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Update on the population of *Phytophthora* sp. agent of cocoa black pod disease in Côte d'Ivoire

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Abstract

Black pod disease is a major constraint on cocoa production in all African cocoa-producing countries, particularly Côte d'Ivoire. In Africa, this disease is caused by two main species of the genus *Phytophthora* (*P. palmivora* and *P. megakarya*). The former is cosmopolitan and less aggressive, while the latter is endemic and very aggressive. Traditionally, only the *P. palmivora* species had been identified on cocoa in Côte d'Ivoire. However, in recent decades, high incidences of the disease have been recorded in several regions of the country, probably due to the emergence of a new species. The aim of this study was to update the population of *Phytophthora* sp. in Côte d'Ivoire by identifying the *Phytophthora* species colonizing the cocoa orchard. To this end, in addition to morphological identification, the PCR-RFLP technique using the Alu I and Hae III restriction enzymes, sequencing of the ITS region, and phylogenetic analysis, was used to characterize *Phytophthora* sp. isolates from the country's main cocoa-growing areas. The results showed that the approximate size of the DNA fragments of the isolates studied was 900 bp. DNA sequencing of a sample of 50 isolates revealed 60% *P. palmivora* and 40% *P. megakarya*. This study provided molecular confirmation of the presence of *P. megakarya* in Côte d'Ivoire. The use of the PCR-RFLP technique therefore enables rapid and precise identification of *Phytophthora* species causing cocoa black pod disease. Hence its importance for decision-making in the management of this disease in Côte d'Ivoire.

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Introduction

On average, Africa produces 75% of the world's cocoa crop, with Côte d'Ivoire and Ghana accounting for 38% and 19%, respectively (Nagel *et al.*, 2013; ICCO, 2022). However, cocoa production in Africa is under significant threat from black pod disease and swollen shoot. Black pod disease is a problem in all cocoa producing countries in Africa (Bailey *et al.*, 2016; Muller *et al.*, 2018; ICCO, 2022). The disease is caused by oomycetes of the genus *Phytophthora* (Brasier and Griffin, 1979; Cooke *et al.*, 2000). It is the most important and widespread disease affecting cocoa pods, accounting for approximately 50% of production losses (Ali *et al.*, 2016; Marelli *et al.*, 2019). Cocoa black pod disease in Africa was initially attributed solely to *P. palmivora* species. However, based on symptoms, morphology, aggressiveness and losses, it became clear that other species were involved (Sansome *et al.*, 1975, 1979). This led to the description of *Phytophthora megakarya* by Brasier and Griffin in 1979. *Phytopthora megakarya* is indeed the more aggressive of the two species and can cause up to 80% losses if no control measures are applied (Ndoumbè-Nkeng *et al.*, 2004; Ali *et al.*, 2016). The species is well established in Cameroon, Gabon, Nigeria and São Tomé and Príncipe (Brasier & Griffin, 1979; Nyasse *et al.*, 1999), where it appears to have largely or completely replaced *Phytophthora palmivora* (Mfegue, 2012; Akrofi, 2015; Bailey *et al.*, 2016). From the Nigeria and Cameroon border zone, *Phytophthora megakarya* spread to Togo in 1982, Ghana in 1985 and Côte d'Ivoire in 2003, where it is still in an invasive phase, coexisting with *Phytophthora palmivora* (Dakwa, 1987; Risterucci *et al.*, 2003). Other *Phytophthora* species have been reported to cause cocoa black pod disease, but so far *Phytophthora megakarya*, *Phytophthora palmivora*, *Phytophthora capsici* and *Phytophthora citrophthora* are considered the most important species (Ali *et al.*, 2016; Marelli *et al.*, 2019). In Brazil, *Phytophthora capsici*, *Phytophthora citrophthora*, *Phytophthora heveae* and *Phytophthora palmivora* have been associated with a high incidence of cocoa black pod disease, but these *Phytophthora* species were only identified by morphological comparisons (Luz *et al.*, 2018; Lessa *et al.*, 2020). The symptoms and progression of cocoa black pod disease depend on the cocoa genotype and the *Phytophthora* species involved (Guest, 2007; Puig *et al.*, 2018). Both are also influenced by climatic factors such as relative humidity, temperature and rainfall (Puig *et al.*, 2018). The threat of *Phytophthora megakarya* in cocoa is of great concern to growers and scientists alike, but the processes underlying the emergence of *Phytophthora megakarya* in cocoa are unknown. Therefore, there is an increasing need for basic knowledge on the diversity and epidemiology of *Phytophthora megakarya* in order to develop effective and sustainable control methods (Akrofi, 2015). This is because studying the spatial and temporal dynamics of plant diseases allows us to understand pathogen dispersal processes and improve control recommendations. To achieve this goal in the case of cocoa black pod disease control, it is essential to know the distribution and identification of *Phytophthora* species in all cocoa production zones.

