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Phenotypic and Molecular Characterizations of Strains of *Lasiodiplodia theobromae* (Pat.) Griffon & Maulk, Pathogen Associated with Black Rot Cocoa Pods in the Tshopo Province, Kisangani Region (DR Congo)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The cocoa tree (*Theobroma cacao* L) is a persistent perennial crop in tropical regions whose production period is sufficiently spread out over the whole year. As a result, it offers pathogens conditions for survival without real disruption of their life cycle. Symptoms of cocoa pod black rot

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disease have been observed in both the Bengamisa cocoa growing area and the Yangambi area in the Democratic Republic of Congo (DRC).
This study aimed to characterize the strains of *Lasiodiplodia theobromae* on cocoa trees in the Kisangani region.
Macroscopic and microscopic observations were made on the pods while the identification of the species was confirmed by a molecular approach based on the sequencing of part of the ribosomal DNA including the ITS regions (internal transcribed spacers) and the 5.8S gene.
The results of this study showed that the phenotypic characteristics of the strains isolated in the two cocoa growing areas were typical of the *L. Theobromae* species. These are in particular the spots of soft rot, initially brown, gradually evolving into soot-black which subsequently produced a sort of whitish powder on the surface of the diseased pod. However, the fruiting of the fungus in the PDA medium gave rise to the latter's mycelia, initially whitish, which darkened as they matured. In addition, PCR amplification followed by sequencing of the fungal strain was beneficial by removing any doubt about the nature of the fungal species isolated in the two cocoa-growing areas.

Keywords: Characterization; strains; Lasiodiplodia theobromae; cocoa tree; Kisangani.

1. INTRODUCTION

It remains indisputable that in recent years, new parasitic diseases are reappearing every day and often gaining spread in all countries of the world [1].

In DR Congo, since 1914 Vermoessen, by describing pod diseases in Mayumbe in Kongo Central (formerly Bas Congo), indicates the presence of brown pod rot in this region [2]. Around 1944, the agent responsible for rotting cocoa pods in the Democratic Republic of Congo (DRC) was identified as *Phytophthora faberi* [2] (Steyaert, 1944). It was around 1962 that in the Democratic Republic of Congo there was talk of the black rot disease caused by *L. theobromae* (INEAC, 1962). No molecular level study had yet been carried out confirming the presence of this pathogen agent.

This disease is recognized as quite widespread, but the damage it causes is generally much lower than that caused by *Phytophtora. palmivora*. Indeed, *L. theobromae* is primarily a wound parasite. However, once it has settled into the pod, it can destroy it completely causing enormous losses in production (INEAC, 1962).

Since the late 1980s, cocoa orchards have been increasingly affected by wasting disease caused by *L. theobromae*. This has been recorded in all orchards of producers in Cameroon affecting 100% of cocoa trees in some farms (Mbenoum et al., 2000). Regardless of age, affected cocoa trees show typical symptoms on trees, leaves on outer twigs turn yellow first, and damage may spread to trees, twigs, and pods.

The black rot fungus was first reported on cocoa in Cameroon in 1895 and has since caused symptoms of black rot. Severe dieback, similar to this has also been described on other crops such as cashew [3]; Prunus [4,5,6]; mango and kumquat in other countries [7,8], *Annona muricata* [9], Papaya [10].

In the DRC, since the introduction of cocoa (*Theobroma cacao*) the main disease has been pod rot caused by fungi of the genus *Phytophthora*.

The designation of *L. theobromae* as the cause of black rot disease of cocoa pods was based, long ago, on the morphological characteristics of pod spots. However, there has never been a study undertaken on the disease in the country because it did not appear to be of concern at the time. The reports of numerous researchers on this disease in the cocoa growing area of Bengamisa aroused the curiosity of researchers from INERA Yangambi who also found signs similar to those encountered on pods in the cocoa growing area of Bengamisa, on a few pods in their experimental fields, but without being alarming.

