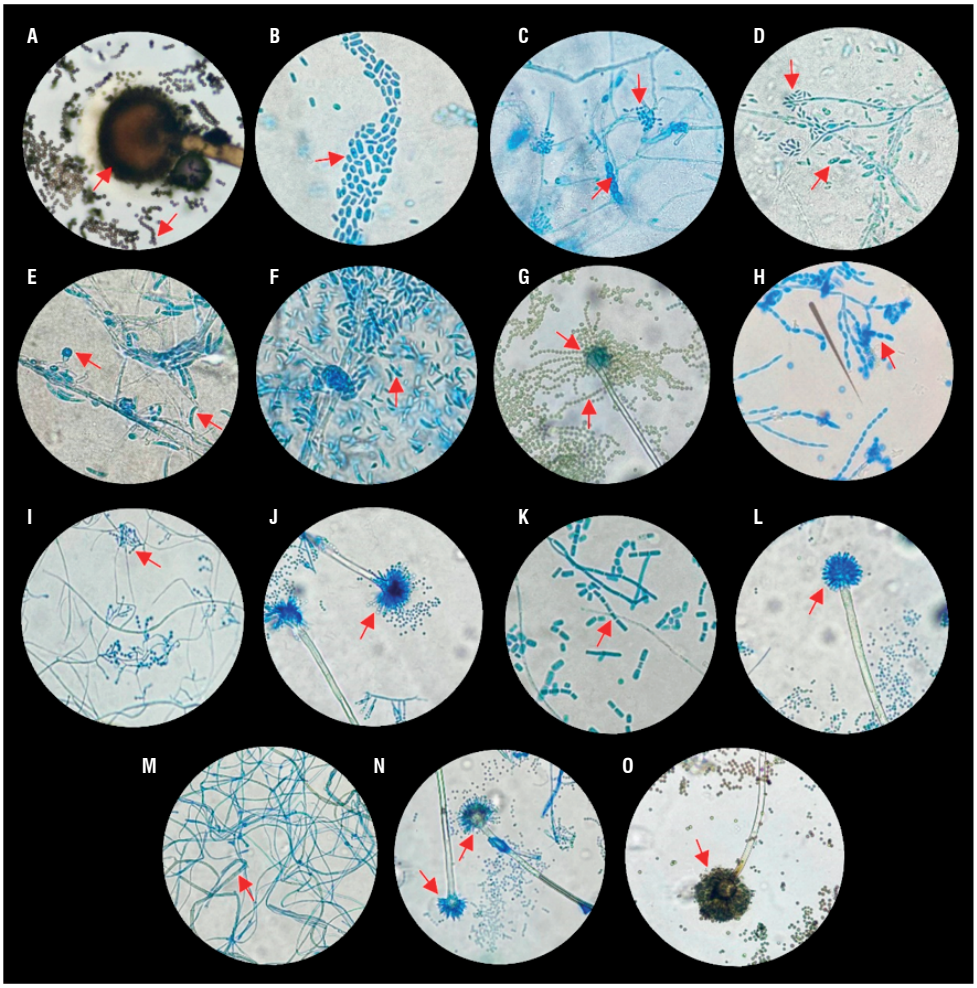

FIGURE 4. Microscopic characterization of strains isolated from S1. The strains are labeled as follows: (A) H1-40X, (B) H2-100X, (C) H3-100X, (D) H4-100X, (E) H5-100X, (F) H6-100X, (G) H7-40X, (H) H8-100X, (I) H9-40X, (J) H10-40X, (K) H11-100X, (L) H12-40X, (M) H13-100X, (N) H14-40X, and (O) H15-40X. The arrows indicate the presence of structures of interest, such as reproductive structures.

In the isolates from site S2, various morphological characteristics were observed (Fig. 5). The H16 and H25 showed conidial structures formed by the fragmentation of hyphae (arthroconidia), with a size between 3 and 6 µm, and a rectangular shape. In H17, we observed septate, pigmented hyphae without conidia. The H18 presented septate hyphae and whorled phialides, with ovoid conidia, 5 to 11 µm long. The H19 showed septate hyphae with secondary branches and ellipsoid conidia between 13 and 20 µm. H20, H21, H22, and H24 presented hyaline, septate hyphae and conidiophores with fusiform conidia between 20 and 30 µm long. On the other hand, the H23 showed septate hyphae, conidiophores with metulas and phialides, and round conidia forming chains 3 to 5 µm long. The H26 showed initially hyaline and then pigmented, septate hyphae, forming pigmented conidia, with three to four septa, with hyaline cells at the ends, 3 µm wide by 10 µm long.

For the molecular study, it was not possible to recover isolates H8, H9, and H13 in subculture, so the first two were only identified at the genus level, and the third was not identified. From the DNA extracted from the remaining 23 isolates and previously phenotypically characterized, a PCR was performed to amplify a fragment of the ITS region (Fig. 6). Amplicons ranging between 450 and 750 bp were obtained.

