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Epidemiology of cocoa pod rots and characterization of causal agents in Cote d'ivoire

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ABSTRACT: Cocoa pod rot poses a real threat to cocoa farming in tropical countries. In Côte d'Ivoire, these diseases are caused by a fungal complex that can lead to severe yield losses of up to 60 %. The aim of this study was to assess the epidemiological parameters of cocoa pod rots and characterize the fungal agents responsible. To this end, a phytosanitary survey was carried out in eight producing areas (Agboville, Aboisso, Abengourou, Bouaflé, Daloa, Méagui, Soubré and Tiassalé). The symptoms observed were described, their severity have been assessed. The fungi associated with these different symptoms were isolated on PDA, then their pathogenicity was assessed after inoculation on asymptomatic pods. Pathogenic fungi were identified morphologically and molecularly. Black and brown rot are the symptoms observed in all the locality. Rot was very severe in Tiassalé with 68.4% black rot and 62.2% brown rot, and less severe in Soubré with 21.6% and 20%. Molecular analyses revealed the presence of *Fusarium falciforme*, *Colletotrichum aenigma*, *Lasiodiplodia theobromae* and *Phytophthora megakarya*, which demonstrated their pathogenic powers.This first exhaustive study of the microflora and knowledge of the fungal species responsible for rotting in Côte d'Ivoire enables us to better orientate control methods.

KEYWORDS: Ivory Coast, cocoa farming, prevalence, fungi, severity

INTRODUCTION

The cacao tree (*Theobroma cacao* L.) is a dense, humid tropical forest tree belonging to the Malvaceae family [1]. The Theobroma genus comprises over 120 species, including *Theobroma cacao* L. the most cultivated in the world. This crop was introduced to Côte d'Ivoire in the south- east region towards the end of the 19th century [2].

The cocoa tree is mainly grown for its beans, which are very important ingredients in theagri- food, cosmetics, pharmaceutical and confectionery industries [3, 4]. This crop is an important pillar in the socio-economic life of the Ivorian populations. In Côte d'Ivoire, cocoa occupies an important place in the socio-economic life of populations. Indeed, this sector contributes 15% of Gross Domestic Product (GDP) and generates around 46% of the country's export earnings [5, 6]. World cocoa bean production was 5.58 million tonnes in 2022 for 11.5 million hectares under cultivation. Production is concentrated in two main countries, Côte d'Ivoire and Ghana [7]. In Africa, the cultivated area is 8.5 million hectares, for an annual production of 4.1 million tonnes in 2022 [7]. Côte d'Ivoire is the leading producer and exporter, with annual production estimated at over 2.2 million tonnes in 2022 from 4.3 million hectares under cultivation [7].

Despite its socio-economic importance, cocoa bean production is increasingly exposed to a diversity of diseases that cause considerable losses for producers. Climate change, declining soil fertility and insect pests are also having an impact on this crop. Faced with all these difficulties, most producers abandon their orchards or convert them to other perennial crops [8]. Among these, fungal diseases are a major constraint for cocoa farming. Fungal infections cause various symptoms of pod rot, leading to very significant losses of up to 20 has 60 %, or even the destruction of orchards [9, 10]. Rot has been defined as anomalies on cocoa pods that correspond to necrosis. Cocoa pods reached by rot show spots on the surface. Over time, a white sporiferous layer covers the necrotic part of the cocoa pod and the beans, which become completely black [11]. Since the explosion ofs cocoa pod rot in Côte d'Ivoire, the disease has been mentioned individually in the work of Pohé et al. [12] and Coulibaly et al. [12]. However, no in-depth study has been carried out on the fungal complex and the characterization of the fungal agents responsible. The aim of this study was to assess the epidemiological parameters of cocoa pod rot in Côte d'Ivoire and to characterize the fungal agents responsible.

