Annual Research & Review in Biology

Volume 39, Issue 6, Page 41-56, 2024; Article no.ARRB.117491
ISSN: 2347-565X, NLM ID: 101632869
(Past name: Annual Review & Research in Biology, Past ISSN: 2231-4776)

Isolation, Identification and Characterization of *Phyllosticta citricarpa* Causing Citrus Black Spot Disease in Benin

Goudjo Habib Toessi [a,b,c*], Rachidatou Sikirou [c], Elisée Georges Dadé Ler-N'ogn Amari [a,b], Oumarou Zoéyandé Dianda [d], Issa Wonni [d] and Mustapha El Bouhssini [e]

[a] African Excellence Center on Climate Change, Biodiversity and Sustainable Agriculture (CEA-CCBAD), Félix Houphouët-Boigny University, 22 BP 582 Abidjan, Côte d'Ivoire.
[b] Laboratory of Biotechnology, Agriculture and Valorization of Biological Resources, UFR Biosciences, Félix Houphouët-Boigny University, 22 BP 582 Abidjan, Côte d'Ivoire.
[c] Laboratory of Crop Protection (LDC), National Institute of Agricultural Research of Benin (INRAB), 01 BP 884 Cotonou, Benin.
[d] Phytopathology Laboratory of the National Fruit and Vegetable Specialisation Centre, Burkina-Faso.
[e] College of Agriculture and Environemental Sciences, Mohammed VI Polytechnic University (UM6P) Benguerir, Morocco.

Authors' contributions

This work was carried out in collaboration among all authors. Authors RS and EGDLA coordinated the research and revised the paper. Author GHT designed the research and wrote the paper. Authors IW, ME and OZD revised the paper. Author GHT collected and analyzed the data. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.9734/arrb/2024/v39i62087

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/117491

Original Research Article

Received: 15/03/2024
Accepted: 22/05/2024
Published: 28/05/2024

**Corresponding author: E-mail: habib.toessi@yahoo.com;*

Cite as: *Toessi, G. H., Sikirou, R., Amari, E. G. D. L.-N., Dianda, O. Z., Wonni, I., & Bouhssini, M. E. (2024). Isolation, Identification and Characterization of Phyllosticta citricarpa Causing Citrus Black Spot Disease in Benin. Annual Research & Review in Biology, 39(6), 41–56. https://doi.org/10.9734/arrb/2024/v39i62087*

ABSTRACT

Citrus black spot is a major constraint to citrus production in Benin. Identification of the pathogen causing citrus black spot disease was carried out in the laboratory on symptomatic fruits. Fruit samples were collected from orchards in 4 citrus-growing agro-ecological zones. A total of 66 representative isolates of *Phyllosticta* sp. were obtained from isolation. Molecular analysis using PCR of the nucleotide sequences of the ITS regions with universal primers ITS1/ITS4 and specific primers GcF1/GcR and the phylogenetic tree showed that the sequences of all isolates obtained in the different agroecological zones were identical to those of *Phyllosticta citricarpa*. The pathogenicity test satisfied Koch's postulates by re-isolation of *Phyllosticta citricarpa* from inoculated fruits. A thorough study of genetic diversity and a full understanding of the behavior of *P. citricarpa* will pave the way for more targeted approaches to the prevention, control and sustainable management of citrus black spot disease in Benin.

Keywords: P. citricarpa; black spot disease; citrus; PCR; phylogenetic analysis; Benin.

1. INTRODUCTION

Citrus black spot disease first reported in Australia in 1895 and caused by *Phyllosticta citricarpa* (Syn. *Guignardia citricarpa*) is one of the major citrus fungal diseases in tropical and subtropical regions of Asia, Africa and America [1]. In most areas of its current distribution, black spot disease is reported to be a destructive disease of citrus causing huge losses in quality and yield and fruit imperfections rendering them unmarketable [2]. Yield losses caused by black spot disease in Ghana were around 80%, followed by severe premature fruit drop [2]. In South Africa, the majority of fruits from unprotected trees infected by black spot disease were declared unfit for export, and losses of more than 80% were frequently reported [3]. All sweet orange varieties (*Citrus sinensis*) and species such as *C. limon*, *C. paradisi*, *C. reticulata* and *C. deliciosa* are susceptible to this disease [4]. According to EFSA et al [1], *Phyllosticta citricarpa*, the causal agent of black spot disease, is a quarantine pathogen that restricts the export of fresh fruit to the European Union. The period of fruit susceptibility to black spot infection extends from fruit set until 4 to 7 months later [5]. Symptoms on fruit generally appear 40 to 360 days after infection, depending on the type of lesion and the stage of fruit development at the time of infection [6]. However, the infection may remain dormant from the time the petals fall until the fruit ripens [7]. Fruit ripening and climatic conditions that favor the pathogen's development bring this dormant period to an end, and symptoms are observed in the form of lesions or hard, virulent, cracked, freckled spots and false melanosis [6]. Concerning leaves and twigs, symptoms are rarely observed but sometimes present in highly susceptible citrus such as Citrus limon L. or on trees in physiological imbalance [2]. The epidemiology of citrus black spot depends on the abondance of *P. citricarpa* inoculum. In areas characterized by a single rainy season, ascospores produced on decaying dead leaves are the main source of disease inoculum. In contrary, in areas with two rainy seasons, pycnidia containing *P. citricarpa* conidia represent important sources of inoculum from fruit left on trees after the previous season [8]. Ascospores are blown by the wind over long distances and colonize susceptible leaves and fruit.

Furthermore, the morphological characteristics used to identify the pathogen are confusing within the species of the *Phyllosticta* genus associated with citrus [9,10,11]. However, five *Phyllosticta* species have been identified as causal agents of citrus fungal diseases such as *Phyllosticta citricarpa*, associated with black spot disease; *Phyllosticta paracitricarpa*, which causes damage to detached sweet orange fruit [12]; *Phyllosticta citriasiana* [13] and *Phyllosticta citrimaxima* [14], associated with brown spot of pomelo; and *Phyllosticta citrichinaensis* which induce symptoms on leaves and fruits of pomelo, sweet orange and mandarin [11]. In addition, *Guignardia mangiferae*, an endophytic species has been reported. It is morphologically very similar to the causal agent of black spot disease and manifests itself in the same way [9]. Therefore, for an accurate and efficient diagnosis of black spot disease suspected to be caused by *Phyllosticta citricarpa*, it is essential to use morphological and molecular data to identify the pathogen [15]. Molecular characteristics help to clarify its species status and avoid confusion with other pathogenic species. According to Cai et al

[16], quarantine decision-making, plant breeding and pathogen management and control rely on the correct identification of fungal pathogens. Despite its high economic impact, no management strategy has been established to control it in Benin. This is a major concern for farmers. The molecular characteristics of *Phyllosticta* isolates obtained from citrus fruits need to be studied in order to develop effective and sustainable management strategies for this disease, and alleviate the burden on farmers in Benin.

This study aims to accurately identify *Phyllosticta* isolates using molecular methods.