The presence of black rot disease in the two main cocoa-growing areas (Bengamisa and Yangambi) of Tshopo province is already a problem to which research is called to provide solutions and these must necessarily involve real knowledge of the species of fungus involved in this condition [11].

The literature reports that it is the genus *Lasiodiplodia* that causes black rot disease in cocoa pods. Confirmation of the fungus species involved in the two cocoa growing areas (Bengamisa and Yangambi) is a prerequisite for any effective control.

Given the similarities of the symptoms of this disease on the pod with those caused by other fungi, it was prudent that we investigated to reassure the scientific community about the pathogen responsible for the disease in the two cocoa-growing areas.

It should also be remembered that the cocoa seeds in the two zones came from different areas. The seeds of CABEN which were also installed in the plantations of the peasants in the family block came from the Ivory Coast while those of INERA Yangambi came from hybrids resulting from crosses between the material of local selection and Upper Amazon hybrids from Central America [12] the report was carried out by the group of cocoa trees from the two stations, INERA Bongabo and INERA Yangambi. In this study, we aimed to characterize the strains of *L. theobromae* in the cocoa-growing areas of the Kisangani region to know the phytopathogenic agent responsible for black rot of cocoa pods in the Kisangani region.

2. MATERIALS AND METHODS

2.1 Study Area

This work was carried out in the experimental field of INERA Yangambi where the samples were taken.

The Bengamisa Cacao (CABEN) is a Congolese state company located at 36 km on the Kisangani-Buta road axis. Its activities extend from 8 km to 128 km.

The Yangambi Research Center is a research institution of the National Institute for Agronomic Studies and Research (INERA) located at 100 km on the Kisangani-Isangi road in the DRC.

2.2 Plant Material

The plant material used in this study consisted of indisputably diseased cocoa pods showing symptoms of black rot disease of cocoa pods harvested and deposited in the Microbiology and Phytopathology laboratory of Faculty of Sciences of the University of Kisangani.

2.3 Fungal Material

The fungal material used in this work consists of strains of *L. theobromae* isolated from samples of the cocoa pods from Bengamisa and Yangambi.

2.3.1 Phenotypic characterization

Macroscopic observations were conducted on the pods. The identification of the species was based on the morphological characterization described by Abdollahzadeh *et al.* [13] and Alves et al. [14]. According to these, *L. Theobromae* produced pycnidia or small subepidermal cavities formed by the interlaced mycelium, a little dark. These very numerous pycnidia, but generally isolated from each other, appeared to the naked eye as small black protuberances which give the infected part a rugged appearance. They soon produce masses of spores which are expelled through a thin orifice piercing the epidermis. These spores agglutinate into a rather long, drooping filament on the side of the pore making the most capricious contortions. Soon after the exit from the pycnidia, the spores ripen, that is to say, the partition thus forming characteristic bicellular spores and become blackish sooty, they are then black with soot. At one point, moreover, these clusters present colors ranging from white to black passing through the gray.

Fig. 1. (A) Symptom of black rot disease on a 4-month-old pod; (B) Pods used as samples to collect strains

These pycnidia, distributed in large numbers over the entire diseased surface of a pod, can cover it after a short time with a veritable layer of grayish or blackish dust depending on the degree of maturity of the spores, especially when they are in a humid place.

For microscopic observations, the direct observation method was used. This consisted of scraping the area of development of the sporocysts, solution of them in sterile distilled water, and direct observation under a microscope.

The microscopic observations were done using a Motic AE21 convex light microscope and compared to the description of *Punthalingan* [15]. This author describes the morphology of the fungus as comprising a colony of dark green or black conidia spreading out from dark brown, striated, ellipsoidal, antiseptic, and produced in ascostromatic pycnidia on PDA medium (Potato Dextrose Agar). Despite all these observations, only molecular confirmation can definitively confirm that a strain belongs to a species [16]. The most recent descriptions of these species, other than morphology, are based on the sequencing of the intergenic spacer regions of rDNA (ITS) and elongation factor 1-alpha (EF-1) [4,10].