MATERIALS AND METHODS

Determination of the phytosanitary status of cocoa tree plantations

Thes prospectings in the middle rural were carried out from June 2023 to early March 2024 in eight major cocoa production localities reparte in six regions of Côte d'Ivoire. These were Agboville and Tiassalé (Agnéby - Tiassa) ; Abengourou (Indénié - Djuablin), Soubré and Méagui (Nawa) ; Aboisso (Sud - Comoé) ; Daloa (Haut - Sassandra) and Bouaflé (Marahoué). These localities are located in two of Côte d'Ivoire seven agro-ecological zones (ZAE). These are the Southern Dense Rainforest Zone (ZAE I) and the Western Dense Rainforest Zone (ZAE II), with average temperatures of 26 °C and 25.5 °C respectively. The southern rainforest zone (ZAE I) covers the localities of Aboisso, Abengourou, Agboville and Tiassalé. The western humid forest zone (ZAE II) covers the localities of Bouaflé, Daloa, Méagui and Soubré. Each locality in the different agro-ecological zones covers four types of season : two rainy seasons and two dry seasons. Symptomatic and asymptomatic cocoa pods were collected and transported to the laboratory of the Unity Sante of Plantes of the University of Nangui ABROGOUA for analysis (Fig. 1). The study of state sanitary was carried out in these various localities in the form of individual interviews. Information on varieties grown, age of planting, type of treatments used and frequency of treatments was collected. A total of 40 producers were interviewed, because of 5 producers of cocoa by locality. The data collected were compared with observations made on the plantations.

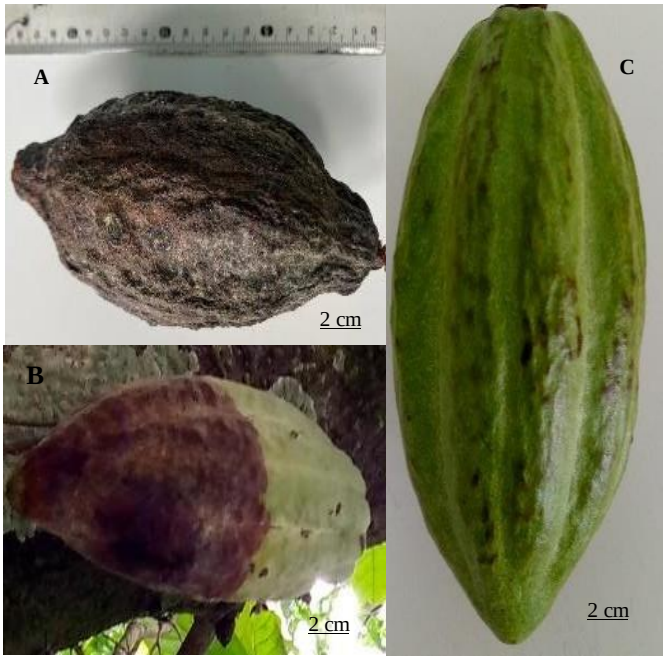

Fig. 1. Cocoa pods collected from the cocoa plantations visited. A : Pod affected by black rot; B : Pod affected by brown rot; C : Healthy pod

Determining the prevalence of rots

Rots prevalence represents the ratio of the total number of plants bearing infected cocoa pods to the total number of plants inspected in each subplot. Prevalence was determined according to the formula of Zokou et al. [14].

$$P(\%) = \frac{NPI}{NPT} x\ 100 \quad (1)$$

when P (%): Prevalence of rot; NPI : Number of plants bearing cocoa pods reached by rot; NPT : Total number of cocoa trees in a sub-plot.

Determining of rot severity

In the delimited sub-plots, 10 cocoa plants were used to assess severity. The severity of different types of pod rot was assessed, and several notes were assigned to infected plants according to the modified rating scale of Iwaro et al. [15].

Rating scale from 0 to 5: 0: no symptoms on cocoa pods; 1: 1 to 20% of pods show symptoms; 2: 21 to 40% of pods show symptoms; 3: 41 to 60% of pods show symptoms; 4: 61 to 80% of pods show symptoms; 5: 81 to 100% of pods show symptoms. The rot severity index was calculated according to the following formula of Cooke [16] cited by Dianda et al. [17].

$$IS(\%) = \frac{\Sigma(Xi \times Ni \times 100)}{N \times Nt} \quad (2)$$

when IS (%): Rot severity index; Xi: Score attributed to a diseased plant ; Ni : number of infected plants; Nt : Total number of plants observed ; N : Highest observed severity score.

Collecting of cocoa pod samples

Symptomatic and asymptomatic pods were randomly collected from the various major cocoa- producing areas visited in Côte d'Ivoire. Indeed, 40 plots of pure cocoa were visited, including five per locality. Within each plot, 5 sub-plots were delimited and sampled randomly in following the device in “X” in order to visit a large number of cocoa plants bearing infected pods.