Molecular approaches combined with morphological data have been shown to be necessary to resolve the phytopathogenic species complex and have revealed previously uncharacterised species affecting different crops (Cooke *et al.*, 2000; Blair *et al.*, 2008; Bezuidenhout *et al.*, 2010; Martin *et al.*, 2014). DNA sequence data from phylogenetic studies have also been used to differentiate *Phytophthora* species. Specific regions that have been examined include large and small ribosomal RNA subunits and rDNA ITS regions (Cooke *et al.*, 2000, Crawford *et al.*, 1996; Förster *et al.*, 2000). More recently, advances in molecular characterization by polymerase chain reaction (PCR) and sequencing or restriction digestion of portions of the ribosomal rRNA repeat gene (Cooke *et al.*, 2000, 2001) have allowed more rapid and objective identification of many *Phytophthora* taxa.

Few studies have been carried out in Côte d'Ivoire that includes all stages of population identification of *Phytophthora* sp. The present study therefore aims to update the population structure of *Phytophthora* sp. in cocoa orchards of Côte d'Ivoire.

Materials and methods

Fungal material

The *Phytophthora* sp. isolates studied originate from cocoa plantations in Côte d'Ivoire. They were isolated from cocoa pods naturally affected by black pod disease and obtained following a systematic collection organized in the form of a phytosanitary survey in the main cocoa-growing regions of Côte d'Ivoire.

Collecting pods affected by black pod disease in the cocoa orchard

In order to update the information on the population of *Phytophthora* sp. in the cocoa fields, surveys were carried out in the production regions of Côte d'Ivoire. In each region, 9 plantations were surveyed and 3 pods naturally affected by brown rot were collected from each plot. The harvested pods were stored in plastic bags with details of the harvest (location, date). The harvested pods were taken to the laboratory for *Phytophthora* isolation.

Cultivation of Phytophthora sp. isolates isolation and purification of Phytophthora sp.

Isolations were made on immature pods affected by black pod disease with necrosis in progress. Necrotic parts were washed with 95% alcohol and flamed for 30 seconds. The sample area was selected and the superficial tissue was removed with a sterile scalpel. Three 7 mm cubic fragments were removed from the subcortical tissue at the level of the necrosis growth front using a sterile punch. The fragments were then placed on 1.5% agar water culture medium in 90 mm diameter Petri dishes. Incubation was carried out in the dark in an oven at 26°C for 7 days. After one week of incubation, the isolates obtained were purified by successive subcultures on pea medium (PP) in 90 mm diameter Petri dishes. The isolates were purified by successive subcultures. During purification, isolates were transferred by sampling a fragment of agar containing very fine mycelial filaments from the growth front.

Cloning of Phytophthora sp. isolates by monozoospore isolation

Phytophthora sp. isolates from mass cultures were cloned by monozoospore isolation using the technique of Babacauh (1980) and Ortiz-Garcia (1996). Isolates cultured on pea medium (PP) in Roux flasks were incubated in total darkness at 26°C for 05 days and then exposed to a 12 h photoperiod for 5 days to induce sporocyst formation. To induce zoospore release from mature sporocysts, the cultures were each flooded with 40 ml of sterile distilled water and placed in a refrigerator (4°C) for 15 minutes. They were then returned to the light of an incandescent lamp (60 W) for 45 minutes at room temperature (26°C). The suspension of zoospores thus obtained in Roux flasks was counted using a Malassez haematometer and calibrated at a concentration of 50-60 zoospores/ml by successive dilutions on an aliquot in which the zoospores had been immobilised by two drops of hydrochloric acid. Using a sterile micropipette, 100 µl of the calibrated suspension was applied to 1.5% agar water in 90 mm diameter Petri dishes. Using a bent glass rod, previously flamed and cooled in sterile distilled water, the suspension was spread by successive passages. After incubation for 12 to 24 hours in the dark at 26°C, germinated zoospores were picked individually under a binocular loupe using a sterile lance-shaped micro-needle and inoculated onto carrot agar medium at a rate of 4 clones per isolate and isolate. After 7 days of incubation in the dark at 26°C, the parental clone was selected for further study.

