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Research Article

Morphological and Molecular Characterization of Lasiodiplodia Spp. and Fusarium Spp. Isolated from Cocoa Pods in the Agneby-tiassa Region of Ivory Coast

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Abstract

Cocoa (*Theobroma cacao* L.) is the main cash crop in Côte d'Ivoire and the primary source of income for producers. However, recent studies have highlighted the harmful effects of pod diseases on local production and the existence of a group of pathogens (*Lasiodiplodia* spp. and *Fusarium* spp.) that require better characterization in order to reorient control efforts. The objective of this study was to perform morphological and molecular characterization of isolates of *Lasiodiplodia* spp. and *Fusarium* spp. isolated from cocoa pods affected by rot. Samples of infected pods and soil from cocoa plantations were collected from seven locations in the Agneby-Tiassa region. After isolation on 11% agar culture medium, the isolates were purified on pea and carrot agar culture medium. Macroscopic and microscopic observations were made on these isolates. After DNA extraction, PCR tests with ITS4 and ITS5 primers were performed for molecular identification of the isolates. The PCR products obtained were transferred to a facility for sequencing. It should be noted that macroscopic and microscopic observations showed that *Lasiodiplodia* spp. isolates are light gray or gray-black in color, with thick-walled septate conidia. Fusarium spp. isolates are whitish in color, with oval microconidia and curved macroconidia. PCR test results showed that the isolates from the southeastern region of Côte d'Ivoire are *Lasiodiplodia theobromae* and *Fusarium solani*. Sequencing showed that these isolates were 100% similar to the species in the gene bank. This study will help to better target the fight against cocoa pod rot in Côte d'Ivoire by implementing sustainable control strategies.

Keywords

Pod Rot, *Lasiodiplodia Theobromae*, *Fusarium Solani*, Cocoa, Molecular Biology

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1. Introduction

Cocoa farming plays an important role in the Ivorian economy, with production estimated at over 2.38 million tons of cocoa beans in 2023 [1]. In macroeconomic terms, it accounts for more than 50% of export earnings and contributes 15% to gross domestic product (GDP) [2]. In addition, it generates two-thirds of direct and indirect jobs, according to the FAO [3]. On a social level, around 600,000 farm managers provide a livelihood for nearly 6 million people. Since 1977, Côte d'Ivoire has been the world's leading producer of cocoa beans, accounting for nearly 40% of global production [4]. The importance of cocoa cultivation lies mainly in the manufacture and trade of chocolate. Chocolate is a highly prized commodity on the world market and is therefore an important commercial focus. However, several constraints pose a real threat to Côte d'Ivoire, which wants to maintain its position as the world's leading producer of cocoa beans. These constraints include the effects of climate change, exacerbated by irregular rainy seasons and increasing biodiversity loss. At the same time, heavy pressure from pests (mirrids) and diseases such as swollen shoot [5] and brown and black rot of cocoa pods [6] are significantly reducing production. These types of rot are caused by fungi of the genus *Phytophthora* spp. *Lasiodiplodia* spp. and *Fusarium* spp. *Lasiodiplodia* spp. and *Fusarium* spp. are fungi that cause significant damage to cocoa crops. Production losses due to *Lasiodiplodia* spp. can reach 50% [7, 8]. The disease manifests itself through the appearance of a soft brown lesion, which then spreads across the entire pod with the appearance of a soot-like coating [9]. Symptoms can also appear on leaves, which turn brown, as well as on branches, twigs, and bark. These two fungi have been identified as pathogens in several crops, notably mango tree decline in Burkina Faso [10].

While the damage caused by P*hytophtora* spp. is widely known and documented, that caused by *Lasiodiplodia* spp. and *Fusarium* spp. remains poorly understood and under-documented in research in Côte d'Ivoire. This is why a study of the morphological and molecular identification of these two pathogens is necessary for effective disease management. The objective of this work was to perform a morphological and molecular characterization of isolates of *Lasiodiplodia* spp. and *Fusarium* spp. isolated from cocoa pods.