In each sub-plot, 4 pods affected by rot and 2 healthy pods were collected from 6 cocoa trees. One hundred and fifty (150) samples of pods were collected, including 100 symptomatic and 50 asymptomatic per locality, for a total of 1,200 samples. Collected samples were kept in plastic bags, labelled, and transferred to the laboratory of the Plant Health Unit of Nangui ABROGOUA University for isolation of fungal strains.

Characterization of fungi associated with cocoa pod rot Isolation of fungal strains from infected cocoa pods

Collected infected cocoa pods were disinfected by immersion for 3 minutes in a 2% sodium hypochlorite solution, then rinsed 3 consecutive times with sterile distilled water. They were then deposit on blotting paper in below laminar flow hood for five minutes to remove the rinsing water. Then, explants were taken from the cocoa pods at the forehead of the rot growth and inoculated onto a PDA (*Potato*

Dextrose Agar) culture medium in 8.5 cm diameter Petri dishes. To do this, in each Petri dish, 4 explants were placed at the edges of the dishes and then incubated at room temperature (25 ± 1°C) for two to three days, to allow for the development of mycelial colonies. The developed mycelial colonies were then subcultured to obtain pure colonies according to the method of Choi et al. [18].

Evaluation of the Pathogenicity of Isolated Fungal Strains

The pathogenicity test of fungal isolates isolated from rot was conducted on healthy, mature pods collected to identify the genera responsible for the rot. Indeed, 25 mature pods were disinfected with 3% sodium hypochlorite for 3 min, then rinsed three times with sterile distilled water. Next, the pods were placed on blotting paper for 10 min under a laminar flow hood to remove rinse water.

The four fungal genera, each 14 days old on PDA (*Potato Dextrose Agar*) culture medium, were used for this pathogenicity test. For each genus, five cocoa pods were used to conduct the experiment. Two superficial wounds, 15 mm in diameter and 5 mm in thickness, were made in the epicarp using a sterilized scalpel blade [19]. The mycelium of the 14-day-old fungal strains was scraped with a sterile scalpel blade separately and then placed on the wounds. The wounds containing the mycelium were covered with slightly damp sterile distilled water-soaked cotton to promote fungal development. The cotton was secured with adhesive tape to protect the wounds on the pods. Control pods were inoculated with PDA medium without fungus (Fig. 2). Finally, for each fungal genus, the experiment was repeated three times. All these cocoa pods were kept in separate container bins for fungal genera at room temperature in the laboratory (25±1 °C) for one week. The parameter assessed was the appearance of symptoms after 7 days. Koch postulate were verified after symptom development on the inoculated pods.

Fig. 2. Pathogenicity Test of Isolated Fungi on Healthy cocoa Pods. A: injueries made on the cocoa pods; B: protection of wounds inoculated with isolated fungi by cotton; C: cotton protection with adhesive tape

Dna extraction

Fungal strains responsible for pods rots aged 7 days obtained from a monospore culture were used for molecular characterization. DNA from fungal strains was extracted using the method of Doyle and Doyle [20] and Tiendrebeogo et al. [21]. Fungal colonies aged 7 days were scraped separately with a sterile spatula using the method of Than et al. [22]. A quantity of 0.2 g of mycelium was weighed and ground in 1 ml of CTAB (Cethyltrimethyl Ammoniac Bromide) buffer preheated at 65°C for 10 minutes. 1ml of croushed from each fungal isolate obtained was transferred to sterile 2ml Eppendorf tubes, then lightly vortexed. Tubes were then incubated for 30 minutes in a water bath at 65°C, homogenizing by inversion every 10 minutes to facilitate cell wall lysis. On exit, tubes were left at room temperature for 2 minutes, then 800 µl of chloroform-isoamyl alcohol (24:1) was added to each tube. Tubes were vortexed and centrifuged at 13500 rpm for 10 minutes. The supernatant (750 µl) was transferred to 2-ml Eppendorf tubes, where a further 500 µl of chloroform was shaken, vortexed and centrifuged at 13000 rpm for 10 minutes. Six hundred and fifty (650 µl) microliters of the supernatant were removed and placed in 1.5-ml Eppendorf tubes, then precipitated with 650 µl of cold (4°C) isopropanol. The solution was mixed by inversion and incubated in the freezer at -20°C for over 2 hours. After incubation, the solution was centrifuged at 13500 rpm for 10 minutes. The pellet of each solution contained in each tube was recovered afterwards and dried on blotting paper, at laboratory room temperature. Next, 500 µl of 70% ethanol was added to each Eppendorf tube and centrifuged for 5 minutes at 13000 rpm. After centrifugation, the supernatant was discarded, retaining the DNA deposit. The tubes were then drained and left to dry at room temperature. Finally, the DNA pellet was recovered in 50 µl TE and stored in the freezer at -20°C.