2.3.2 Molecular characterization

Strains were isolated from mesocarp fragments taken from diseased cocoa, pruned beforehand using a Scarpelli, and then disinfected with bleach for two minutes then rinsed in sterile distilled water. Using anatomical forceps, the pieces were inoculated on PDA culture media (39g/L) already solidified in Petri dishes.

These dishes were taped with parafilm and then incubated at 25 ° C in the dark. The samples were sent simultaneously to two different labs: The University of Aberdeen laboratory in Scotland and the University of Ghent in Belgium.

Molecular characterization focused on PCR amplification and sequencing of the internal transcribed spacers (ITS), ITS4 and ITS5 regions, the 5.8S gene of ribosomal DNA and β-tubulin (BT2). The strains analyzed were chosen according to the morphological characteristics.

The following steps were carried out for the molecular characterization: obtaining the monospore strains, seeding on PBS liquid medium, DNA extraction, quantification of the DNA concentration, PCR amplification, analysis of the PCR product on agarose gel, UV visualization, and sequencing.

DNA was extracted using the CTAB method of *Stewart* and *Via* (1993), modified for use with fungi, and then diluted 10x before PCR amplification.

Direct sequencing was carried out on 10 isolates, previously purified, by the Sanger method in the laboratory, with the PCR product diluted 10 times in distilled water. The sequencing of each fragment was carried out separately with the forward and reverse primers. The mixture to be sequenced was placed in an Eppendorf tube. The complete segment of DNA was obtained by assembly of the contiguous sequences (Vector NTI Advence11, Invitrogen). Alignment of the obtained sequences was carried out via Clustal W by including the sequences of the ITS regions of the ribosomal DNA of *L. theobromae* accessions available in the Gen Bank. NCBI Blast was performed on the nucleotide sequences.

3. RESULTS AND DISCUSSION

3.1 Morphological Characteristics

Observations conducted on diseased pods and those made on fruiting bodies under a microscope identified *L. theobromae* as the causative agent of black pod rot disease in cocoa areas of Bengamisa and Yangambi.

It may be seen from Fig. 2 that, on the diseased pod, the spots of soft rot, initially brown, gradually evolving to black with soot were observed. These spots then produce a sort of whitish powder on the surface of the diseased pod.

Similar spots had already been described by Abdollahzadeh *et al.* [13] and *Alves et al.* [14].

Fruiting of the fungus in the PDA medium gave rise to the latter's mycelia, initially whitish, which darkened as they matured. After twelve days, the entire Petri dish was full. Colonies in the culture medium are moderately dense, with aerial mycelium, starting white, turning gray-olive color at 7 days, and overtime acquiring a black color. The growth temperature of *L. theobromae* was maintained at 25 ° C in the Microbiology and Phytopathology laboratory of the Faculty of

Sciences of the University of Kisangani. This situation had also been described by *Slippers et al.* [17] and *Alves et al.* [14] who, for them, this growth temperature was 15 ºC minimum, 28 ºC optimally, and 40 ºC maximum. Sporulation was made possible by culturing the strain on V8 1/5 + Beta-sitosterol medium, for 3 days in total darkness at 24 ° C then 7 days in indirect light at 24 ° C.

Saha et al. [18] found that the sporulation of the fungus is favored by photoperiods of more than 16 hours of exposure to light, which allows the formation of pycnidia ; on the contrary, an exposure of fewer than 4 hours of daily light, over 23 days, inhibits sporulation. The presence of nitrogen in the culture medium promotes sporulation.

Also they evaluated the nitrogen concentration in various culture media, finding that PDA medium with added tea root extract induces rapid growth and higher mycelium, in addition to a higher spore concentration than the rest of the environments evaluated.