2. Materials and Methods

2.1. Materials

2.1.1. Study Site

This study was conducted in seven sub-prefectures in the Agneby-Tiassa region of Côte d'Ivoire: Azaguie, Agboville, Cechi, Grand-Morie, Loviguie, Oress-Krobou, and Rubino. This region is located in the southeast of Côte d'Ivoire and belongs to agroecological zone I as defined by [11]. It is a humid forest area with an altitude ranging from 0 to 200 m, annual rainfall between 1,400 and 2,500 mm, and an average temperature of 29 ℃. The soil is clayey and of the weakly desaturated ferralitic type. The plantations are aging and characterized by high disease pressure [12].

2.1.2. Plant Material

The plant material used consisted of immature green pods naturally affected by brown and black rot. Most of these pods came from cocoa trees on village plantations consisting of Ghanaian cocoa, French cocoa, and selected hybrid cocoa trees (Mercedes) that are widely grown by farmers in Côte d'Ivoire. These are Amelonado and Criollo.

2.1.3. Fungal Material

The pathogenic fungal material consisted of isolates of *Lasiodiplodia* spp. and *Fusarium* spp. isolated from pods affected by brown and black rot and from the soil under cocoa trees in village plantations in seven localities in Agneby-Tiassa, Côte d'Ivoire.

2.2. Methods

2.2.1. Sampling

Sampling took place mainly in seven sub-prefectures (localities) in the region (Figure 1). In each of the seven localities, five cocoa plantations were selected (sampling area). In each plantation, three immature green pods affected by brown and black rot were collected during the periods from June to July 2017 and from September to October 2018, which are periods of disease proliferation [13]. Soil samples were taken from homogeneous areas of the cocoa plantations. Samples were taken around the trunk at a distance of 60 to 80 cm. Samples were taken from the top ten centimeters of soil, which is the area most conducive to microbial activity and, above all, the initial host/parasite relationships [14]. After clearing the surface of the soil of dead leaves and plant debris, 150 g of soil was collected using a sterile knife flamed with 90% alcohol to prevent contamination between samples. Five samples were taken per cocoa tree plantation, following one of the diagonals of the plot. These samples were placed in sterile plastic bags and stored at 26 ℃ in a dry place in the laboratory. The geographical coordinates of each site were recorded for the purpose of mapping.