Amplification of dna fragments

Universal ITS1 sense (TCCGTAGGTGAACCTGCGC) and ITS4 antisense (TCCTCCGCTTATTGATATGC) primers from White et al. [23] and Tiendrebeogo et al. [21] were used. Both primers are indicated in the 5'->3' direction and are located at the 18S (ITS1) and 28S (ITS4) subunits of ribosomal DNA genes. PCR was performed in a reaction volume of 25 µl, containing 4 µl of DNA, 2 µl of each primer (ITS1 and ITS4), 4.5 µl of ultrapure water and 12.5 µl, GoTaq® G2 Hot Start Green Master Mix. A witness containing all PCR components except DNA was added to the samples. DNA fragments were amplified using a thermal cycler (T 100 Bio Rad). The amplification program compound an initial denaturation cycle at 94°C for 5 min,

followed by a series of 35 cycles. The cycle consisted of denaturation at 94°C for 35 s, hybridization at 60°C for 35 s, elongation at 72°C for 1 min 30 s and final elongation at 72°C for 7 min.

Electrophoresis and pcr product purification

Amplification products were separated by electrophoresis on a 1% agarose gel. The gel was stained with 0.5 ng/ml ethidium bromide and immersed in 0.5X TBE buffer. A 2 µl volume of size marker (Bench Top 100 bp DNA ladder, Promega, USA) was deposited in the wells at both ends to determine the size of the various amplified DNA fragments. A volume of 5 µl of PCR product was then deposited in the agarose gel wells. Electrophoretic migration was performed at 80 V for 60 minutes. Finally, amplicons were visualized in the gel under UV light using a gel reader (EBOX VX5, France). Amplified DNA stained with ethidium bromide appeared as a band under UV light at 360 nm. Positive samples migrated at a molecular weight of 600 base pairs. PCR amplicons obtained with the primers (ITS1 and ITS4) were sequenced using the Sanger sequencing method by Macrogen Europe (Netherlands). The 5.8 S - ITS region of each sequence was amplified.

Statistical analysis of the collected data

The data was recorded and graphs were realized using Excel 2021 software. The data (prevalence and severity of rots) collected in the various locations surveyed were subjected to a one-factor analysis of variance (zones). Data on the rate speed of propagation of pathogenic strains collected in the laboratory were subjected to a one-factor analysis of variance (fungi). All data collected were analyzed using RStudio software version 4.3.0. In the event of a significant difference at the 5% level, Fischer's LSD test was used to identify prevalent zones and fungal virulence.

RESULTS AND DISCUSSION

Symptoms observed on cocoa pods

A diversity of symptoms was observed in the different areas of cocoa-growing visited. In each locality visited, the most common symptom on cocoa pods was organ alteration. It was characterized by the development of black spots and/or brown spots on the surface of the pods (Fig. 3). The black lesions on the pods are soft to the touch, and those with brownish parts were covered with white mycelium. The sections of the pods adorned with brown spots feel firm to the touch. These symptoms are characteristic of cocoa pod rot. Sanitary surveys of cocoa plantations in the eight localities surveyed revealed the presence of a variety of organ alteration symptoms, such as soft-brown rot, soft-black rot, dry-black rot, and dry-brown rot on pods. This diversity of symptoms could be due to the pathogen complexes associated with tissue destruction. Additionally, one or more fungal agents could be the cause of this diversity of cocoa pod rot symptoms.