Molecular analyses allowed the identification of 23 isolates at the species level, all with identity values of 100% in both databases (Genbank & Mycobank).

The phylogenetic analysis of the described species is shown in Figure 7. It represented the evolutionary relationship between the different recovered fungal isolates and several reference species. As an "outgroup", an ITS

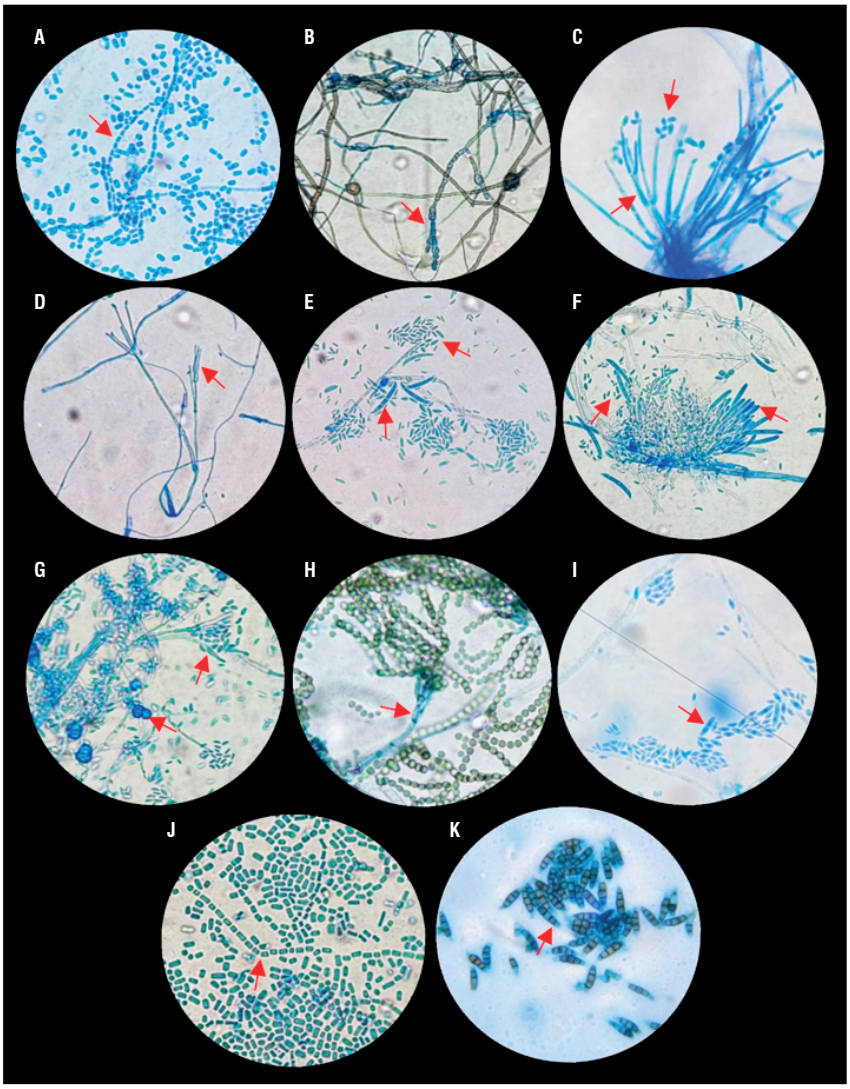

FIGURE 5. Microscopic characterization of strains isolated from S2. The strains are labeled as follows: (A) H16-100X, (B) H17100X, (C) H18-100X, (D) H19-40X, (E) H20-100X, (F) H21-100X, (G) H22-100X, (H) H23-100X, (I) H24-100X, (J) H25-100X, (K) H26-100X.