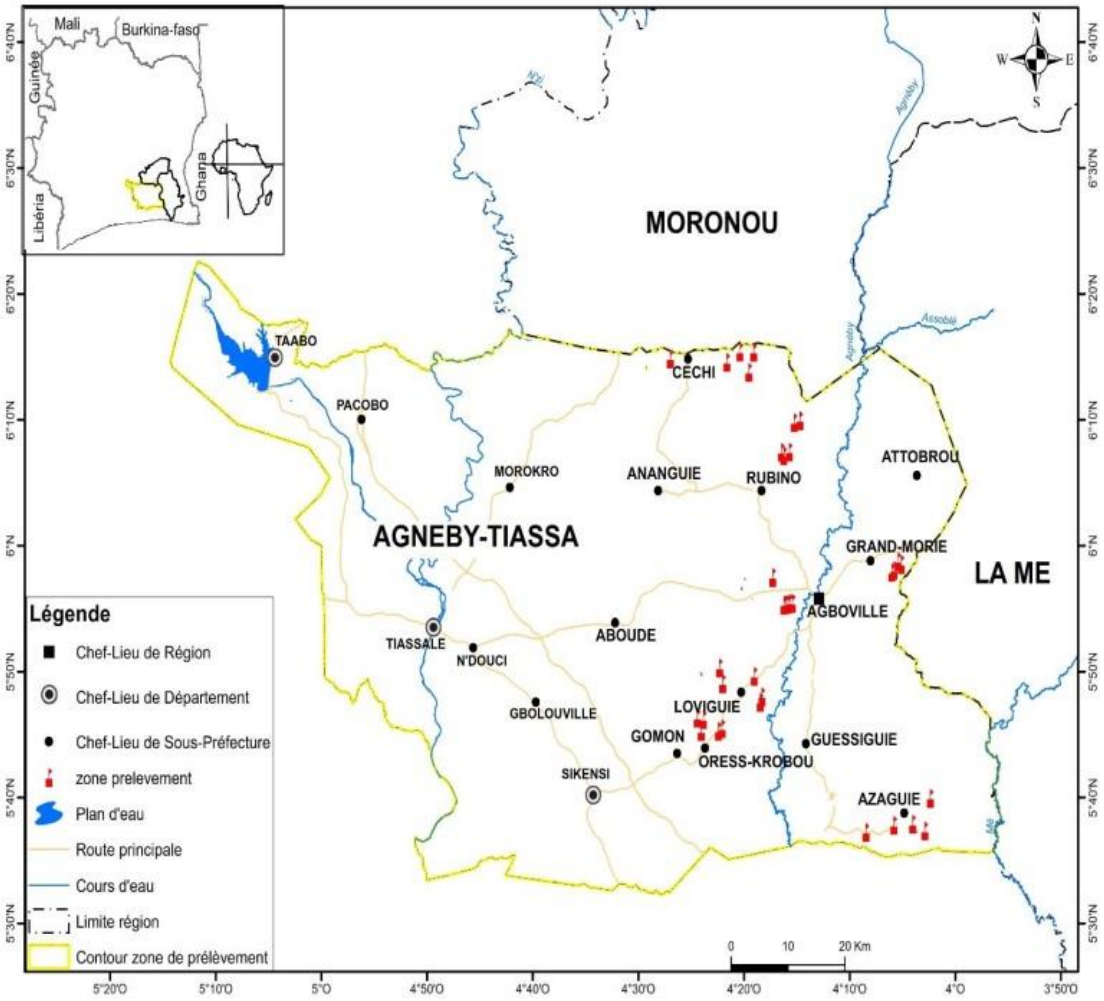

Figure 1. *Sampling sites for cocoa pods and soil under cocoa trees.*

2.2.2. Sampling Isolation of Fungal Pathogens from Pods Affected by Back and Black Rot

The pathogens were isolated from immature green pods showing symptoms of brown and black rot on a culture medium consisting of 11% agarized water. To do this, the pod was washed with tap water, then disinfected and flamed for 15 seconds with 90% alcohol. Next, a cube-shaped fragment was taken from the growth front of the rot, i.e., between the infected part of the pod and the healthy part (Figure 2). Three fragments were then placed on the agar culture medium, and the cultures were incubated at 26 ℃ in the dark for 4 days. After the thallus had formed, a mycelial fragment was taken from the growth front and placed on the pea agar culture medium [15]. Fragments of mycelium were inoculated several times onto pea agar culture medium in order to obtain pure colonies. After isolation, purification, and single-spore culture, 150 isolates were transferred to Montpellier (France) for molecular characterization. Four isolates out of 60 of *Lasiodiplodia* spp. and *Fusarium* spp. were identified.

The collected isolates were inoculated onto V8 agar culture medium. They were then cultured on liquid V8 medium for seven days.

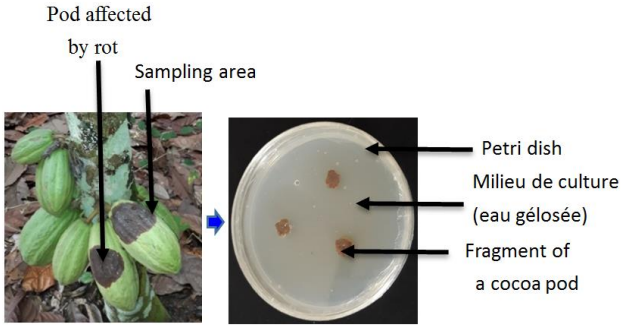

Figure 2. *Green pods affected by rot (left) and pod fragments on agar culture medium (right).*

2.2.3. Isolation of Soil Fungal Parasites

Isolation was carried out using the suspension-dilution technique. The principle consists of suspending the sample in sterile distilled water, then incorporating the various dilutions

of this suspension into the isolation [16, 17]. Thus, 10 g of soil sample is poured into 100 ml of sterile distilled water contained in a jar. The mixture is then homogenized using a magnetic stirrer for 30 minutes. The resulting suspension constitutes the 10^{-2} dilution. Successive 1 ml samples from this suspension, then from the subsequent suspensions added each time to 9 ml of sterile distilled water, constituted a series of dilutions from 10^{-3} to 10^{-9}. 10 µl of each final suspension was placed on semi-selective agar medium solidified in Petri dishes. For each dilution and each isolation medium, two replicates were performed. The Petri dishes were incubated in the dark at 26 ℃ in an incubator for 3 to 7 days.