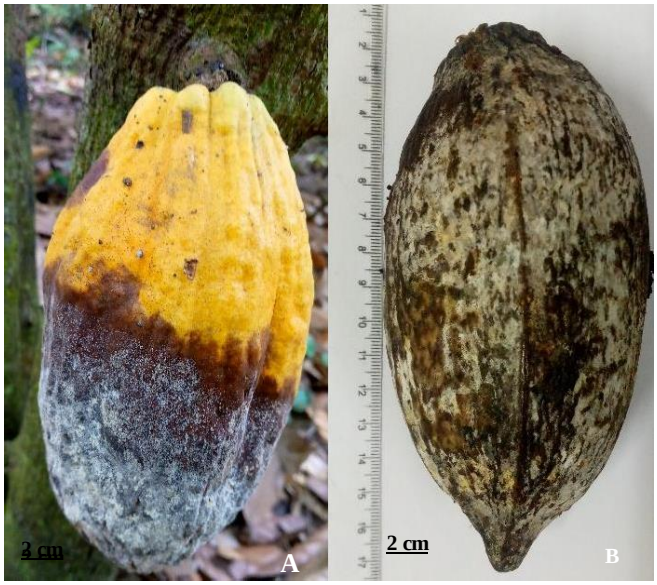

Fig. 3. Different types of cocoa pod rot. A: characteristic symptoms of brown rot; B: characteristic symptoms of black rot

Prevalence of the different types of rot Prevalence of black cocoa pod rot

The prevalence of black pod rot varied from 60% to 91% depending on the surveyed localities. The analysis of the data on the prevalence of rot showed a significant difference between the localities (P = 0.015). Indeed, the locality of Tiassalé presented the highest prevalence with an average of 91%, while the locality of Soubré presented the lowest prevalence (60%) (Fig. 4).

Prevalence of brown cocoa pod rot

The prevalence of brown pod rot varied from 58% to 87% depending on the surveyed localities. The analysis of the data on the prevalence of rot showed a significant difference between the localities (P = 0.003). Indeed, the locality of Tiassalé presented the highest prevalence with an average of 87%, while the locality of Abengourou was low with a prevalence of 58% (Fig. 4).

Severity of the different types of rot Severity index of black pod rot

The severity indices for black pod rot varied significantly

from 21.6% to 68.3% depending on the surveyed localities. The rot was the most severe in the locality of Tiassalé, with a severity index of 68.3%. In contrast to the locality of Soubré, the rot was the lowest, with an average of 21.6%. The statistical analyses carried out showed a significant difference between the severity indices of black pod rot of the surveyed localities ($P < 0.05$) (Fig. 5).

Severity index for brown pod rot

The severity indices of brown pod rot varied significantly from 20.4% to 62.2% depending on the surveyed localities. Brown rot was the most severe in the locality of Tiassalé, with a severity index of 62.2%. Compared to the locality of soubré, the rot was the lows, with an average of 20.4%. The statistical analyses revealed a highly significant difference between the severity indices of brown rot in the surveyed localities ($P < 0.05$) (Fig. 5).

During this study, the prevalence and severity index of the two types of rot were higher in the Tiassalé locality than in the other surveyed localities. The environmental characteristics such as temperature, rainfall and relative humidity of this forest area in Côte d'Ivoire could explain this severity. In addition, this high severity could be explained by the age of the plantations visited. Indeed, the age of the plantations in the locality varied from 7 to 20 years, which could explain its weak defense system. The high humidity and shade conditions prevailing in cocoa plantations would favour the development of the pathogen or disease. Given that all these conditions are present in this locality, the pathogen could multiply and spread, resulting in a high prevalence and variation in the severity of necrosis. These results corroborate those of Pohé et al. [24] and Ndoungue [25]. In the course of their work, these authors showed that in cocoa crops, fungal development is favored by shading, heat and high humidity in these crops. Moreover, conditions are also conducive to the spread of the disease in the humid forest zones of Cameroon.

Fungal strains isolated from infected cocoa pods

Four fungal genera were isolated from the cocoa pods. These were *Lasiodiplodia* sp., *Collectotrichum* sp., *Phytophthora* sp., and *Fusarium* sp. Table and Fig. 6 exhibit the macroscopic and microscopic characteristics used to identify them.

Pathogenic power of the isolated fungal genera

The four fungal genera (*Lasiodiplodia* sp., *Fusarium* sp., *Collectotrichum* sp., and *Phytophthora* sp.) caused different types of rot symptoms on cocoa pods after inoculation. Organ deterioration was observed on these pods after inoculation, with a significant difference in aggressiveness level between the genera tested (Fig. 7).