2. METHODOLOGY

2.1 Isolation of the Pathogen from Infected Fruits

Citrus fruits showing symptoms of black spot disease were collected from four citrus-growing agro-ecological zones in Benin. The symptomatic fruits were collected in zone V: cotton region of middle Benin (7°21'N, 1°56'E), zone VI: zone dominated by ferralitic soils (7°9'N, 2°15'E), zone VII: depressions zone (6°49'N, 2°60'E), and zone VIII: fisheries zone (6°36'N, 1°57'E). The sampled fruits were sent to the phytopathology laboratory of the Centre National de Spécialisation en Fruits et Légumes (CNS-FL)/INERA in Burkina-Faso (11°60' N, 11°60' W; altitude: 405 m) to isolate the pathogens associated with symptoms. PDA (Potato Dextrose Agar) culture medium was used for pathogen isolation and purification. Fruits tissue fragments taken from the spot growth front were disinfected with 70% alcohol for 1 min, 1% sodium hypochlorite for 30 s, rinsed twice with sterile distilled water and dried with absorbent paper. After drying, the fragments were placed in 9 cm diameter Petri dishes containing Whatman paper moistened with sterile distilled water and incubated for 7 days at 25 ± 2 °C and 12 h photoperiod. Single spores of fungal species obtained by binocular examination after incubation were plated onto PDA culture medium using a drawn glass capillary (Pasteur pipette). The plates were then incubated for 7 days at 25 ± 2 °C, with a 12 h photoperiod to promote mycelial development. The successive subculturing technique was used to obtain the apparently pure isolates. The isolates thus obtained were kept cold (-80°C) in 2 ml Eppendorf tubes containing glycerol solution (50%) and glucose (1%) with sterile distilled water (a mixture of 1 ml glycerol and 0.5 ml glucose) for subsequent studies.

2.2 Morphological and Cultural Study of *Phyllosticta* sp.

Macroscopic observation of fungal isolates was carried out with the naked eye or binocular on 7-day cultures after purification. Diagnosis was based on cultural characteristics of the isolates, such as radial growth, color and shape of colonies, appearance of aerial mycelium, pigmentation, presence or absence of microsclerotia, surface and reverse side of cultures [17]. Microscopic observation took into account the color, shape and size of spores, the presence or absence of chlamydiospores, septation, mycelium ramification or not, the presence of appendices and the number of septa.

2.3 Mycelial Growth

To determine the pathogen's mycelial growth, 5 mm-diameter mycelial disks were taken from active sporulation zones near the growth front of seven-day cultures of each isolate. These discs were then transferred to PDA culture medium and incubated at 25 ± 2°C. Colony diameter was measured daily using a double decimeter for 14 days. Two perpendicular lines were traced on the reverse side of the Petri dishes, through the center of the mycelial explant. Mycelial growth was obtained from the average of the two diameters over 14 days of daily growth (millimeters per day). The length and width of spores from each isolate were also measured using digital microscope software.

$$D = \frac{d1 + d2}{2}$$

$$\begin{cases} D: average\ diameter\ of\ isolate\ in\ a\ petri\ dish \\ d1\ et\ d2: measures\ of\ the\ two\ perpendicular\ lines \end{cases}$$

2.4 Molecular Characterization of *Phyllosticta* sp.

2.4.1 DNA extraction

DNA from isolates was extracted using the protocol of Al-*Sadi* et al. [18] and Thompson, [19] followed by slight modification. Extraction was performed on a total of 66 isolates from two orange varieties. The extraction kit was the DNeasy® Plant Mini Kit (Qiagen), using the AP1 lysis buffer supplied by the manufacturer (cf Dossier LNR de validation de la méthode MIAM 005). DNA concentration and quality were determined by assay using the NanoDrop 2000c

spectrophotometer (Thermo Fisher Scientific, Walthum, MA) and visualized on a 1% agarose gel stained with SYBR Safe DNA gel stain under ultraviolet light (UVP Bio Imaging Systems, Upland, CA) to facilitate polymerase chain reaction (PCR). DNA quantification was determined by spectrophotometer by measuring the absorbance of each sample against DNA-free distilled water at wavelengths of 260 and 280 nm [20]. DNA preparations with A260/A280=1.8-2.0 were considered sufficient quality for PCR. DNA was stored at -20°C for future use.

2.4.2 Polymerase chain reaction (PCR)

DNA obtained from the isolates served as a template for PCR, which was carried out using two primer pairs: ITS1/ITS4 (ITS1 5' TCCGTAGGTGAACCTGCGG 3'/ ITS4 5' TCCTCCGCTTATTGATATGC 3'), to amplify the entire ITS region and Gc-F1/Gc-R1(Gc-F1 5' GGT GAT GGA AGG GAG GCC T 3'/ Gc-R1 5' GCA ACA TGG TAG ATA CAC AAG GGT 3'), specific to the ITS region of *Phyllosticta citricarpa*. The protocol developed by Van Gent-Pelzer et al [21] was used for real-time PCR.

The specificity of the Gc-F1/Gc-R1 primers was evaluated with DNA from 66 isolates of *Phyllosticta* sp. and 3 isolates of *Colletotrichum* sp. The PCR mixture, with a total reaction volume of 25 µl, was prepared using 2 µl of DNA extracted from the isolates, 1 µl of each primer, 5 µl of Master Mix and 16 µl of deionized distilled water. Amplifications were performed in a thermal cycler (Eppendorf® Mastercycler ep Gradient), with (i) initial denaturation at 95°C for 10 min, (ii) 35 cycles comprising denaturation at 94°C for 60 sec; hybridization at 60°C for 30 sec; extension at 72°C for 60 sec and (iii) a final extension cycle at 72°C for 5 min. PCR products were separated by 1.5% agarose gel electrophoresis (Ultra Pure TM agarose, Invitrogen, Spain) in 0.5X 1 x Tris-Acetate-EDTA (TAE) buffer and visualized under UV light. The 100-bp DNA ladder plus was used as a molecular weight marker (Fermentas, St. Leon-Rot, Germany) for PCR amplicons.

2.4.3 Sequencing

Extracted fragments were forward and reverse sequenced (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). Purified fragments were analyzed on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific) for each reaction in each sample. DNASTAR was used to analyze the ab1 files generated by the ABI 3500XL genetic analyzer, and results were obtained using a BLAST search (NCBI).

2.4.4 Phylogenetic analysis

Sequences from the ITS region of the representative isolates in this study were aligned with reference sequences (Table 1) downloaded from the NCBI GenBank using ClustalX 2.0 in accordance with recent publications [22,23]. Alignments were performed using default parameters and manually enhanced with MEGA v.11.0 [24]. The Maximum Likelihood (ML) phylogenetic analysis was performed using 1000 bootstrap replicates, and the trees were visualized in figtree 1. (http://tree.bio.ed.ac.uk/software/figtree). To confirm the molecular diagnosis of the pathogen, pathogenicity testing was carried out using the identified isolates.

2.5 Pathogenicity Test

The pathogenicity test was carried out in the laboratory on apparently healthy fruit (no spots) with 5 identified isolates. These fruits were washed and surface disinfected with 70% alcohol, then rinsed twice with sterile distilled water. The spore suspension was prepared by adding 10 ml of sterile distilled water to 20-day-old pure cultures. The conidial surface was scraped with a pasteur pipette, then the solution was transferred to a test tube before being vortexed for homogenization. The resulting suspension was filtered through sterile muslin to separate conidia from mycelial fragments. The suspension was then diluted with sterile distilled water and the final concentration of 10^5 conidia/ml was determined using the Malassez cell counting chamber.