3.2 Molecular Characteristics

After extraction of DNA from the strains, amplification of the region of ribosomal DNA between the primers ITS4 and ITS5 was performed by PCR. The DNA concentrations of different samples of fungal strains studied are presented in Table 1 while the results of the amplification of the strains are illustrated by Fig. 4. It appears that for the ITS region, an amplicon of approximately 580 bp has been obtained.

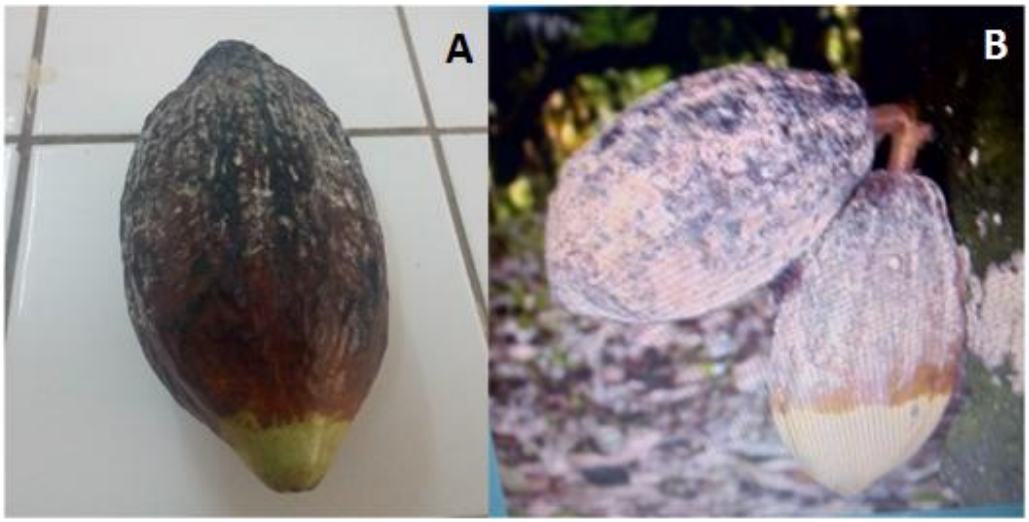

Fig. 2. (A) Cocoa pod show the characteristic symptoms of cocoa pod black rot; (B) Whitish mycelial felting on the surface of the affected pod

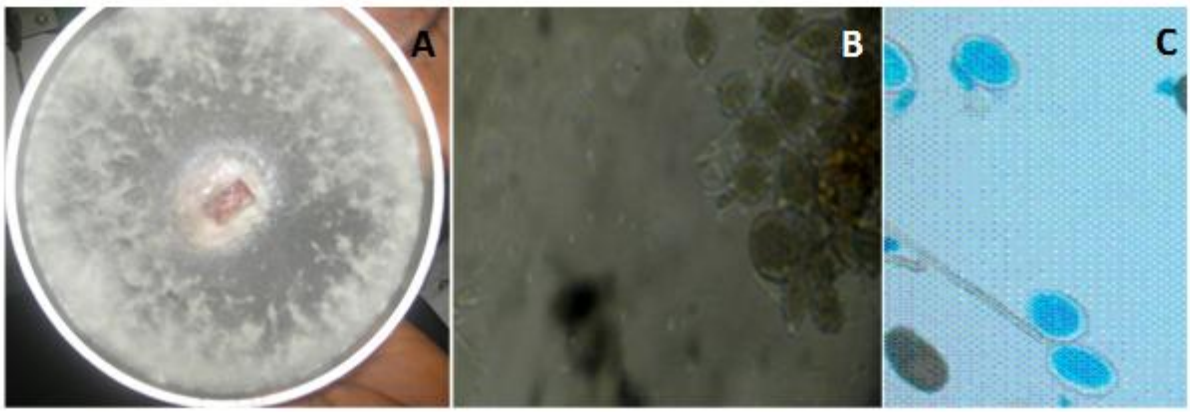

Fig. 3*. L. theobromae* strain growing on PDA medium; (A) Fruiting of the fungus ; (B) Mycelium and pycnidia ; (C) Spores and sporangium