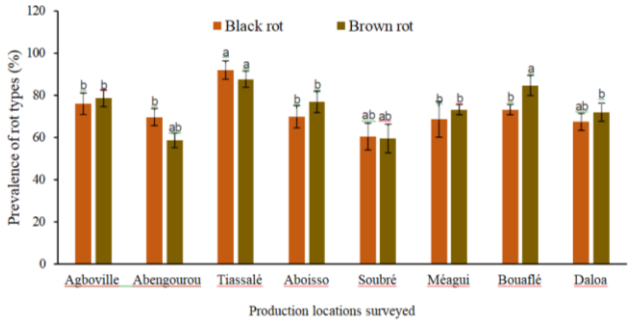

Fig. 4. Prevalence of different types of cocoa pods rots in the production areas surveyed. The histograms with the same letter are statistically identical at the 5% threshold according to Fisher's LSD test

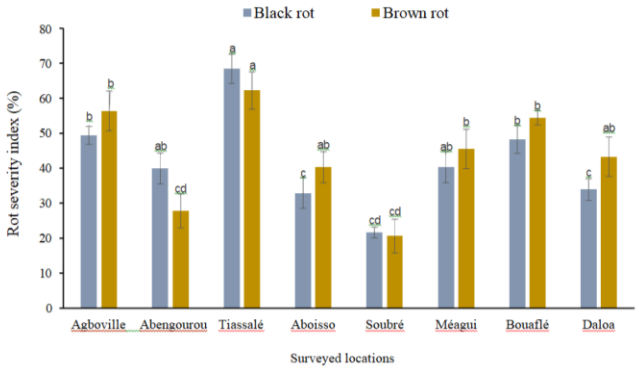

Fig. 5. Average severity index of the different types of cocoa pod rot in the surveyed production localities

Table . Cultural and microscopic character of fungi identified from cocoa pods affected by rots.

Fungi isolated	Macroscopic character	Microscopic character
Lasiodiplodia sp.	**Color**: grey **Appearance**: flaky **Size**: widespread **Relief**: high	**Conidia**: cylindrical bicellular united partitioned at the rounded ends on one hand and elongated on the other hand
Colletotrichum sp.	**Color**: violet on front and back **Appearance**: cottony **Size**: widespread **Relief**: flat	**Conidia**: non-partitioned cylindrical hyaline rounded at both ends
Phytophthora sp.	**Color**: white on both sides **Appearance**: flaky **Size**: widespread **Relief**: flat	Spores are globose and smooth, with phiallids at the ends of the conidiophores.
Fusarium sp.	**Color**: pink on both sides **Appearance**: cottony **Size**: widespread **Relief**: flat	**Macroconidia** and **Microconidia** Fusiform curved multi-partitioned