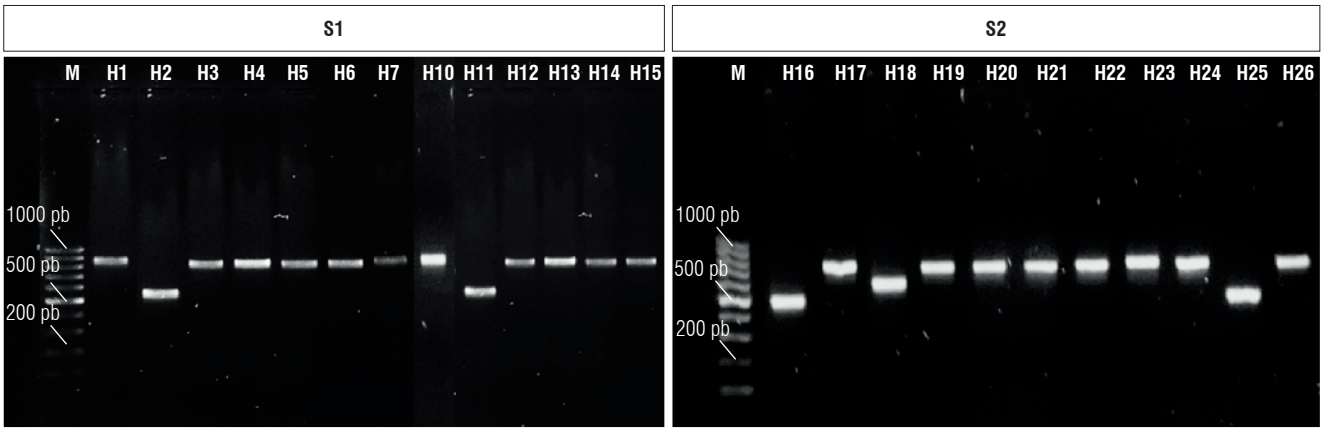

FIGURE 6. The 1.5% agarose gel electrophoresis product of the ITS region amplicons obtained from samples S1 and S2.

sequence corresponding to the Basidiomycete fungus *Amanita muscaria* was used to root the phylogenetic tree. The results showed that all isolates belong to the phylum Ascomycota, grouped into the classes Sordariomycetes, Dothideomycetes, Eurotiomycetes, and Saccharomycetes, distributed in five orders: Hypocreales, Amphisphaeriales, Botryosphaeriales, Eurotiales, and Saccharomycetales. A clear separation was observed between the different fungal species identified, with coherent groupings by genus, which highlighted the effectiveness of this marker for the taxonomic classification of fungi. The group formed by the isolates identified as *Fusarium solani* (H20, H21, H22, H24, H3, H4, H6) was grouped with high support values (bootstrap of up to 95), forming a consistent clade corresponding to *Fusarium solani species complex* (FSSC), which showed a close phylogenetic relationship between these isolates. This group, in turn, is related to related genera such as *Clonostachys* and *Bionectria*.

On the other hand, the isolates belonging to the genus *Aspergillus* (H12, H14, H10, H1, H15, H7) were grouped in different well-supported clades (bootstrap between 94 and 99), showing a clear separation between species such as *A. niger, A. flavus, A. versicolor,* and *A. amoenus*. Likewise, *Penicillium paneum* (H23) was positioned as a close sister group to the clade of *Aspergillus*, which agreed with its belonging to the same taxonomic family. The isolates classified as *Geotrichum candidum* (H2, H11, H16, H25) formed a clade with a high support (bootstrap up to 99), reflecting a close evolutionary relationship between them. As for the isolates H26, H17, and H5, identified as *Pestalotiopsis microspora, Neofusicoccum parvum,* and *Neocosmospora tonkinensis,* respectively; these formed independent clades, which indicated that they corresponded to differentiated species, a result that was also supported by high percentages of identity in the comparison with reference sequences. However, their location within the tree did not have high bootstrap values (19-29), so it was not possible to establish solid evolutionary inferences for these branches, although their identity was recognized at a specific level. Finally, the tree was rooted using *Amanita muscaria*, a basidiomycete fungus, as an outgroup, which allowed establishing the evolutionary direction of the lineages analyzed, all belonging to the phylum Ascomycota.

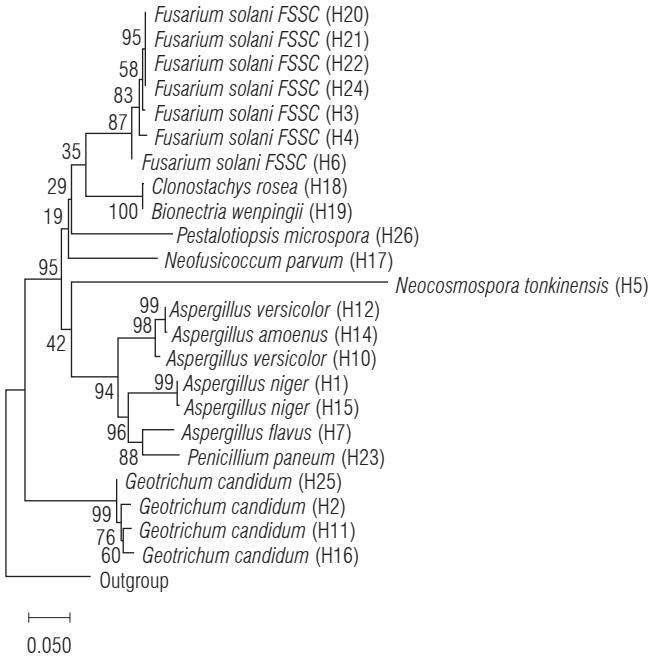

FIGURE 7. Phylogram of the amplified sequences of the ITS region of the species obtained from S1 and S2 using the Neighbor-Joining method. The values above the branches represent the bootstrap support percentages.