In addition, 12 inoculation points were created on each fruit, which received 5 µl of suspension respectively. Inoculation was performed by injection with a sterile hypodermic needle to a depth of around 2 mm in the albedo (the area of white pith just below the skin). Control fruits were inoculated with sterile water. The inoculation points on each fruit were circled with a permanent marker. Inoculated fruits were incubated at 25 ± 2°C, under a lighting system providing a 12-hour photoperiod. Lesion development was assessed on days 5, 15 and 25 after inoculation. Koch's postulate was verified by re-isolation of isolates from lesions on inoculated fruit.

Table 1. Fungal strains and GenBank accession numbers of the sequences used for the phylogenetic analyses in this study

Species	Strain	Host plant	Country	Gen Bank number
				ITS
P. capitalensis	CBS 173.77	*Citrus aurantiifolia*	New Zealand	KF206179
P. capitalensis	CBS 100175	*Citrus* sp.	Brazil	FJ538320
P. capitalensis	CPC 16592	*Citrus limon*	Argentina	KF206187
P. citriasiana	CBS 120486	*Citrus maxima*	Thailand	FJ538360
P. citriasiana	CBS 123371	*Citrus maxima*	Vietnam	FJ538356
P. citricarpa	CBS 102374	*Citrus aurantium*	Brazil	FJ538313
P. citricarpa	CBS 120489	*Citrus sinensis*	Brazil	FJ538315
P. citricarpa	CBS 127454	*Citrus limon*	Australia	JF343583
P. citriasiana	CBS 123370	*Citrus maxima*	Vietnam	FJ538355
P. citriasiana	CBS 123393	*Citrus maxima*	Vietnam	FJ538358
P. citricarpa	CBS 102373	*Citrus aurantium*	Brazil	FJ538312
P. citricarpa	CBS 122384	*Citrus limon*	South Africa	FJ538316
P. citricarpa	CBS 122482	*Citrus sinensis*	Zimbabwe	FJ538317
P. owaniana	CBS 776.97	*Brabejum stellatifolium*	South Africa	FJ538368
P. owaniana	CPC 14901	*Brabejum stellatifolium*	South Africa	JF261462
P. aloeicola	CPC 21020	*Aloe ferox*	South Africa	KF154280
P. aloeicola	CPC 21021	*Aloe ferox*	South Africa	KF154281
P. citricarpa	PhZ5	*Citrus sinensis*	Benin	OR673558
P. citricarpa	PhZ5R	*Citrus sinensis*	Benin	OR673559
P. citricarpa	PhZ6R	*Citrus sinensis*	Benin	OR673560
P. citricarpa	PhZ6	*Citrus sinensis*	Benin	OR673561
P. citricarpa	PhZ7R	*Citrus sinensis*	Benin	OR673562
P. citricarpa	PhZ8	*Citrus sinensis*	Benin	OR673563
P. citricarpa	PhZ8R	*Citrus sinensis*	Benin	OR673564
P. citricarpa	PhZ7	*Citrus sinensis*	Benin	OR673565
C. gloeosporioides	CBS 953.97	*Citrus sinensis*	Italy	JN121209

2.6 Statistical analysis

Data were entered using Excel 2013. R software was used for all study analyses. ANOVA was used to analyze data on conidial size, colony growth diameters and isolate pathogenicity. The Newman-Keuls test with a threshold of 5% was used to compare the variable means of the different isolates concerned.

3. RESULTS

3.1 Morphological Identification of *Phyllosticta* sp.

The colonies of the isolated fungus from orange leaves and fruits on PDA plates were clear at emergency and grew slowly with irregular edged colonies surrounded by a larger translucent zone of immersed clear mycelium (Fig. 1). Centre of the colonies was composed of grey aerial mycelium forming a plectenchymatous rind. The reverse had a very dark centre surrounded by grey and beige areas. Average colony diameter ranged from 7.60 cm to 8.40 cm after 20 days at 25 ± 2°C.

Colonies showed hard, black mass stromas after 7 to 8 days. Ripe pycnidia contained conidia after 10 to 14 days (Fig. 2). Conidia were 9.4 - 12.7 µm long and 5.0 - 8.5 µm wide, ellipsoid to obovoid with a truncate base, hyaline, guttulate, aseptate with a tiny apical mucoid and translucent layer appendage, 3-10 µm long and 1.5 µm wide (Fig. 3).

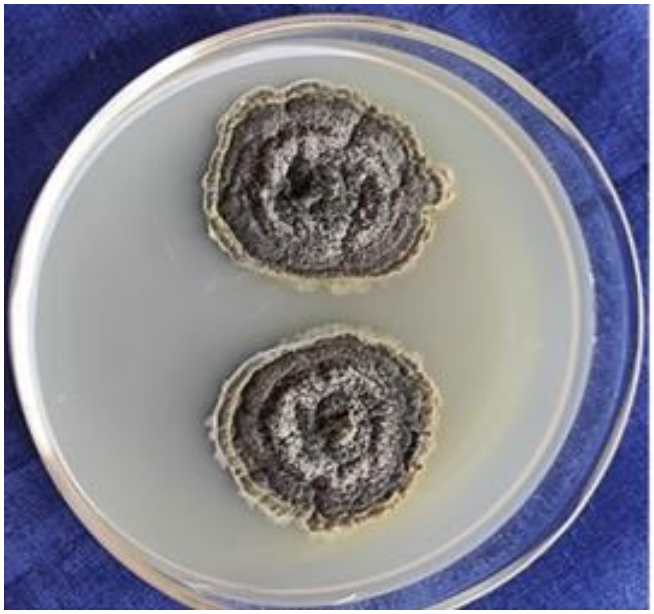

Fig. 1. Morphological aspect of *Phyllosticta* sp. on PDA culture medium

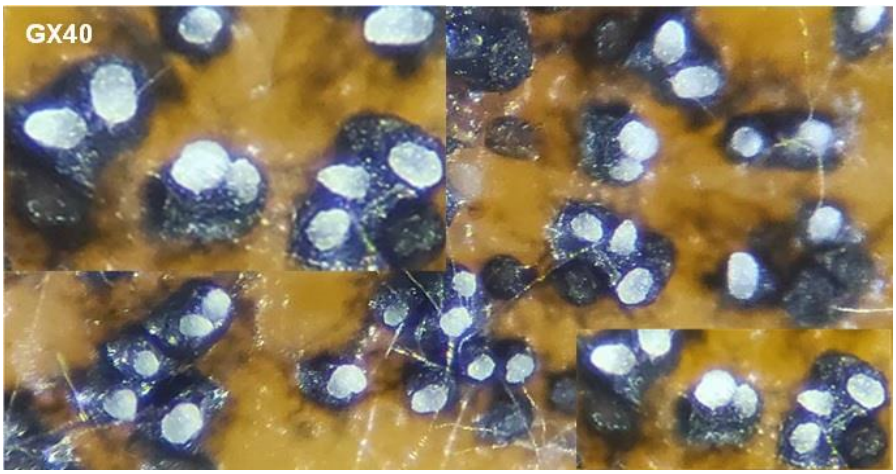

Fig. 2. Binocular observation of *Phyllosticta* sp.: Pycnidia sporulating on surface of citrus fruit