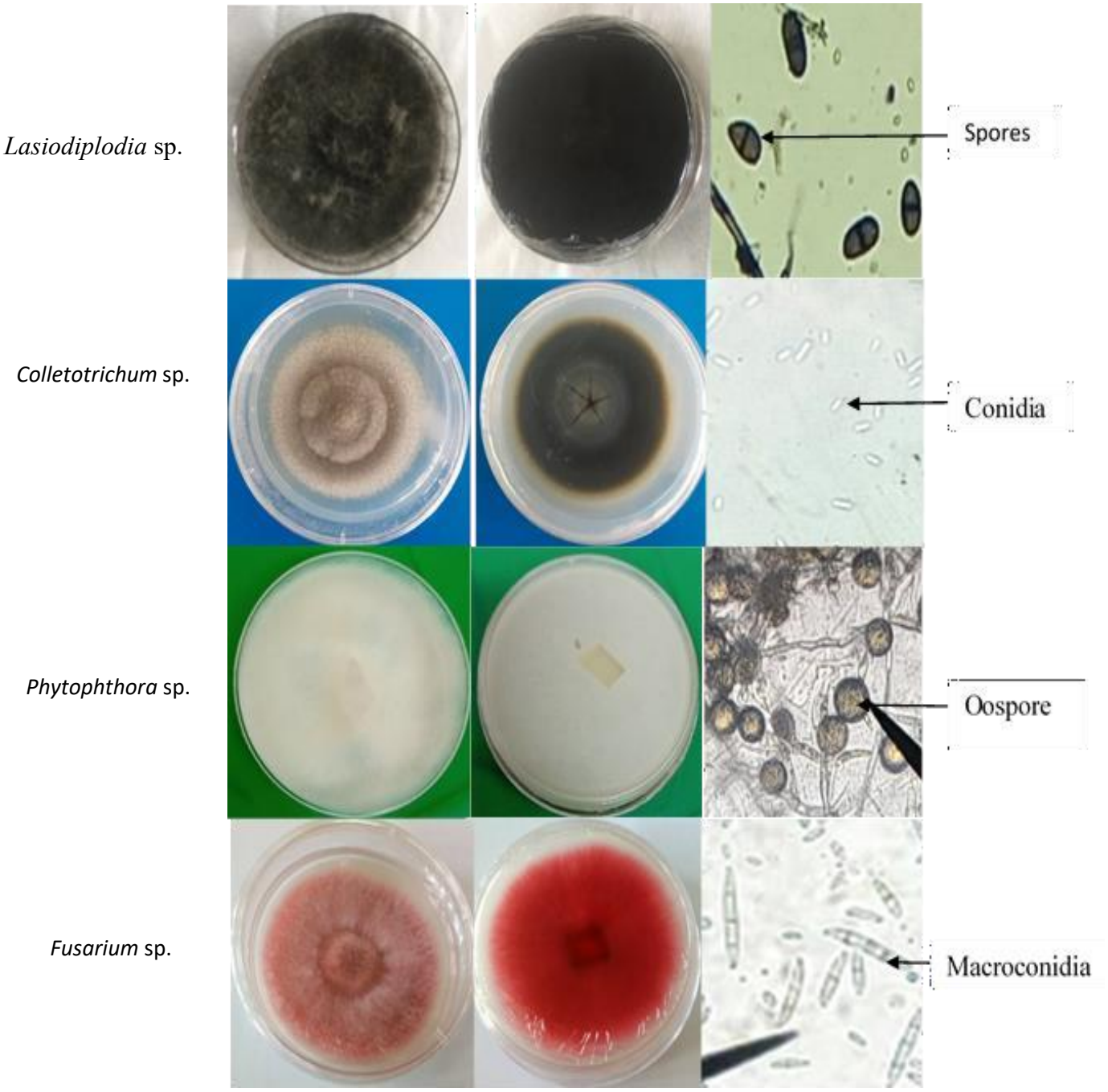

Fig. 6. Macroscopic and microscopic characteristics of various fungi isolated from infected cocoa pods

Fig. 7. Induced symptoms of the four fungal genera isolés. A: *Fusarium* sp., B: *Collectotrichum* sp., C: *Lasiodiplodia* sp., D: *Phytophthora* sp.

Four fungal genera were isolated from cocoa pods reached by rot. This fungal diversity on cocoa pods could be justified by the fact that fungi are dependent on certain substrates or supports for their development, for adhesion or nutrition. Their presence can lead to morphological or physiological modifications. Moreover, this fungal diversity may be linked to the age of the crop, whose defense systems are ineffective against the virulence of these fungal genera. Indeed, some of these fungal agents have been for rots are frequently isolated from cocoa pods by several researchers. These results are in line with those of Fofana et al. [26] and Coulibaly et al. [13]. These authors also isolated some of these fungal genera in their work on cocoa pod rot. However, some genera of fungi could be either different or morphologically identical, hence the need for molecular analysis.

Koch's postulate

Verification of Koch's postulate was carried out after the onset of symptoms. For this purpose, the associated strains used to inoculate asymptomatic cocoa pods caused brown and black rot symptoms. Pathogens associated with pod-induced symptoms, after isolation, showed morphological characteristics identical to those inoculated.

Speed of propagation of rot a function of fungal strain

A variation in rot propagation speeds ranging from 4.89 cm/j to 7.60 cm/j was observed, depending on the strain. These lesions appeared three days after pod inoculation and developed on the pods over time. *Phytophthora* sp. and *Lasiodiplodia* sp. strains recorded the highest rate of lesion propagation compared with *Fusarium* sp. and *Collectotrichum* sp. strains, with rot propagation rates of 7.60 and 6.9 cm/j respectively. *Fusarium* sp. and *Collectotrichum* sp. strains showed low rot speeds of 4.89 and 5.1 cm/j respectively. Statistical analysis of the average rot propagation speeds showed a highly significant difference between the propagation speeds of the pathogenic strains, with $P < 0.05$ (Fig. 8).