Table 1. DNA concentration (ng / µL) extracted from fungal strains

N°Samples	1	2	3	4	5	6	7	8	9	10	Average
DNA concentration (ng/µL)	1,6	3,7	4,1	14	8,5	12	30	46	9	12	14,11

This table indicates that sample # 8 has a higher DNA concentration, i.e. 46 ng / µL, while sample # 1 has a very low concentration, i.e. 1.62 ng / µL.

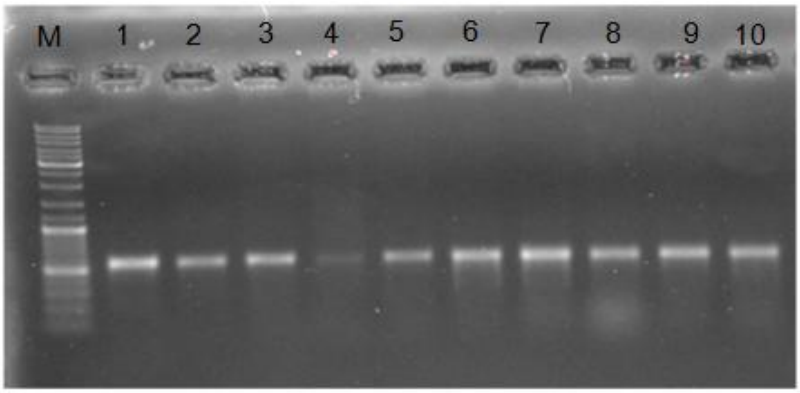

Fig. 4. Visualization of amplicons obtained after amplification of DNA extracted from fungal strains of *L. theobromae*

After analysis the number of base pairs of all fungal strains analyzed is 580bp, referring to the standard DNA primer.

The DNA concentration after amplification and purification of DNA extracted from fungal strains of *L. theobromae is* shown in Table 2.

After PCR, purification, and sequencing of 10 samples and using the National Center for Biotechnology Information, NCBI blast database, the following sequence of sample # 5 was found for illustrative purposes:

CCGCCGAGGTCTTTGAGGCGCGTCCGCAGT
GAGGACGGTGCCCAATTCCAAGCAGAGCTT
GAGGGTTGTAATGACGCTCGAACAGGCATG
CCCCCCGGAATACCAAGGGGCGCAATGTGC
GTTCAAAGATTCGATGATTCACTGAATTCTGC
AATTCACATTACTTATCGCATTTCGCTGCGTT
CTTCATCGATGCCAGAACCAAGAGATCCGTT
GTTGAAAGTTTTAGTTTATTAACTTGTTTATCA
GACGTCTGCGTTTACTGACTGGAG

The results of identification of the fungal strain used are presented in Table 3.

This table indicated that the fungal strain studied is *L. theobromae*. After sequencing the strain studied, it was discovered that *L. theobromae* is the fungal agent responsible for black pod rot in cocoa pods in the Kisangani region (Yangambi and Bengamisa).

Similar results have been found in Sao Tome and Principe [19], Cameroon [20], in Ghana and in Ivory coast [7,8] on pods and other organs of the cocoa tree.

This discovery is the first in the Kisangani region.

Like other researchers, this fungus has already been isolated in the tropics and subtropics. First reported on cocoa in Cameroon in 1985 [7,8], *L. theobromae* is a soil fungus that causes rots and diebacks on various plants, whether cultivated or not. More than 280 species have been reported as susceptible. It has been detected in several countries in Europe (France, Spain), Asia (India, Pakistan, Philippines), Africa (Cameroon, Cote d'Ivoire, Ghana), Americas (Cuba, United States, Canada, Ecuador, Brazil, Chile), and Oceania (Australia, Western Samoa).