Discussion

Through this study, it was possible to identify 2 genera and 23 species of fungi from diseased fruits of *Theobroma cacao* in the harvest phase from two regions of Colombia. Among the isolates obtained from a cocoa farm located in the department of Norte de Santander (S1), it is worth highlighting that of *Moniliophthora* spp., although it could not be characterized molecularly because it was difficult to recover in synthetic culture medium, its phenotypic characteristics corresponded to *Moniliophthora roreri*, which was previously reported by Villamizar-Gallardo *et al.* (2019) from cocoa fruits obtained from the exact geographical location. This pathogen is characterized by producing the disease called moniliasis, which affects only fruits and is endemic to this crop (ICCO, 2021). Aime and Phillips-Mora (2005) note that cocoa moniliasis may have occurred for the first time in Colombia, specifically in the department of Norte de Santander, in 1817. This could explain its isolation in the S1 zone and not in S2, although its distribution is ample in the national territory. The presence of this disease in only one of the study areas, despite having favorable environmental conditions in both, could be due to several factors, including the presence of susceptible plant material, the degree of incidence of the disease, or inadequate crop management by farmers.

In contrast, in zone S2, the isolation of the species *Neofusicoccum parvum* was achieved for the first time in the department of Bolívar in Colombia. This strain was deposited in GenBank and can be evidenced through the accession number PQ721376.1. This species stands out for its high growth rate, 22.48 mm d^{-1}, which is related to

its ability to adapt to the environment and its pathogenic potential. Recently, this pathogen has been identified as the causal agent of pod rot in cocoa crops in Hawaii (Puig *et al*., 2019, 2021). In Colombia, this disease is usually associated with *Phytophthora palmivora*, which affects not only the pods but also the root and stem of the cocoa tree, causing significant losses of up to 30% of production (Rodríguez-Polanco *et al*., 2020). Therefore, the identification of *Neofusicoccum parvum* as the etiological agent of this pathology in the country generates new knowledge for the agroecology of the crop.

In the culture phase, it is also common to find endophytic fungi of the genus *Aspergillus* spp*., Fusarium* spp*., Penicillium* spp*., Pestalotiopsis* spp., as reported by Delgado-Ospina and collaborators (Delgado-Ospina *et al*., 2021). In this study, 7 of the 26 isolates, which are about 27% of the total, corresponded to the *Fusarium solani* complex (FSCS) with 100% identity percentages. More than 60 phylogenetically distinct species belonging to this complex are known for their pathogenic action in plants (Coleman, 2016; Hamzah *et al*., 2018), particularly in cocoa cultivation. *Fusarium solani* was described by Sosa del Castillo *et al*. (2016) as a species characterized by aggressive behavior. Villamizar-Gallardo *et al*. (2017) isolated and molecularly characterized various species of pathogenic fungi in cocoa and reported *F. solani* as a secondary pathogen of this crop. Kagezi *et al*. (2017) show this species comes from beetles, and they inoculated it into cocoa seedlings, observing that it caused wilting. This finding allowed us to identify that *F. solani* can establish symbiotic relationships with vectors that can transmit this disease to this crop. Likewise, Huda-Shakirah *et al*. (2024) identify and associate *F. solani* as the etiological agent of stem cancer, a disease that affects cocoa.

The second most abundant group of fungi in this research corresponds to the genus *Aspergillus,* comprising six species that account for approximately 23% of the total isolates, including *A. niger*, *A. flavus*, *A. versicolor*, and *A. amoenus*. The presence and impact of those fungi are indirect, since it has been shown that they significantly affect the quality of the final product, but not the development of the crop. Delgado-Ospina *et al*. (2022) report the presence of mycotoxins, specifically Ochratoxin A, produced by *Aspergillus niger* in cocoa beans. Similarly, *A. flavus* is reported as a fungus that affects cocoa cultivation in the post-harvest phase (Sukmawati *et al*., 2024), while *A. versicolor* is reported by Romero-Cortes *et al*. (2019) as a fungus responsible for general food spoilage. In general terms, the presence of all these fungal species is related to their mycotoxigenic potential, with a carcinogenic effect when they reach the consumer through the final processed product. The species identified as *Penicillium paneum* also represents a risk for the crop, especially during the grain fermentation process. This fungus, like the *Aspergillus* species mentioned above, produces mycotoxins, which represent a public health problem as reported by Subroto *et al.* (2023).

In addition, we identified *Pestalotiopsis microspor*a. This is an endophytic fungus characterized by its ability to remain dormant in the plant until it is stressed. At this time, it adopts a pathogenic lifestyle, triggering the development of diseases. EFSA Panel on Plant Health (PLH) *et al.* (2023) reported more than 100 endophytic fungi, including *P. microspora*. It evaluated their effect on stimulating or inhibiting the growth of two high-impact diseases in cocoa: moniliasis and Witch's Broom. We found that *P. microspora* promotes the growth of cocoa fruits when it behaves as an endophytic fungus but also promotes the development of moniliasis when it changes its behavior to a pathogenic mode.