2.2.4. Morphological and Molecular Characterization of Fungal Parasites of Cocoa Trees

(i) Monospore Culture of Isolates of *Lasiodiplodia* Spp. and *Fusarium* Spp.

The isolates were grown on V8 agar medium. Incubation was carried out in total darkness at 26 ℃ for 4 days, and the Petri dishes were then exposed to a 12-hour photoperiod for 7 days to induce spore formation. To induce spore release, each culture was flooded with 10 ml of sterile distilled water. The *Lasiodiplodia* spp. and *Fusarium* spp. cultures were kept at laboratory room temperature (26 ℃) for 2 hours. The spores obtained were counted using a Malassez cell. A concentration of 50 to 60 spores ml-1 was obtained by successive dilution of an aliquot in which the spores were immobilized by two drops of 10% hydrochloric acid. One hundred microliters of the calibrated suspension were spread on 1.5% V8 agar medium using a curved glass rod, which had been flamed and cooled in sterile distilled water. Incubation was carried out in the dark at 26 ℃ for 12 to 24 hours. After incubation, the germinated spores were collected individually under a binocular magnifying glass with a sterile needle, then seeded in carrot agar culture medium at a rate of four single spores per isolate. After 7 days of incubation in the dark at 26 ℃, clones identical to the parental phenotype were selected for further study.

(ii) Morphological Identification

All morphological studies were performed on a 22% pea agar extract in distilled water. The isolates were purified by successive transfer to pea agar culture medium. After purification of the isolates on nutrient medium [15], a 7 mm diameter explant was taken from the growing tip of the fungus using a punch, then placed in the center of each Petri dish. After 7 days of incubation at 26 ℃ in the dark, macroscopic and microscopic observations were made. The macroscopic description of the isolates focused on the appearance and color of the thallus and the spores from Petri dish cultures. After the morphological characterization of each isolate, the mycelium from a 7-day-old culture was collected using a needle and placed on a glass slide. Microscopic observations were made using an optical microscope. These observations focused on the appearance of the mycelium and the morphology and shape of the spores. Macroscopic and microscopic descriptions and the recognition of characteristic features that could be used to identify the species made it possible to distinguish between groups of fungi using the identification keys provided by a number of authors [18-23].

(iii) Molecular Characterization of *Lasiodiplodia* Spp. and *Fusarium* Spp.

Cultivation, extraction, and quantification of DNA

The isolates were transferred to the CIRAD laboratory in France for analysis. DNA extractions were performed following the steps provided in the CTAB (cetyl trimethylammonium bromide) DNA extraction protocol described by a number of authors [24-26]. The total genomic DNA obtained was diluted with distilled water for PCR amplification. The different steps for extraction are as follows: wall digestion by digestion buffer, extralysis, cell lysis by cetyl trimethylammonium bromide (CTAB), purification by CIAA, precipitation by isopropanol, washing with ethanol, and recovery of DNA in distilled water. The DNA obtained was measured using a Nanodrop 2000.

Polymerase chain reaction (PCR) of DNA

After titration, a volume Vx of DNA from the stock solution was taken from each sample to form aliquots concentrated at 20 ng/µl in Eppendorf tubes (e.g., prepare 20 ng/µl DNA solutions, one plate = 100 samples, 3 µl/PCR). The volume Vx of DNA to be taken depends on the concentration obtained after titration. PCR was performed with the primers ITS5-FGGAAGTAAAAGTCGTAACAAGG and ITS4-R TCCTCCGCTTATTGATATGC for sequencing. The labeled primers (Forward), at 10 nmol and supplied in lyophilized form, were diluted as described by a number of authors [27-29]. PCR was performed in a reaction volume of 40 µl. Amplification was carried out using an Eppendorf thermocycler (Biometra, An Analytic Jena Company).