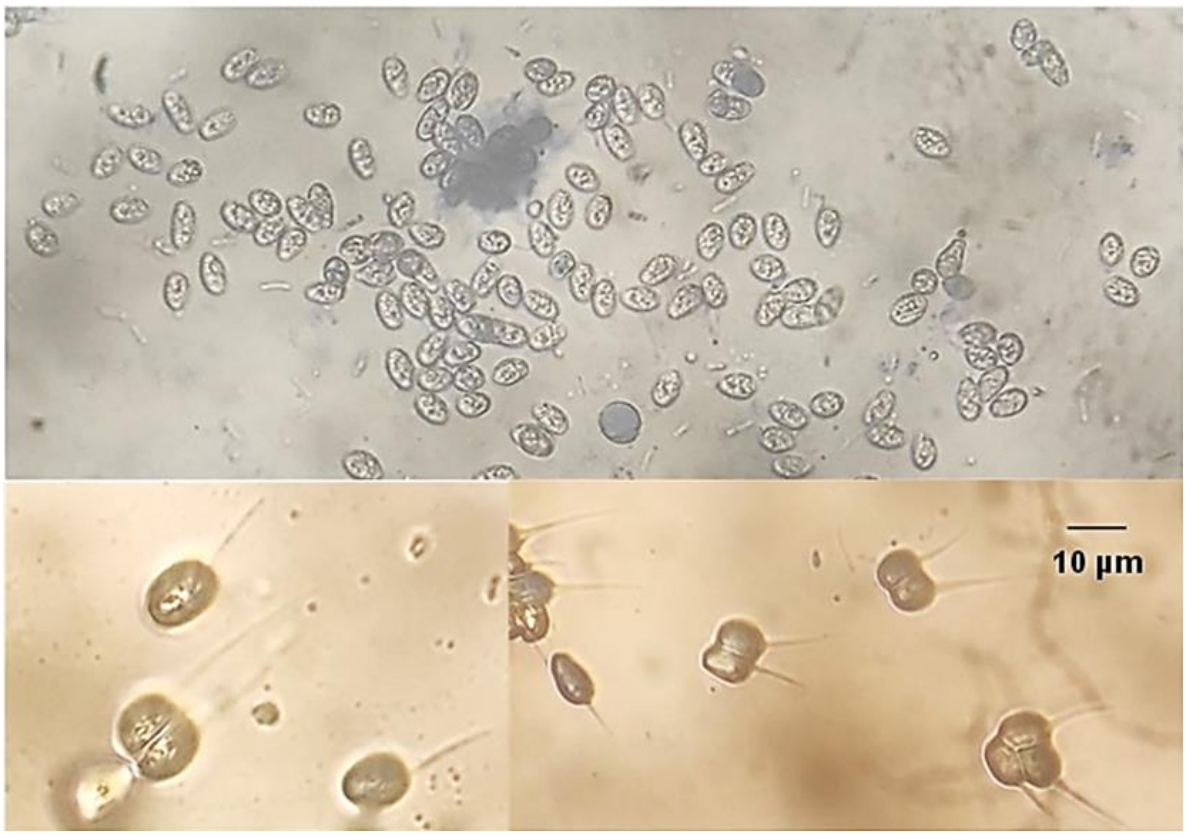

Fig. 3. Microscopic observation of *Phyllosticta* sp.: Conidia with mucoid sheaths and apical appendages

3.2 Molecular Characterization of *Phyllosticta* sp.

Based on rDNA ITS regions, all 66 *Phyllosticta* sp. isolates tested with ITS1/ITS4 primers (Table 2) generated around 550 bp of DNA fragments (Fig. 2). Real-time PCR testing using primers specific to *Phyllosticta citricarpa* showed a positive reaction for *Phyllosticta* sp. isolates and a negative reaction for *Colletotrichum* sp. isolates. Amplicons from primers GcF1/GcR1 produced a molecular weight of around 69 bp (Fig. 3).

3.3 Phylogenetic Analysis

All sequences used in this study were submitted to GenBank (Accession Nos. OR673558 - OR673565). Comparison of these sequences with those of reference strains using the BLASTn search analysis in National Center for Biotechnology Information (NCBI) GenBank showed a similarity with those of *P. citricarpa*. The ITS sequences of the isolates were aligned with various *Phyllosticta* species deposited in the fungal databases of the Centraal Bureau voor Schimmelcultures (CBS) and NCBI GenBank. The phylogenetic tree revealed that 8 isolates representative of the 66 isolates obtained in this study have an ITS rDNA sequence identical to strains of *P. citricarpa* CBS127454 (from *Citrus limon* fruit in Australia), *P. citricarpa* CBS122482 (from *Citrus aurantium* fruit in Zimbabwe) and *P. citricarpa* CBS102373 (from *Citrus sinensis* fruit in Brazil) (Fig. 4).

3.4 Pathogenicity Test

Fruits tested for pathogenicity revealed the pathogenic potential of all isolates. Inoculated fruit showed typical symptoms of fungus at the inoculation point after 7 days (Fig. 5). No spots were observed on control fruits. Isolates were systematically re-isolated from lesions occurred on inoculated fruit, satisfying Koch's postulates (Fig. 6).

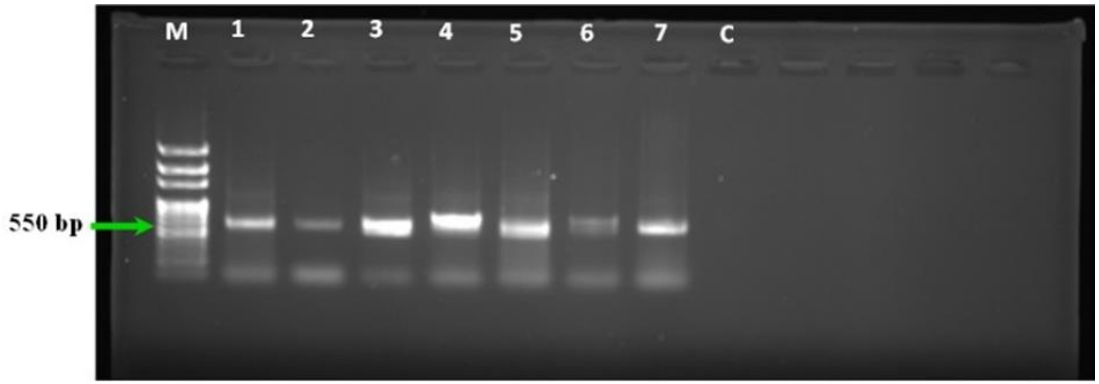

Fig. 4. Polymerase Chain Reaction (PCR) amplification of 550 bp of *P. citricarpa* DNA with ITS5/ITS4 primers; C = Negative control; M = Size marker (100 bp)

Table 2. PCR amplification of isolates

N° d'ordre	Isolates code	Isolates	Plant part	Host Plant	Localities	ITS1/ ITS4	Gc-F1/ Gc-R1
1	Bo1VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Bopa	+	+
2	Bo1PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Bopa	+	+
3	Bo2VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Bopa	+	+
4	Bo2PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Bopa	+	+
5	Bo3PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Bopa	+	+
6	Do1VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Dogbo	+	+
7	Do2VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Dogbo	+	+
8	Do2PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Dogbo	+	+
9	To2VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Toviklin	+	+
10	To3VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Toviklin	+	+
11	To3PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Toviklin	+	+
12	La1PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Lalo	+	+
13	La2VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Lalo	+	+
14	La3PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Lalo	+	+
15	La3VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Lalo	+	+
16	Kl1PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Klouékanmey	+	+
17	Kl1PCo	*Collectotrichum* sp.	Fruit	*Citrus sinensis*	Klouékanmey	+	-
18	Kl2PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Klouékanmey	+	+