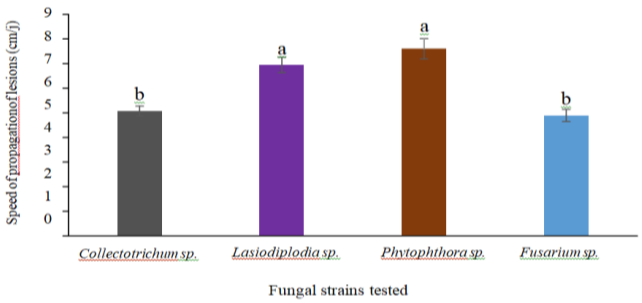

Fig. 8. Lesion propagation speed of fungal strains

Molecular characteristics of fungal dna strains

Cocoa pod rot fungal pathogens whose DNA was extracted and successfully amplified by the ITS1 / ITS4 universal primer pair. The DNAs migrated at 800 base pairs, characteristic of the fungi (Fig. 9). The sequencing results revealed a specific diversity of fungi responsible for cocoa pod rot. Comparison of the sequences of pathogenic cocoa fungi in Côte d'Ivoire with those in the NCBI (National Center for Biotechnology Information) database, through BLAST, revealed that the sequences of the strains in the present study have homology rates of 100% (Table II). Species such as *Fusarium falciforme*, *Colletotrichum aenigma*, *Lasiodiplodia theobromae* and *Phytophthora megakarya* were identified.

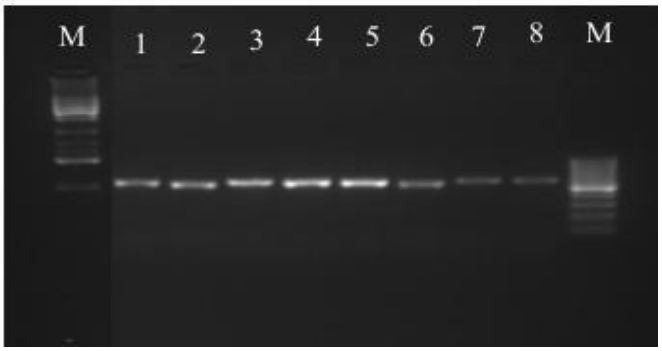

Fig. 9. Electrophoretic profile of fungal DNA amplification products using primers ITS1 and ITS4. M: molecular weight marker; 1 to 8: pathogenic fungal DNA samples

Table II. Homology rate of DNA sequences of cocoa pods rots fungi isolated from Ivorian cocoa orchards and those available on Genbank.

Fungal strains	Sequenced species name	Homology rate (%)	Accession number
Lasiodiplodia sp.	*Lasiodiplodia theobromae*	100	PQ157650
Phytophthora sp.	*Phytophthora megakarya*	100	PQ157645
Colletotrichum sp.	*Colletotrichum aenigma*	100	PQ157646
Fusarium sp.	*Fusarium falciforme*	100	MN907536.1

Molecular analysis of the DNA sequences revealed the presence of four cocoa pod pathogenic species with 100% identity with GenBank reference strains: *Fusarium falciforme*, *Colletotrichum aenigma*, *Lasiodiplodia theobromae* and *Phytophthora megakarya*. Indeed, according to Crouch et al. [27], ITS could be a good marker of inter-generic differentiation. These results are in line with those of Ghorri [28] and El kharazi [29]. In their work, these authors characterized a diversity of Trichoderma sp. species with 95-100% similarity to the Trichoderma genus present in soil. These results could be explained by the fact that fungi are highly adaptable to different climatic conditions and are also widespread in nature. However, knowledge of the different pathogenic species could better guide control methods for these agents.

CONCLUSIONS

The aim of this study was to assess the epidemiological parameters of cocoa pod rot and characterize the fungal agents responsible. At the end of the study, rot was present in all the localities surveyed, and was most severe in Tiassalé. Four fungal genera were isolated from rotten pods. These were *Collectotrichum aenigma, Lasiodiplodia theobromae*, *Fusarium falciforme* and *Phytophthora megakarya*. Pathogenicity tests carried out *in vitro* demonstrated the pathogenicity of these four fungal species on pods. Knowledge of these species could help guide the timing of biological control methods.

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Authorship Contributions

A. B. C. N., K. S., M. E., H. A. D., methodology design. A. B. C. N. data collection with support from K. S., M. E., Y. Y. F. R. K. data analysis and drafting of the manuscript A. B. C. N., A. J. N., Y. Y. F. R. K., H. A. D. All authors have read and approved the final manuscript.

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Ethical Approval

Not applicable to this paper

Consent for publication

Not applicable.

Competing interests

The authors declared that there is no conflict of interest.

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