Microscopic features of Phytophthora sp. isolates

Sexual sign

The sexual sign of *Phytophthora* sp. isolates was determined on carrot agar. Two purified isolates of *Phytophthora* sp. were compared in the same Petri dish. One of the isolates came from the survey and the other was a *Phytophthora palmivora* strain called BL7.11.2, sexual type A2, which served as a reference strain. The isolates were separated by 6 cm, depending on the diameter of the Petri dishes. The dishes were then incubated in the dark at 25°C for 10 days. A thallus sample was then taken with a lanceolate needle from the contact zone between the growth fronts of the two thalluses, placed on a microscope slide in a drop of cotton blue, covered with a coverslip and observed under a light microscope at 400x magnification. The observations focused on the presence or absence of oospores in relation to the type of sexual sign.

Molecular characterization of Phytophthora sp. isolates

Isolate culture and DNA extraction

After 7 days of incubation in the dark at 26°C, the thallus of each isolate was harvested with a sterile spatula and lightly dehydrated on absorbent paper. It was then transferred to sterile 2 ml Eppendorf microtubes and stored at 4°C. DNA extraction was performed using the Zymo Research DNA Extraction Kit. 200 g of mycelium was weighed and placed into ZR BashingBead Lysis Tubes to which 750 µl of lysis buffer was added. The mixture was then subjected to two 20-second grinding cycles in a vibratory mill, with a 5-minute interval between each grinding cycle. The ZR BashingBead Lysis Tube was then centrifuged at 10,000 rpm in a microcentrifuge. After centrifugation, 400 µl of the supernatant was transferred to a ZymoSpin IV filter in a 2 ml tube and centrifuged again at 7,000 rpm. 1200 µl of Fungal/Bacterial DNA Binding Buffer was added to the filtrate. Then 800 µl of the mixture obtained was transferred to a Zymo IIC column placed in a tube and centrifuged at 10,000 rpm. The fraction that had passed through the column was removed and the step was repeated. Next, 200 µl of Pre-wash DNA Buffer was added to the Zymo spin IIC column, placed in a new 2 ml tube and centrifuged at 10,000 rpm. The Plant/Seed DNA Wash Buffer was added to the Zymo spin IIC column and the mixture was centrifuged at 10,000 rpm. The Zymo spin IIC column was then transferred to a new 1.5 ml tube and 100 µl of DNA buffer solution was added directly to the column. Finally, the whole was centrifuged at 1000 rpm for 30 seconds to elute the DNA. The DNA extracts obtained were stored at -20°C.

DNA amplification by PCR

PCR amplification of the ITS region of DNA was performed using primers ITS1 and ITS4 as described by White *et al.* (1990). Amplification was performed on a Thermo Scientific Savant DNA 120 thermal cycler. It was carried out in a 10 µl reaction volume consisting of 5.4 µl deionised water, 1 µl 10X PCR buffer, 0.8 µl dNTPs (at 10 mM each), 0.8 µl $MgCl_2$ at 25 mM, 0.2 µl each of ITS1 and ITS4 primers (at 10 µM) and 2 µl DNA. The amplification cycle parameters used were initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, hybridisation at 55°C for 45 seconds and extension at 72°C for 2 minutes. This was followed by an extension at 72°C for 6 minutes. A volume of 2 µl of bromophenol blue was then mixed with 3 µl of DNA solution. This was taken up with a micropipette set to the appropriate volume and added to the wells of the agarose gel flooded with buffer solution (TBE 1X). The tank was then sealed and energized at 100 V for 45 min. The gel, removed from the migration tank, is immersed in BET solution (1 µg/ml) for at least 15 min, then rinsed in distilled water and finally exposed to UV light using an 'Alpha imager' reading system. Fluorescent DNA bands were photographed using a digital system connected to a computer. UVP doc-ItLS® software was used to visualize the fluorescent bands.