Sequencing

The single-stranded sequences obtained during sequencing with the ITS4 and ITS5 primers were corrected based on the interpretation of the electropherograms using Codoncode Align software in order to clean up the sequences. Similarity searches in the ITS4 and ITS5 regions of the genome were performed using BLAST software in the NCBI gene bank. The isolates were compared to those listed in the GenBank database of the National Center for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Searches performed by BLAST (Basic Local Alignment and Search Tool) at NCBI provide a list of probable results corresponding to our sequence, with sequence similarity values. Sequence comparison programs aim to identify areas where two sequences are identical or very similar and to deduce which of these are significant and correspond to a biological meaning among those observed in international databases. BLAST detects short segments that are locally homologous to the unknown sequence [30]. The results of sequence comparisons are presented as the homology ratio between the sequences obtained and the closest reference sequences.

3. Results

3.1. Morphological Characteristics of Fungal Parasites

Macroscopic and microscopic characteristics of isolates

During this study, more than 200 isolates were obtained from pods affected by rot and from the soil under cocoa trees in the seven locations surveyed. The isolates consisted of 45% *Lasiodiplodia* spp. and 15% *Fusarium* spp. (Figure 3). These observations also showed that *Lasiodiplodia* spp. isolates are light gray or gray-black in color, with thick-walled septate conidia. *Fusarium* spp. isolates are whitish in color, with oval microconidia and curved macroconidia (Table 1).

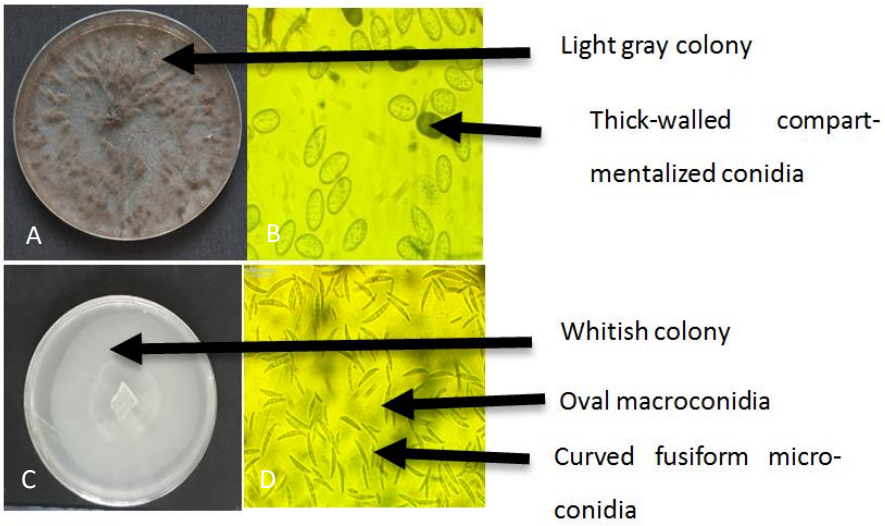

Figure 3. *Macroscopic and microscopic characteristics of isolates.*

A: Macroscopic characteristics of *Lasiodiplodia* spp; B: Microscopic characteristics of *Lasiodiplodia* spp; C: Macroscopic characteristics of *Fusarium* spp; D: Microscopic characteristics of *Fusarium* spp

Table 1. *Morphological identification of isolates.*

	Camps	Mushroom codes	Color of the thallus	Appearance of the thallus
Agboville	Scaf	AGS1	light gray	Cotton-like Radiated stripe
		AGS2	whitish	
		AGS3	light gray	
Agboville	Mure	AGM1	whitish	Cotton-like
		AGBM2	whitish	
		AGM3	whitish	
Agboville	Gbalekro	AGG1	light gray	Cotton-like Radiated stripe
		AGG2	light gray	
		AGG3	whitish	
Cechi	Ndampo	CND1	light gray	Cotton-like Radiated stripe
		CND2	light gray	
		CND3	light gray	
Cechi	Kouamekro	CKO1	whitish	Cotton-like