N° d'ordre	Isolates code	Isolates	Plant part	Host Plant	Localities	ITS1/ ITS4	Gc-F1/ Gc-R1
19	Kl2VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Klouékanmey	+	+
20	Kl3PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Klouékanmey	+	+
21	Ap1PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Aplahoué	+	+
22	Ap2VCo	*Collectotrichum* sp.	Fruit	*Citrus sinensis*	Aplahoué	+	-
23	Ap2VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Aplahoué	+	+
24	Ap3PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Aplahoué	+	+
25	Ap3VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Aplahoué	+	+
26	Zg1PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Zangnanado	+	+
27	Zg2PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Zangnanado	+	+
28	Zg3VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Zangnanado	+	+
29	Zp1PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Zakpota	+	+
30	Zp2PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Zakpota	+	+
31	Zp2PCo	*Collectotrichum* sp.	Fruit	*Citrus sinensis*	Zakpota	+	-
32	Zp3PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Zakpota	+	+
33	Zp3VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Zakpota	+	+
34	Ag1PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Agbangnizoun	+	+
35	Ag2PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Agbangnizoun	+	+
36	Ag2VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Agbangnizoun	+	+
37	Ag3PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Agbangnizoun	+	+
38	Co1PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Covè	+	+
39	Co1VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Covè	+	+
40	Co2PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Covè	+	+
41	Co3VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Covè	+	+
42	Zo1PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Zogbodomey	+	+
43	Zo2PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Zogbodomey	+	+
44	Zo2VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Zogbodomey	+	+
45	Dj1VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Djidja	+	+
46	Dj2VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Djidja	+	+
47	Dj3PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Djidja	+	+
48	At3VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Athiémé	+	+
49	Lo1VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Lokossa	+	+
50	Lo2PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Lokossa	+	+
51	Al1PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Allada	+	+
52	Al1VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Allada	+	+
53	Tr1VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Tori-Bossito	+	+
54	Tr1PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Tori-Bossito	+	+

N° d'ordre	Isolates code	Isolates	Plant part	Host Plant	Localities	ITS1/ ITS4	Gc-F1/ Gc-R1
55	Ze1PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Zè	+	+
56	Ze1VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Zè	+	+
57	Ke1PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Kétou	+	+
58	Ke2PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Kétou	+	+
59	Ke3VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Kétou	+	+
60	Sa1PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Sakété	+	+
61	Sa2PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Sakété	+	+
62	Po1PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Pobè	+	+
63	Po2PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Pobè	+	+
64	Tf1PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Toffo	+	+
65	Tf2VP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Toffo	+	+
66	Tf3PP	*Phyllosticta* sp.	Fruit	*Citrus sinensis*	Toffo	+	+

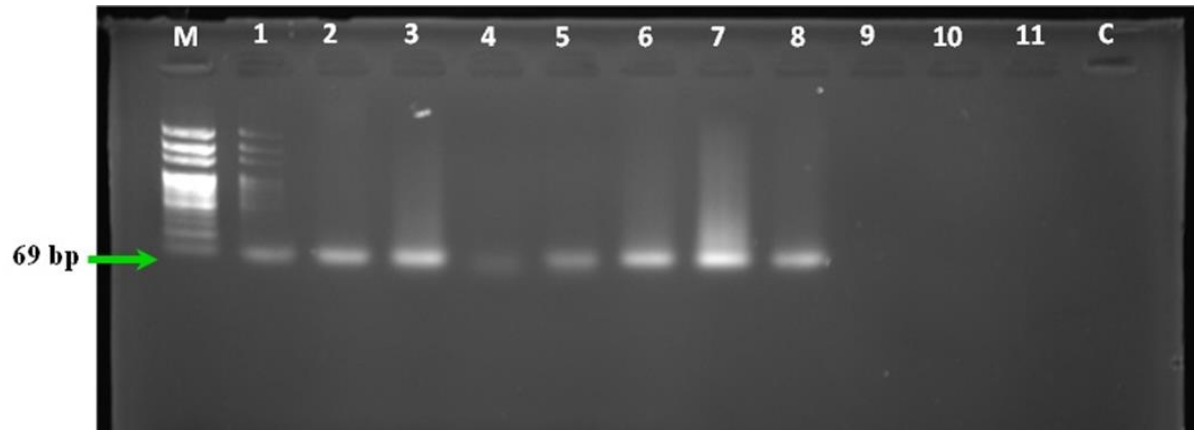

Fig. 5. Polymerase Chain Reaction (PCR) with specific primers Gc-F1/Gc-R1: 1-8, *P. citricarpa* (69 bp); 9-11, *Colletotrichum* sp.; C = Negative control; M = Size marker (100 bp)