Restriction enzyme digestion of the ITS region

Restriction enzyme digestion of the ITS region was performed to highlight the diversity within our isolates by DNA restriction mapping. The PCR products were digested with two restriction enzymes known to identify *Phytophthora* species. These were the restriction enzymes Hae III and Alu I. The restriction enzyme digestion was carried out in a total volume of 15 µl, consisting of 8 µl sterile deionised water, 1.5 µl 10X buffer supplied with the enzymes, 0.5 µl enzyme and 5 µl PCR product. The reaction medium in sterile Eppendorf microtubes was incubated in a water bath at 37°C for 24 hours. The digestion products were separated by electrophoresis on a 1% agarose gel in 1X TBE buffer at 80 V for 3 hours. The gel, removed from the migration tank, was visualized using the same procedure as above. When it was difficult to read the molecular weight of certain fragments directly on the gel, a curve of the migration distance (cm) as a function of the logarithm of the molecular weight of the DNA fragments was drawn using EXCEL software, and an equation of the type $y = ax+b$ was used to estimate the size of fragments of unknown molecular weight. The MESURIM Pro software was used to calculate the migration distance in pixels (10 ft = 26.458 mm).

Results

Microscopic study

Sexual sign of Phytophthora sp. isolates

The study of the sexual sign of the isolates revealed the existence of two sexual types within the population studied. Indeed, isolates placed in *in vitro* confrontation with the *Phytophthora palmivora* strain (BL7.11.2); (Ouattara *et al*., 2022) of sexual type A2 produced, after fertilization, amphigyne or paragyne oospores. These were designated as sexual signs A1. In the other isolates no signs of fertilization were observed and they were designated as sexual type A2. Thus, of the 115 isolates compared, 44 isolates, i.e. 38.26% of the total number, were of sexual sign A1 and therefore related to *Phytophthora megakarya*, while 71, i.e. 61.78%, were of sexual sign A2 and therefore related to *Phytophthora palmivora*.

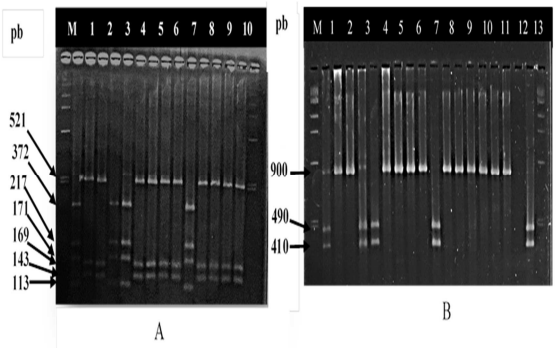

Fig. 1. PCR-RFLP profiles of *Phytophthora* spp., isolates after digestion with Alu I (A) and Hae III (B) restriction enzymes.

Molecular characteristics of Phytophthora sp. isolates

Polymerase chain reaction (PCR) results

The migration results obtained show a polymorphism in restriction fragment length for the two restriction enzymes used in this study (Table 1, Fig. 1).

Table 1. Results of DNA sequence digestion of *Phytophthora* spp., isolates with Alu I and Hae III restriction enzymes

Restriction enzyme	Approximate sizes (bp) of PCR products	PCR-RFLP fragment sizes obtained with restriction enzymes
Alu I	≈ 900	372 ; 217 ; 171 ; 113 521 ; 169 ; 143
Hae III	≈ 900	900 410 ; 490

PCR amplification of the ITS region of *Phytophthora* spp., isolates produced a DNA fragment with an approximate size of 900 bp. This value was confirmed by calculating the sum of the different restriction fragments produced by the restriction enzymes used. The ITS regions of all isolates were digested by the restriction enzymes (Hae III and Alu I), indicating that the ITS regions of the *Phytophthora* spp., isolates studied contain recognition sites for these enzymes. In the restriction profiling analysis, the Alu I endonuclease generated 4 restriction fragments for isolates with sexual sign A1. The size of the generated restriction fragments was estimated to be 372 bp, 217 bp, 171 bp and 113 bp. On the other hand, three restriction fragments were generated for isolates with sexual sign A2. The size of the restriction fragments generated was 521 bp, 169 bp and 143 bp.