	Camps	Mushroom codes	Color of the thallus	Appearance of the thallus
		CKO2	light gray	Radiated stripe
		CKO3	light gray	
		CNG1	whitish	
Cechi	Nguessan kouamekro	CNG2	light gray	Cotton-like Radiated stripe
		CNG3	whitish	
		GMN1	whitish	
Grand-Morie	Ntchago	GMN2	light gray	Cotton-like Radiated stripe
		GMN3	whitish	
		GGM1	whitish	
Grand-Morie	Grand-Moutcho	GGM2	Gray-black	Cotton-like
		GGM3	light gray	
		GMA1	light gray	
Grand-Morie	Akpessenou	GMA2	light gray	Cotton-like Radiated stripe
		GMA3	light gray	
		RND1	Gray-black	
Rubino	Ndampo	RND2	light gray	Cotton-like Radiated stripe
		RND3	light gray	
		RAL1	Gray-black	
Rubino	Allepila 2	RAL2	light gray	Cotton-like Radiated stripe
		RAL3	Gray-black	
		RMB1	whitish	
Rubino	Mbotchimpo	RMB2	whitish	Cotton-like Radiated stripe
		RMB3	whitish	
		OKAb1	whitish	
Oress-krobou	Piste Aboude	OKAb2	whitish	Radiated stripe
		OKAb3	whitish	
		OKAg1	light gray	
Oress-krobou	Piste Agboville	OKAg2	Gray-black	Cotton-like Radiated stripe
		OKAg3	Gray-black	
		OAb2 1	light gray	
Oress-krobou	Piste Aboude 2	OAb2 2	whitish	Cotton-like Radiated stripe
		OAb2 3	whitish	
		LOL1	light gray	
Loviguie	Loviguie 2	LOL2	Gray-black	Cotton-like
		LOL3	Gray-black	
Loviguie	Whain	LOW1	light gray	Cotton-like

	Camps	Mushroom codes	Color of the thallus	Appearance of the thallus
Loviguie	Loviguie1	LOW2	light gray	Radiated stripe
		LOW3	Gray-black	
		LOV1	light gray	Cotton-like
		LOV2	light gray	Radiated stripe
		LOV3	light gray	
Azaguie	Abbe	AZAb1	whitish	Cotton-like
		AZAb2	whitish	Radiated stripe
		AZAb3	whitish	
Azaguie	Mankoudje	AZM1	light gray	Cotton-like
		AZM2	light gray	Radiated stripe
		AZM3	light gray	
Azaguie	Gare	AZG1	light gray	Cotton-like
		AZG2	whitish	Radiated stripe
		AZG3	Gray-black	

3.2. Molecular Characteristics of Isolates from Infected Pods

Assays using the NANODROP 2000 showed that the quantity of DNA obtained is pure and sufficient for molecular studies. The quantity of DNA ranged from 50 to 491 ng/µl.

The results of the analysis of PCR products on agarose gel reflect the successful amplification of DNA by PCR. Conventional PCR amplification tests with ITS4 and ITS5 primers for sequencing *Fusarium* spp. and *Lasiodiplodia* spp. isolates revealed the presence of amplicons in each of the isolates with an approximate amplification size of 540 bp for *Lasiodiplodia* spp. and *Fusarium* spp. (Figures 4 and 5).

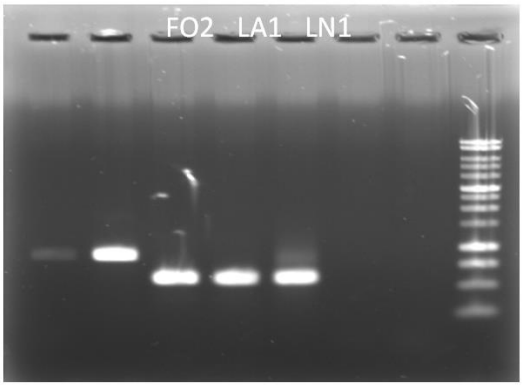

Figure 4. *Agarose gel containing isolates of Fusarium solani and Lasiodiplodia theobromae.*