In contrast, the epiphytic species *Clonostachys rosea* is identified, coinciding with the reports made by Krauss *et al.* (2013), who report the presence of the same pathogen and characterize it as the native mycoparasite most commonly isolated in cocoa trees. The species identified as *Geotrichum candidum* has been reported in the literature and is usually found on the surface of cocoa fruits (Copetti *et al.,* 2011; Oussou *et al*., 2022). The genus *Scopulariopsis* spp. is been identified as the anamorphic phase of the genus *Microascus*, both of which are recognized as agents responsible for biodeterioration, characterized by being infectious opportunists (Paterson & Lima, 2015). In cocoa crop, this genus can be present in different stages of processing, especially during fermentation and drying (Schwan *et al*., 2014). Finally, the genus *Bionectria* spp. is described as the teleomorphic phase of the genus *Clonostachys* (Han *et al*., 2020), which explains the closeness obtained in the phylogram carried out, which groups them into the same clade. *Bionectria* is found in the necrotic tissue of plants. In its latest taxonomic classifications, *Bionectria* is reported as a subgenus of *Clonostachys* (Moreira *et al*., 2016).

Conclusions

In the present study, various species of phytopathogenic, endophytic, and epiphytic fungi were identified associated with diseased fruits and in the harvest phase of the *Theobroma cacao* crop in the Norte de Santander and Bolívar departments, regions characterized by their low

productivity. In Norte de Santander, the presence of *Moniliophthora roreri*, a fungus responsible for moniliasis, one of the most devastating diseases for this crop, was highlighted, which reaffirms its endemic nature and historical impact in this region. On the other hand, in Bolívar, the species *Neofusicoccum parvum*, recognized as responsible for black ear rot, was identified and characterized for the first time. This is a novel and highly relevant finding, since it expands the knowledge we have about the etiological agents responsible for diseases associated with cocoa cultivation in Colombia, traditionally attributed to oomycetes of the genus *Phytophthora*. This discovery has significant implications at a regional and national scale for the management and control of diseases in this crop. The identification of the *F. solani* complex, as well as the different species of *Aspergillus* and *Penicillium*, supports the need for integrated management of the crop, to avoid potential public health risks, due to the presence of traces of mycotoxins that can resist the grain benefit process and reach the final consumer in the transformed product. In contrast, the identification of *Geotrichum candidum*, *Clonostachys rosea*, and *Bionectria wenpingii* demonstrated the presence of fungi with beneficial functions for the crop, either contributing to plant growth or as potential biocontrollers against pathogens.

Acknowledgments

All authors would like to thank the cocoa producers of Norte de Santander and Bolívar, who provided data and the biological material to carry out this research.

Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author´s contributions

Article writing: OJPP, RAVG, RGC. Experimental design: RAVG, RGC, FHH. Experimental setups: OJPP, ECM. Data analysis: OJPP, RAVG, RGC, FHH. All authors reviewed the final version of the manuscript.

Literature cited

Aime, M. C., & Phillips-Mora, W. (2005). The causal agents of witches' broom and frosty pod rot of cacao (chocolate, *Theobroma cacao*) form a new lineage of Marasmiaceae. *Mycologia*, *97*(5), 1012–1022. https://doi.org/10.1080/15572536.2006.11832751

Albores Flores, V. J., Gómez Rodríguez, L., López García, J. A., & Grajales Conesa, J. (2022). Mecanismos de infección endógena en frutos de cacao con *Moniliophthora roreri*. *Polibotánica*, (53), 197–209. https://doi.org/10.18387/polibotanica.53.13

Amaro, T. M. M. M., Cope, J., & Franco-Orozco, B. (2021). Current status of plant pathogens of agricultural importance for Colombia. A review. *Revista Colombiana de Ciencias Horticolas*, *15*(2), Article e12425. https://doi.org/10.17584/rcch.2021v15i2.12425

Antolinez Sandoval, E. Y., Almanza Merchán, P. J., Baraona Rodriguez, A. F., Polanco Díaz, E., & Serrano Cely, P. A. (2020). Estado actual de la cacaocultura: una revisión de sus principales limitantes. *Ciencia y Agricultura*, *17*(2), 1–11. https://doi.org/10.19053/01228420.v17.n2.2020.10729

Arvelo, M. Á., Delgado, T., Maroto, S., Rivera, J., Higuera, I., & Navarro, A. (2016). *Estado actual sobre la producción, el comercio y cultivo del cacao en América*. Instituto Interamericano de Cooperación para la Agricultura, San José, Costa Rica,. http://hdl.handle.net/11159/3773