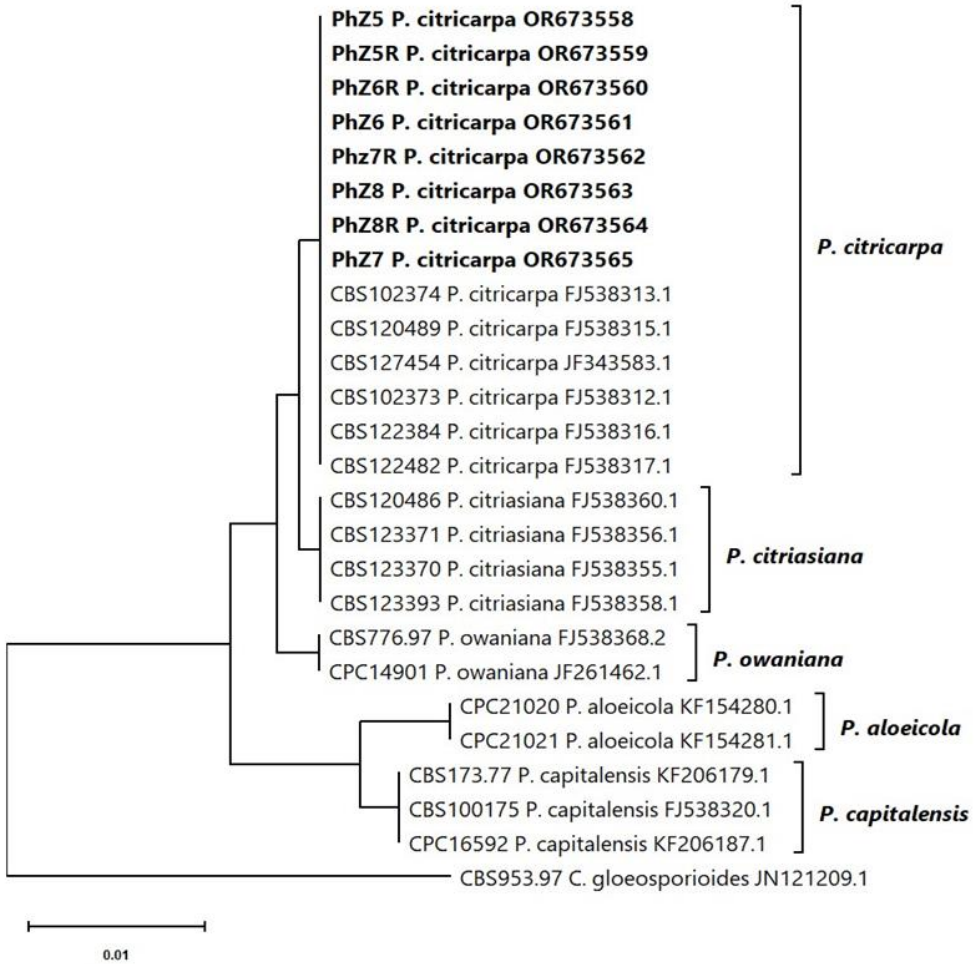

Fig. 6. Phylogenetic tree based on ITS sequences of *Phyllosticta* isolates. The evolutionary history was inferred using the Neighbor-Joining method. The related sequences from NCBI were constructed by MEGA 11.0. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. *Colletotrichum gloesoporioides* as outgroup. Isolates from this study are in blod.

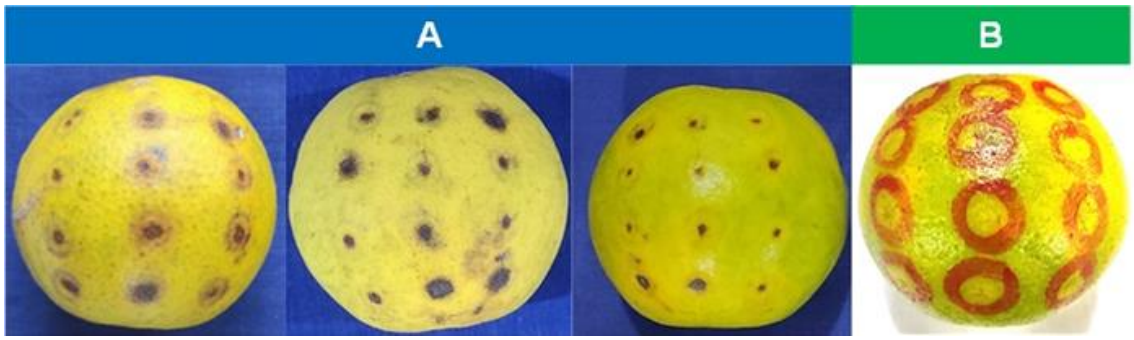

Fig. 7. Pathogenicity test. A symptoms of *P. citricarpa* on inoculated *Citrus sinensis* fruit; B control fruit

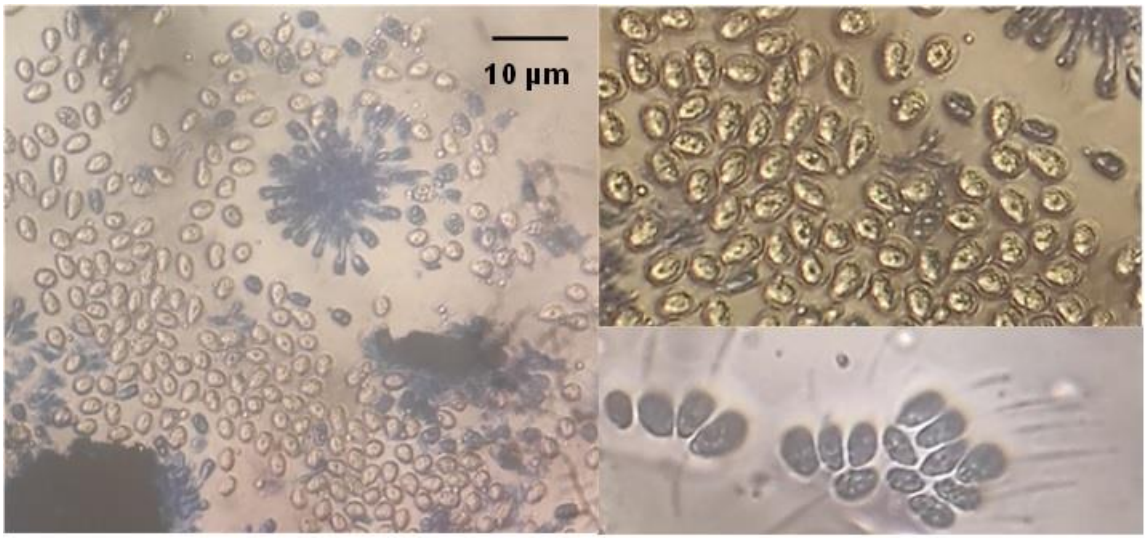

Fig. 8. Microscopic observation of *P. citricarpa* after Pathogenicity test

4. DISCUSSION

Black spot is a major constraint to citrus production in Benin. The morphological characteristics of the disease pathogen correspond to those of *P. citricarpa* [12,25,26,27]. Mycelial growth of the fungus is slow on PDA medium. These results are in line with those of previous studies, which reported the slowly growth of *P. citricarpa* on PDA medium and is generally overgrown by faster-growing fungi such as *C. gloeosporioides* [2,4,9,15]. However, many recent publications have shown that morphological characters alone are not sufficient to accurately identify a pathogen, as they are highly dependent on the environment [28,29].

Morphological identification of *Phyllosticta* sp. was confirmed by molecular analysis and a phylogenetic approach. Real-time PCR used in this study with primers GcF1 and GcR1 enabled rapid and specific identification of *P. citricarpa*. The results of this method showed a positive reaction only for isolates of *Phyllosticta* sp. with 69 bp and a negative reaction for isolates of Colletotrichum sp. These results confirm those of Van Gent-Pelzer et al [21] who reported that real-time PCR with primers GcF1 and GcR1, is a rapid method for the specific detection of P. citricarpa generating much smaller amplicons of 69 bp. Faganello et al [30] reported that real-time PCR is a robust and more sensitive method than conventional PCR and allows detection of *P. citricarpa* in asymptomatic plant tissues. According to Schirmacher et al [31], this method can detect and distinguish *P. citricarpa* from other pathogens without the need for sequencing. Amplification of ITS regions using ITS1/ITS4 primers produced amplicons for all isolates used. The size of DNA fragments 550 bp from *Phyllosticta* sp. isolates generated by ITS1/ITS4 in the present study is similar to that observed by Baayen et al [9].

Phylogenetic analysis of the nucleotide sequences of ITS regions showed that all isolates obtained in this study belong to *P. citricarpa*. These results suggest that the same species (*P. citricarpa*) is present in orchards of all agro-ecological zones. Moreover, its presence in all agro-ecological zones suggests that either climatic conditions are favorable to the pathogen, and facilitate its spread, or it has an intrinsic potential that enables it adaptation to climatic conditions in all agro-ecological zones. This pathogen dynamic can lead to a more extensive dissemination of the disease, an increase of damages and resistance to control methods, and make management more complex. The same observation was made by Boughalleb-M'Hamdi et al [26], who recently confirmed that black spot disease caused by *P. citricarpa* was spreading rapidly in the main citrus-growing areas of Tunisia. According to Zajc et al [32], as *P. citricarpa* spreads across a region, the extent of damage increases and the scope for eradication or control decreases considerably. The European Union (EU) under phytosanitary regulations 2016/2031 and 2019/2072, considers *P. citricarpa* a quarantine pathogen because of the economic, environmental and social impact it is likely to cause [32]. It is also included in the list of priority harmful organisms, for which annual surveys by member states are mandatory, under Regulation 2019/1702. Previous studies have shown that black spot disease can cause up to 80% of yield losses in citrus orchards [2,7]. According to EPPO [33], black spot is responsible for huge economic losses in several citrus-growing countries around the world, including South Africa, Mozambique, Swaziland, Zimbabwe and Namibia, Ghana, China, Angola and India. The economic importance of this disease is also justified by the fact that fruits with black spots are not exported [34]. Consequently, research into the genetics of the pathogen is needed to better understand its variability and behavior.