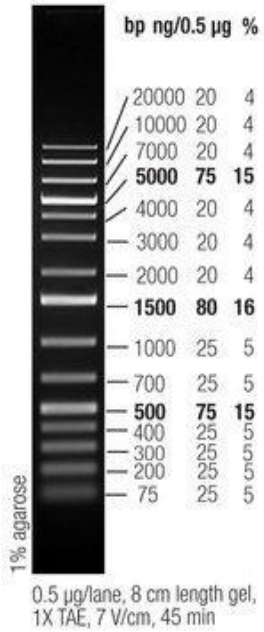

Figure 5. *Promega molecular weight marker.*

The PCR products from the four isolates were transferred to GENWIZ for sequencing. The DNA sequences obtained were edited, corrected, and then aligned using Codoncode Aligner software, before being compared with those in the GENBANK databases. The BLAST software identified a 100% similarity with the nucleotide sequences studied and indicates that isolates GRMFL1 and AGBFO2 are similar to *Fusarium solani* and RUBLN1 and LOVLA1 to *Lasiodiplodia theo-*

bromae. The sequence similarity searches performed between the ITS4 and ITS5 primers and the sequences in the NCBI databases are presented in Table 2.

Table 2. *Information on DNA sequences of Lasiodiplodia theobromae and Fusarium solani from cocoa orchards.*

Localities	Isolated codes	Sequences	Species	GENBANK reference
Grand-Morie	GRMFL1	Seq1	*Fusarium solani*	KX385048 1
Agboville	AGBFO2	Seq2	*Fusarium solani*	KX385048 2
Loviguie	LOVLA1	Seq3	*Lasiodiplodia theobromae*	MG870594 3
Rubino	RUBLN1	Seq4	*Lasiodiplodia theobromae*	MG870594 4

4. Discussion

Macroscopic and microscopic analysis identified two types of facies with light gray and gray-black mycelium colors for *Lasiodiplodia theobromae* and a whitish color for the *Fusarium solani* species. In addition, the conidia were thick-walled and septate for *Lasiodiplodia theobromae*, and for *Fusarium solani*, the macroconidia were oval, while the microconidia were fusiform and curved. These results corroborate those obtained by several authors [6, 32]. Black pod rot is caused by two major species of *Phytophthora* (*P. palmivora* and *P. megakarya*) in Côte d'Ivoire. In this study, only the species *P. palmivora* was identified in the localities studied. *P. palmivora* is the dominant species in Ivorian cocoa orchards, according to these author [31, 22]. In addition to this species, there are two other species (*Lasiodiplodia theobromae* and *Fusarium solani*). These are fungi that parasitize many fruits. *Lasiodiplodia* spp. or *Botriodiplodia theobromae* is responsible for black pod disease in cocoa pods, and *Fusarium solani* attacks pods after they have been infected by *Phytophthora* spp. Similar results were reported by the authors [25, 10], who showed that these pathogens are responsible for the rot of dessert bananas and many other fruits.

Furthermore, the author [6] showed that the sequences of strains from Côte d'Ivoire were 100% similar to those found in databases. This could be explained by the fact that there is little or no interspecific variability between species for ITS primers. The molecular weight marker generated 520 base pairs for both species. Our results contradict those of the authors [32], whose molecular weight marker generated base pairs of less than 520.

5. Conclusions

Morphological and molecular characterization identified pods in seven locations in the Agneby-Tiassa region. This study will enable producers to refocus their efforts to combat pod rot in order to minimize production losses and increase their income.

Abbreviations

DDT	Dichloro-diphenyl-trichloroethane
dNTP	Deoxyribonucleotide Triphosphates
EDTA	Ethylenediaminetetraacetic Acid
ITS	Internal Transcribed Spacer
Kpb	Kilo pair of Bases
Pb	Pair of Bases
PCR	Polymerase Chain Reaction
PDA	Patato Dextrose Agar
TAE	Tris-acetate EDTA
TBE	Borate EDTA Tris

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Author Contributions

Kone Nahoua: Methodology, Visualization, Data Curation, Investigation.

Silue Napkalo: Data Curation, Funding acquisition.

Fofana Balakissa: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Writing – original draft, Writing – review & editing.

Zouzou Michel: Conceptualization, Validation.

Kone Daouda: Conceptualization, Methodology, Project

administration, Supervision, Validation.

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Conflicts of Interest

The authors declare no conflicts of interest.

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