Bailey, B. A., & Meinhardt, L. W. (Eds.) (2016). *Cacao diseases: A history of old enemies and new encounters*. https://doi.org/10.1007/978-3-319-24789-2

Coleman, J. J. (2016). The *Fusarium solani* species complex: Ubiquitous pathogens of agricultural importance. *Molecular Plant Pathology*, *17*(2), 146–158. https://doi.org/10.1111/mpp.12289

Copetti, M. V., Iamanaka, B. T., Frisvad, J. C., Pereira, J. L., & Taniwaki, M. H. (2011). Mycobiota of cocoa: From farm to chocolate. *Food Microbiology*, *28*(8), 1499–1504. https://doi.org/10.1016/j.fm.2011.08.005

De Hoog, G. S., Guarro, J., Gené, J., Ahmed, S. A., Al Hatmi, A. S. S., Figueras, M. J., & Vitale, R. G. (2020). *Atlas of clinical fungi* (4th ed.). https://www.atlasclinicalfungi.org

Delgado-Ospina, J., Molina-Hernández, J. B., Chaves-López, C., Romanazzi, G., & Paparella, A. (2021). The role of fungi in the cocoa production chain and the challenge of climate change. *Journal of Fungi*, *7*(3), Article 202. https://doi.org/10.3390/jof7030202

Delgado-Ospina, J., Molina-Hernandez, J. B., Viteritti, E., Maggio, F., Fernández-Daza, F. F., Sciarra, P., Serio, A., Rossi, C., Paparella, A., & Chaves-López, C. (2022). Advances in understanding the enzymatic potential and production of ochratoxin A of filamentous fungi isolated from cocoa fermented beans. *Food Microbiology*, *104*, Article 103990. https://www.sciencedirect.com/science/article/abs/pii/S0740002022000144

EFSA Panel on Plant Health (PLH), Bragard, C., Baptista, P., Chatzivassiliou, E., Di Serio, F., Gonthier, P., Miret, J. A. J., Justesen, A. F., MacLeod, A., Magnusson, C. S., Milonas, P., Navas-Cortes, J. A., Parnell, S., Potting, R., Stefani, E., Thulke, H. H., Van der Werf, W., Civera, A. V., Yuen, J., …, & Reignault, P. L. (2023). Pest categorisation of *Pestalotiopsis microspora*. *EFSA Journal*, *21*(12), Article e8493. https://doi.org/10.2903/j.efsa.2023.8493

Fedecacao. (2021a). *Caracterización de productores de cacao 2017-2021*. Federacion Nacional de Cacaoteros. https://www.fedecacao.com.co/documentos-tecnicos

Fedecacao. (2021b). *Economía nacional*. Federacion Nacional de Cacaoteros. https://www.fedecacao.com.co/economianacional

Fedecacao. (2025). *Colombia reportó en 2024 producción histórica de cacao*. Federacion Nacional de Cacaoteros. https://www.fedecacao.com.co/post/colombia-report%C3%B3-en-2024-producci%C3%B3n-hist%C3%B3rica-de-cacao

Fernández-Niño, M., Rodríguez-Cubillos, M. J., Herrera-Rocha, F., Anzola, J. M., Cepeda-Hernández, M. L., Aguirre Mejía, J. L., Chica, M. J., Olarte, H. H., Rodríguez-López, C., Calderón, D., Ramírez-Rojas, A., Portillo, P., Restrepo, S., & González Barrios, A. F. (2021). Dissecting industrial fermentations of fine flavour cocoa through metagenomic analysis. *Scientific Reports*, *11*, Article 8638. https://doi.org/10.1038/s41598-021-88048-3

Garcés de Granada, E., Correa de Restrepo, M., Coba de Gutierrez, B., Orozco de Amézquita, B., Zapata, A. C., Anacona Chingana, A., & Sabogal, S. P. (2015). *Morfología y clasificación de los hongos*. Universidad Nacional de Colombia. https://repositorio.unal.edu.co/handle/unal/79887

Hamzah, T. N. T., Lee, S. Y., Hidayat, A., Terhem, R., Faridah-Hanum, I., & Mohamed, R. (2018). Diversity and characterization of endophytic fungi isolated from the tropical mangrove species, *Rhizophora mucronata*, and identification of potential antagonists against the soil-borne fungus, *Fusarium solani*. *Frontiers in Microbiology*, *9*, Article 1707. https://doi.org/10.3389/fmicb.2018.01707

Han, P., Zhang, X., Xu, D., Zhang, B., Lai, D., & Zhou, L. (2020). Metabolites from *Clonostachys* fungi and their biological activities. *Journal of Fungi*, *6*(4), Article 229. https://doi.org/10.3390/jof6040229