The results of the pathogenicity test demonstrated the pathogenic potential of all the *P. citricarpa* isolates used. By reproducing the symptoms of black spot disease on inoculated fruits and recovering the pathogen after inoculations, the present study satisfied Koch's postulates for *P. citricarpa*. The same observations were also made by several previous authors [2,3,4,25,26] who re-isolated *P. citricarpa* on citrus fruit after a few days of inoculation.

Furthermore, the control of citrus black spot is essentially based on the use of chemical pesticides [35]. The application of these pesticides is a major threat to the environment and human health. However, alternative techniques (cultural practices, biological control) have been developed to combat citrus black spot disease effectively and sustainably [36]. Biological control using endophytes or biopesticides makes it possible to effectively prevent and control black spot disease in line with sustainable development objectives. Using these endophytes is promising and represents a economical and low-impact option, as they provide benefits to the host and colonise the same niche as the pathogens [37].

5. CONCLUSION

The isolates of *P. citricarpa* of Benin collected from *citrus sinensis* diseased fruits in the for agroecological zones are genetically homogenous. Based on morphological characterization, PCR and phylogenetic analyses it is demonstrated that *P. citricarpa* is the causal agent of citrus black spot disease in Benin. The pathogenicity test demonstrated the pathogenic power of *P. citricarpa* to cause infections on citrus fruit. The knowledge on the behavior of *P. citricarpa* and the development of an appropriate and sustainable of citrus black spot disease management will be the follow step.

A thorough study of genetic diversity and a full understanding of the behavior of *P. citricarpa* will pave the way for more targeted approaches to the prevention, control and sustainable management of citrus black spot disease in Benin.

AVAILABILITY OF SUPPORTING DATA

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

FUNDING

This work was supported by Partnership for Skills in Applied Sciences, Engineering and Technology (PASET) and Regional Scholarship and Innovation Funds (RSIF) programmer.

ACKNOWLEDGEMENTS

The authors thank the Partnership for Skills in Applied Sciences, Engineering and Technology (PASET) and Regional Scholarship and

Innovation Funds (RSIF) programme, the Université Félix Houphouët-Boigny of Côte d'Ivoire, the Institut National des Recherches Agricoles du Bénin (INRAB), Phytopathology laboratory of the Centre National de Spécialisation en Fruits et Légumes (CNS-FL)/INERA/Burkina-Faso and African Centre of Excellence of Climate Change, Biodiversity and Sustainable Agriculture (CEA-CCBAD/Côte d'Ivoire) for financial and technical support and providing working facility of the research study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. EFSA (European Food Safety Authority), Parnell S, Schenk MSG, Vicent A, Delbianco A, Vos S. Pest survey card on Phyllosticta citricarpa. EFSA Supporting Publications. 2020;17(6). DOI:https://doi.org/10.2903/sp.efsa.2020.en-1863
2. Brentu FC, Oduro KA, Offei SK, Odamtten GT, Vicent A, Peres NA, Timmer LW. Crop loss, aetiology, and epidemiology of citrus black spot in Ghana. European Journal of Plant Pathology. 2012;133(3):657–670. DOI:https://doi.org/10.1007/s10658-012-9944-1
3. Fialho RO, Moyo P, Fourie PH, Lanza FE, Machado FJ, Amorim L, Silva-Junior GJ. Citrus black spot intensity and yield losses on sweet orange are affected by Phyllosticta citricarpa inoculum concentration and fruit developmental stage. Plant Pathology. 2022;71(7):1606–1618. DOI:https://doi.org/10.1111/ppa.13586
4. Baldassari RB, Wickert E, De Goes A. Pathogenicity, colony morphology and diversity of isolates of Guignardia citricarpa and G. mangiferae isolated from Citrus spp. European Journal of Plant Pathology. 2008;120(2): 103–110. DOI:https://doi.org/10.1007/s10658-007-9182-0
5. Lanza FE, Metzker TG, Vinhas T, Behlau F, Silva Junior GJ. Critical fungicide spray period for citrus black spot control in São Paulo state, Brazil. Plant Disease. 2018;102(2):334–340. DOI:https://doi.org/10.1094/PDIS-04-17-0537-RE
6. Frare GF, Silva-Junior GJ, Lanza FE, Bassanezi RB, Ramires TG, Amorim L. Sweet orange fruit age and inoculum concentration affect the expression of citrus black spot symptoms. Plant Disease. 2019;103(5):913–921. DOI:https://doi.org/10.1094/PDIS-03-18-0492-RE
7. Baldassari RB, Reis RF, De Goes A. Susceptibility of fruits of the "Valência" and "Natal" sweet orange varieties to Guignardia citricarpa and the influence of the coexistence of healthy and symptomatic fruits. Fitopatologia Brasileira. 2006;31(4):337–341. DOI:https://doi.org/10.1590/S0100-41582006000400002
8. Spósito MB, Amorim L, Bassanezi RB, Yamamoto PT, Felippe MR, Czermainski ABC. Relative importance of inoculum sources of Guignardia citricarpa on the citrus black spot epidemic in Brazil. Crop Protection. 2011;30(12):1546–1552. DOI:https://doi.org/10.1016/j.cropro.2011.08.007
9. Baayen RP, Bonants PJM, Verkley G, Carroll GC, Van der Aa HA, De Weerdt M, Van Brouwershaven IR, Schutte GC, Maccheroni W, Glienke de Blanco C, Azevedo JL. Nonpathogenic isolates of the citrus black spot fungus, Guignardia citricarpa, identified as a cosmopolitan endophyte of woody plants, G. mangiferae (Phyllosticta capitalensis). Phytopathology. 2002;92(5):464–477. DOI:https://doi.org/10.1094/PHYTO.2002.92.5.464
10. Glienke C, Pereira OL, Stringari D, Fabris J, Kava-Cordeiro V, Galli-Terasawa L, Cunnington J, Shivas RG, Groenewald JZ, Crous PW. Endophytic and pathogenic Phyllosticta species, with reference to those associated with Citrus Black spot. Persoonia: Molecular Phylogeny and Evolution of Fungi. 2011;26:47–56. DOI:https://doi.org/10.3767/003158511X569169
11. Wang X, Chen G, Huang F, Zhang J, Hyde KD, Li H. Phyllosticta species associated with citrus diseases in China. Fungal Diversity. 2012;52:209–224. DOI:https://doi.org/10.1007/s13225-011-0140-y
12. Guarnaccia V, Groenewald JZ, Li H,