Table 2. DNA concentration of fungal strains for sequencing

N° Samples	1	2	3	4	5	6	7	8	9	10	Average
DNA concentration (ng/µL)	10	6,6	6,6	2,17	7,5	16	14	19	11	11	10,39

It may be seen from this table that sample n ° 8 has a higher concentration of DNA, ie 19ng / µL, while sample n ° 4 has a lower concentration, ie 2.17ng / µL

Table 3. Identification of the fungal strain after sequencing

Description	Max score	Total score	Frequency	Percentage of identification
Lasiodiplodia mahajangana isolate L-1539/2013 interal trenscribed spacer A	503	503	100	100
Lasiodiplodia theobromae isolate L-1003/2013 interal trenscribed spacer A	503	503	100	100
Diplodia cajani isolate L-998/2013 internal transcribed	503	503	100	100
Lasiodiplodia pseudotheobromae isolate L-850/2013 internal trascribed spcer 1	503	503	100	100
Lasiodiplodia pseudotheobromae isolate L-239/2013 internal trascribed spcer 1	503	503	100	100
Lasiodiplodi parva isolate L-100/2013 internal trnscribed spacer 1	503	503	100	100
Lasiodiplodia pseudotheobromae isolate L-3361/2012 internal trascribed spcer 1	503	503	100	100
Lasiodiplodia mahajangana isolate L-3138/2012 interal trenscribed spacer A	503	503	100	100
Lasiodiplodi parva isolate L-3101/2012 internal trnscribed spacer 1	503	503	100	100
Lasiodiplodi stercliae isolate L-2969/2012 internal trnscribed spacer 1	503	503	100	100
Lasiodiplodia mahajangana isolate L-2622/2012 interal trenscribed spacer A	503	503	100	100
Lasiodiplodia pseudotheobromae isolate PAK-7 internal trascribed spcer 1	503	503	100	100
Lasiodiplodia theobromae isolate TN-R-3 interal trenscribed spacer A	503	503	100	100
Lasiodiplodia pseudotheobromae isolate TTHF5-2 internal trascribed spcer 1	503	503	100	100
Lasiodiplodia pseudotheobromae isolate TTHF4-4 internal trascribed spcer 2	503	503	100	100
Lasiodiplodia theobromae PSU-NK01 DNA, intenal transcribed space 1	503	503	100	100
Lasiodiplodia theobromae PSU-SK01 DNA, intenal transcribed space 1	503	503	100	100

L. theobromae is a pathogen that can cause tree death overtime [21].

Thus, the decline of the cocoa tree due to *L. theobromae* constitutes a permanent danger not only in the aforementioned countries but also in the Democratic Republic of Congo and more particularly in the Kisangani region, according to our results (Table 3).

4. CONCLUSION

The main objective of this study was to characterize the strains of *L. theobromae* to know not only the phytopathogenic agent responsible for black rot of cocoa pods in the cocoa zone of Bengamisa and that of Yangambi in the province of Tshopo but also its phenotypic and molecular characteristics.

Phenotypic observations were made on diseased pods and confirmed by a molecular approach based on the sequencing of part of the ribosomal DNA including the ITS regions and the 5.8S gene.

It emerged from this study that the phenotypic characteristics of the strains isolated in the two cocoa growing areas are typical of the *L. Theobromae* species. Soft rot spots, initially brown, gradually evolving to soot-black were observed.

The latter subsequently produced a sort of whitish powder on the surface of the diseased pod. Fruiting of the fungus in the PDA medium revealed whitish mycelia which darkened as they matured. Regarding molecular analyzes, PCR amplification followed by sequencing of the fungal strain showed that the fungal species responsible for black rot of cocoa pods in the cocoa-growing areas of Bengamisa and Yangambi is beautiful and well *L. theobromae*.

DISCLAIMER

The products used for this research are predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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