Huda-Shakirah, A. R., Zakaria, L., & Mohd, M. H. (2024). Morphology, phylogeny and pathogenicity of fungal species associated with leaf blight and stem canker of *Theobroma cacao* in Malaysia. *Plant Pathology*, *73*(6), 1382–1399. https://doi.org/10.1111/ppa.13900

ICCO. (2021). *Pests & diseases*. International Cocoa Organization. https://www.icco.org/pests-diseases/#toggle-id-39

Jain, A., Sarsaiya, S., Wu, Q., Lu, Y., & Shi, J. (2019). A review of plant leaf fungal diseases and its environment speciation. *Bioengineered*, *10*(1), 409–424. https://doi.org/10.1080/21655979.2019.1649520

Kagezi, G. H., Sseruyange, J., Kucel, P., Kobusinge, J., Nakibuule, L., Kabole, C., & Wagoire, W. W. (2017). *Fusarium* spp. associated with *Xylosandrus compactus* causing wilting in cocoa. *Journal of Plant Pathology*, *99*(2), 542–542. https://www.jstor.org/stable/44686824

Krauss, U., ten Hoopen, M., Rees, R., Stirrup, T., Argyle, T., George, A., Arroyo, C., Corrales, E., & Casanoves, F. (2013). Mycoparasitism by *Clonostachys byssicola* and *Clonostachys rosea* on *Trichoderma* spp. from cocoa (*Theobroma cacao*) and implication for the design of mixed biocontrol agents. *Biological Control*, *67*(3), 317–327. https://doi.org/10.1016/j.biocontrol.2013.09.011

Luo, G., & Mitchell, T. G. (2002). Rapid identification of pathogenic fungi directly from cultures by using multiplex PCR. *Journal of Clinical Microbiology, 40*(8), 2860–2865. https://pmc.ncbi.nlm.nih.gov/articles/PMC120665/pdf/1281.pdf

MADR. (2021). *Cadena de cacao. Dirección de Cadenas Agrícolas y Forestales*. Ministerio de Agricultura y Desarrollo Rural. https://sioc.minagricultura.gov.co/Cacao/Documentos/2021-03-31%20Cifras%20Sectoriales.pdf

Marelli, J. P., Guest, D. I., Bailey, B. A., Evans, H. C., Brown, J. K., Junaid, M., Barreto, R. W., Lisboa, D. O., & Puig, A. S. (2019). Chocolate under threat from old and new cacao diseases. *Phytopathology*, *109*(8), 1331–1343. https://doi.org/10.1094/PHYTO-12-18-0477-RVW

Ministerio de Desarrollo Agrario y Riego. (2023). *Observatorio de commodities de cacao*. Boletín trimestral No. 1. Ministerio de Desarrollo Agrario y Riego. https://cdn.www.gob.pe/uploads/document/file/5264502/ Commodities Cacao%3A ene-mar 2023.pdf?v=1697059698#:~:text=En el primer semestre del,%2C Junín%2C Huánuco y Cusco

Moreira, G. M., Abreu, L. M., Carvalho, V. G., Schroers, H. J., & Pfenning, L. H. (2016). Multilocus phylogeny of *Clonostachys* subgenus *Bionectria* from Brazil and description of *Clonostachys chloroleuca* sp. nov. *Mycological Progress*, *15*(10–11), 1031–1039. https://doi.org/10.1007/s11557-016-1224-6

Muñoz, V., Cisterna, V., & France, A. (2020). Aislamiento de microorganismos fitopatogenos. In J. F. Castro (Ed.), *Conformación de colecciones de cultivos microbianos* (Boletín INIA No. 428, pp. 75–91). Instituto Nacional de Investigaciones Agropecuarias, Chile. https://hdl.handle.net/20.500.14001/6945

Oussou, K. F., Guclu, G., Kelebek, H., & Selli, S. (2022). Elucidating the contribution of microorganisms to the spontaneous fermentation and the quality of Ivorian cacao (*Theobroma cacao*) beans: The quality of Ivorian cacao (*Theobroma cacao*) beans. *Quality Assurance and Safety of Crops & Foods*, *14*(4), 23–35. https://doi.org/10.15586/qas.v14i4.1078

Paterson, R. R. M., & Lima, N. (Eds.) (2015). *Molecular biology of food and water borne mycotoxigenic and mycotic fungi*. CRC Press. https://doi.org/10.1201/b18645

Puig, A. S., Keith, L. M., Matsumoto, T. K., Gutierrez, O. A., & Marelli, J. P. (2021). Virulence tests of *Neofusicoccum parvum*, *Lasiodiplodia theobromae*, and *Phytophthora palmivora* on *Theobroma cacao*. *European Journal of Plant Pathology*, *159*(4), 851–862. https://doi.org/10.1007/s10658-021-02210-1