Glienke C, Carstens E, Hattingh V, Fourie PH, Crous PW. First report of Phyllosticta citricarpa and description of two new species, P. paracapitalensis and P. paracitricarpa, from citrus in Europe. Studies in Mycology. 2017;87:161–185. DOI:https://doi.org/10.1016/j.simyco.2017.05.003

13. Wulandari NF, To-anun C, Hyde KD, Duong LM, De Gruyter J, Meffert JP, Groenewald JZ, Crous PW. Phyllosticta citriasiana sp. nov., the cause of Citrus tan spot of Citrus maxima in Asia. Fungal Diversity. 2009;34:23–39.
14. Wikee S, Lombard L, Nakashima C, Motohashi K, Chukeatirote E, Cheewangkoon R, McKenzie EHC, Hyde KD, Crous PW. A phylogenetic re-evaluation of Phyllosticta (Botryosphaeriales). Studies in Mycology. 2013;76:1–29. DOI:https://doi.org/10.3114/sim0019
15. Peres NA, Harakava R, Carroll GC, Adaskaveg JE, Timmer LW. Comparison of molecular procedures for detection and identification of Guignardia citricarpa and G. mangiferae. Plant Disease. 2007; 91(5):525–531. DOI:https://doi.org/10.1094/PDIS-91-5-0525
16. Cai L, Hyde KD, Taylor P. A polyphasic approach for studying Colletotrichum Fungal Diversity A polyphasic approach for studying Colletotrichum Colletotrichum Corda is one of the eco- nomically most important genera of fungi. 2009;1-16.
17. Martin-Lapierre A. Application de composts et de fumigants pour lutter contre la verticilliose (verticillium dahliae) du fraisier. 2011;108.
18. Al-Sadi AM, Al-Said FA, Al-Jabri AH, Al-Mahmooli IH, Al-Hinai AH, de Cock AWAM. Occurrence and characterization of fungi and oomycetes transmitted via potting mixtures and organic manures. Crop Protection. 2011;30(1):38–44. DOI:https://doi.org/10.1016/J.CROPRO.2010.09.015
19. Thompson WF. Rapid isolation of higher weight DNA. 2014. DOI:https://doi.org/10.1093/nar/8.19.4321
20. Creager ANH. Recipes for recombining DNA: A history of Molecular Cloning: A Laboratory Manual . BJHS Themes. 2020;5:225–243. DOI:https://doi.org/10.1017/bjt.2020.5
21. Van Gent-Pelzer MPE, Van Brouwershaven IR, Kox LFF, Bonants PJM. A TaqMan PCR method for routine diagnosis of the quarantine fungus Guignardia citricarpa on citrus fruit. Journal of Phytopathology. 2007;155(6):357–363. DOI:https://doi.org/10.1111/j.1439-0434.2007.01244.x
22. Hattori Y. Taxonomical studies on Botryosphaeriales in Japan. 2021. Available:https://mie-u.repo.nii.ac.jp/?action=repository_uri&item_id=14730&file_id=17&file_no=1
23. Zhang Z, Liu X, Zhang X, Meng Z. Morphological and phylogenetic analyses reveal two new species and a new record of Phyllosticta (Botryosphaeriales, Phyllostictaceae) from Hainan, China. MycoKeys. 2022;91:1–23. DOI:https://doi.org/10.3897/mycokeys.91.84803
24. Tamura K, Stecher G, Kumar S. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Molecular Biology and Evolution. 2021;38(7):3022–3027. DOI:https://doi.org/10.1093/molbev/msab120
25. Bassimba DDM, Nzambi N, Paixão MIS, Katula IG, Vicent A. First report of citrus black spot caused by Phyllosticta citricarpa in Angola. Plant Disease. 2018;102(3):683. DOI:https://doi.org/10.1094/PDIS-09-17-1374-PDN
26. Boughalleb-M'Hamdi N, Fathallah A, Benfradj N, Mahmoud S, Ben ABH, Medhioub L, Jaouadi I, Huber J, Jeande C, loos R. First report of citrus black spot disease caused by Phyllosticta citricarpa on Citrus limon and C. sinensis in Tunisia . New Disease Reports. 2020;41(1):8–8. DOI:https://doi.org/10.5197/j.2044-0588.2020.041.008
27. Toessi HG, Ler-N'Ogn Dadé Amari GE, Sikirou R, Kone D. First report of citrus black spot disease caused by Phyllosticta citricarpa in Benin. New Disease Reports. 2023;47:e12145. DOI:https://doi.org/10.1002/ndr2.12145.
28. Phoulivong S, Cai L, Chen H, McKenzie EHC, Abdelsalam K, Chukeatirote E, Hyde KD. Colletotrichum gloeosporioides is not a common pathogen on tropical fruits. Fungal Diversity. 2010;44:33–43. DOI:https://doi.org/10.1007/s13225-010-0046-0
29. Honger JO, Offei SK, Oduro KA, Odamtten GT, Nyaku AST. PhenotyPic and

Molecular characterisation of the causal agent of Mango anthracnose disease in ghana. Ghana J. Sci. 2014;54:71–82.

30. Faganello FDS, Carrer Filho R, Dias VD, Morello RMSC, Cunha MGDA. Molecular diagnosis of Guignardia citricarpa in asymptomatic sweet orange tissue. Revista Brasileira de Fruticultura. 2017;39(4). DOI:https://doi.org/10.1590/0100-29452017518.
31. Schirmacher AM, Tomlinson JA, Barnes AV, Barton VC. Species-specific real-time PCR for diagnosis of Phyllosticta citricarpa on Citrus species. EPPO Bulletin. 2019;49(2):306–313. DOI:https://doi.org/10.1111/EPP.12555.
32. Zajc J, Kogej Zwitter Z, Fišer S, Gostinčar C, Vicent A, Domenech AG, Riccioni L, Boonham N, Ravnikar M, Kogovšek P. Highly specific qPCR and amplicon sequencing method for detection of quarantine citrus pathogen Phyllosticta citricarpa applicable for air samples. Plant Pathology. 2023;72(3): 548–563. DOI:https://doi.org/10.1111/ppa.13679.
33. EPPO. Phyllosticta citricarpa, EPPO Bull. 2022;50(3):440–461. DOI:10.1111/EPP.12700.
34. Moyo P, Cook G, Basson E, Steyn C, Bester R, Olivier C, Fourie PH. Monitoring benzimidazole resistance in Phyllosticta citricarpa using a molecular assay targeting mutations in codons 198 and 200 of the β-tubulin gene. Plant Disease. 2022;106(5):1374-1380. DOI:https://doi.org/10.1094/PDIS-07-21-1459-RE
35. Schutte GC, Beeton KV, Kotzé JM. Rind stippling on Valencia oranges by copper fungicides used for control of citrus black spot in South Africa, Plant Dis. 1997;81(8): 851–854. DOI:10.1094/PDIS.1997.81.8.851.
36. Niu JZ, Sanders HH, Zhang YX, Lin JZ, Dou W, Wang JJ. Biological control of arthropod pests in citrus orchands in China. Biol. Control. 2014;68:15–22.
37. Miller KI, Qing C, Sze DMY, Roufogalis BD, Neilan BA. Culturable endophytes of medicinal plants and the genetic basis for their bioactivity. Microb. Ecol. 2012 ;64:431–449.

Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/117491