Puig, A. S., Marelli, J. P., Matsumoto, T. K., Keith, L. M., & Gutierrez, O. A. (2019). First report of *Neofusicoccum parvum* causing pod rot on cacao in Hawaii. *Plant Disease*, *103*(6), 1416–1416. https://doi.org/10.1094/PDIS-10-18-1719-PDN

Rodríguez-Polanco, E., Morales, J. G., Muñoz-Agudelo, M., Segura, J. D., & Carrero, M. L. (2020). Morphological, molecular and pathogenic characterization of *Phytophthora palmivora* isolates causing black pod rot of cacao in Colombia. *Spanish Journal of Agricultural Research*, *18*(2), Article e1003. https://doi.org/10.5424/sjar/2020182-15147

Rodriguez Polanco, E., Parra Alferes, E. B., Bermeo Fuquene, P. A., Segura Amaya, J. D., & Rodríguez Polanco, L. A. (2020). Manejo de la pudrición parda de la mazorca (*Phytophthora palmivora*) en cacao por aplicación conjunta de prácticas culturales y químicas. *Revista Facultad de Ciencias Básicas*, *16*(1), 79–94. https://doi.org/10.18359/rfcb.4887

Romero-Cortes, T., López-Pérez, P. A., Pérez España, V. H., Medina-Toledo, A. K., Aparicio-Burgos, J. E., & Cuervo-Parra, J. A. (2019). Confrontation of *Trichoderma asperellum* VSL80 against *Aspergillus niger* via the effect of enzymatic production. *Chilean Journal of Agricultural and Animal Sciences*, *35*(1), 68–80. https://doi.org/10.4067/s0719-38902019005000202

Schwan, R. F., Pereira, G. V. M., & Fleet, G. H. (2014). Microbial activities during cocoa fermentation. In R. F. Schwan, & G. H. Fleet (Eds.), *Cocoa and coffee fermentations* (pp. 129–192). CRC Press. https://doi.org/10.1201/b17536

Sosa del Castillo, D. S., Parra, D., Noceda, C., & Pérez-Martínez, S. (2016). Co-occurrence of pathogenic and non-pathogenic *Fusarium decemcellulare* and *Lasiodiplodia theobromae* isolates in cushion galls disease of cacao (*Theobroma cacao* L.). *Journal of Plant Protection Research*, *56*(2), 129–138. https://www.plantprotection.pl/Co-occurrence-of-pathogenic-and-non-pathogenic-Fusarium-decemcellulare-and-Lasiodiplodia,94178,0,2.html

Suárez Contreras, L. Y., & Peñaranda Figueredo, F. A. (2022). Identificación molecular de hongos filamentosos y su potencial biotecnológico. *Biotecnología en el Sector Agropecuario e Industrial*, *20*(1), 194–206. https://doi.org/10.18684/rbsaa.v20.n1.2022.1914

Subroto, E., Djali, M., Indiarto, R., Lembong, E., & Baiti, N. (2023). Microbiological activity affects post-harvest quality of cocoa (*Theobroma cacao* L.) beans. *Horticulturae*, *9*(7), Article 805. https://doi.org/10.3390/horticulturae9070805

Sukmawati, D., Firhandini, A., Nurkhasanah, S., Khumaiya, U., Gunadi, S. A., Supiyani, A., Nida Al Husna, S., El Enshasy, H., Dailin, D. J., & Sriherwanto, C. (2024). The effect of yeast antagonist isolated from the fermentation of cocoa beans (*Theobroma cacao*) from Lampung, Indonesia, on the growth of *Aspergillus flavus* UNJCC F-55. *KnE Life Sciences*, *8*(1), 75–84. https://doi.org/10.18502/kls.v8i1.15398

Tekpinar, A. D., & Kalmer, A. (2019). Utility of various molecular markers in fungal identification and phylogeny. *Nova Hedwigia*, *109*(1–2), 187–224. https://doi.org/10.1127/nova_hedwigia/2019/0528

Villamizar-Gallardo, R. A., Ortíz-Rodriguez, O. O., & Escobar, J. W. (2017). Symbiotic and endophytic fungi as biocontrols against cocoa (*Theobroma cacao* L.) phytopathogens. *Summa Phytopathologica*, *43*(2), 87–93. https://doi.org/10.1590/0100-5405/2175

Villamizar-Gallardo, R., Osma, J. F., & Ortíz-Rodriguez, O. O. (2019). Regional evaluation of fungal pathogen incidence in Colombian cocoa crops. *Agriculture*, *9*(3), Article 44. https://doi.org/10.3390/agriculture9030044

Zhao, W., Hong, S.-Y., Kim, J.-Y., & Om, A.-S. (2024). Effects of temperature, pH, and relative humidity on the growth of *Penicillium paneum* OM1 isolated from pears and its patulin production. *Fungal Biology*, *128*(4), 1885–1897. https://doi.org/10.1016/j.funbio.2024.05.005