The Hae III endonuclease produced 2 restriction fragments for A1 sex-sign isolates with estimated lengths of 410 bp and 490 bp. However, no digestion was observed for A2 isolates, resulting in 900 bp fragments. The restriction profiles generated by the restriction enzymes on the basis of restriction numbers and sizes successfully identified the *Phytophthora* spp., isolates studied. In our study, each restriction enzyme generated profiles that correlated with the sexual characteristics of the isolates studied.

rDNA segment sequencing and phylogenetic analyses

Fifty (50) sequences were obtained from PCR analysis using the ITS1/ITS4 primer pair. These sequences were aligned in the NCBI GeneBank database to confirm their similarity to *Phytophthora* spp. Data in Table 2 indicate that the sequences identified ranged in size from 745 bp (CIV225.31) to 852 bp (CIV225.1; CIV225.7; CIV225.8 and CIV225.8). Of the 50 isolates analyzed, 30 isolates or 60% were identified as *Phytophthora palmivora* with the reference controls MT644188_*P. palmivora*, MT113313_*P. palmivora* and MT052675_*P. palmivora*, compared with 20 isolates or 40% identified as *Phytophthora megakarya* with the reference controls MZ541882_*P. megakarya*, MG865534_*P. megakarya* and KR818149_*P. megakarya*.

Table 2. Description of DNA sequences of *Phytophthora* spp. isolates identified in orchards affected by black pod disease in Côte d'Ivoire

Isolates code	Sequences ID/ GenBank	Species	Sequences size (pb)	% nucleotide identity
CIV225.1	*MZ541882.1*	*P. megakarya*	852	87.52
CIV225.2	*MG865534.1*	*P. megakarya*	818	97.47
CIV225.3	*MG865534.1*	*P. megakarya*	818	97.87
CIV225.4	MT644188.1	*P. palmivora*	786	100
CIV225.5	*MG865534.1*	*P. megakarya*	818	99.74
CIV225.6	MG865534.1	*P. megakarya*	818	100
CIV225.7	*MZ541882.1*	*P. megakarya*	852	100
CIV225.8	*MZ541882.1*	*P. megakarya*	852	99.63
CIV225.9	*MT644188.1*	*P. palmivora*	786	100
CIV225.10	MG865534.1	*P. megakarya*	818	100
CIV225.11	*MT644188.1*	*P. palmivora*	786	100
CIV225.12	*ON711505.1*	*P. palmivora*	814	99.74
CIV225.13	MT644188.1	*P. palmivora*	786	100
CIV225.14	*D:JX198559.1*	*P. palmivora*	814	99.87
CIV225.15	MG865534.1	*P. megakarya*	818	100
CIV225.16	MT644188.1	*P. palmivora*	786	100
CIV225.17	MT113313.1	*P. palmivora*	810	99.63
CIV225.18	MT644188.1	*P. palmivora*	786	100
CIV225.19	MT644188.1	*P. palmivora*	786	100
CIV225.20	MG865533.1	*P. megakarya*	821	100
CIV225.21	MG865534.1	*P. megakarya*	818	100
CIV225.22	MT644188.1	*P. palmivora*	786	100
CIV225.23	MT644188.1	*P. palmivora*	786	99.87
CIV225.24	MT644188.1	*P. palmivora*	786	100
CIV225.25	MZ541882.1	*P. megakarya*	852	99
CIV225.26	MT113313.1	*P. palmivora*	810	99.75
CIV225.27	MT113313.1	*P. palmivora*	810	100
CIV225.28	MG865534.1	*P. megakarya*	818	100
CIV225.29	JX198555.1	*P. palmivora*	766	99,87
CIV225.30	MT644188.1	*P. palmivora*	786	100
CIV225.31	ON711524.1	*Phytophthora* sp./ *(P. megakarya 99,73%)*	745	100
CIV225.32	JX198555.1	*P. palmivora*	766	99,87
CIV225.33	JX198555.1	*P. palmivora*	766	99.34
CIV225.34	MT644188.1	*P. palmivora*	786	99
CIV225.35	ON711529.1	*P. palmivora*	748	100
CIV225.36	JX198555.1	*P. palmivora*	766	100
CIV225.37	MG865534.1	*P. megakarya*	818	99
CIV225.38	MT113313.1	*P. palmivora*	810	100
CIV225.39	JX315262.1	*P. megakarya*	824	100
CIV225.40	MT644188.1	*P. palmivora*	786	99,87
CIV225.41	JX198555.1	*P. palmivora*	747	100
CIV225.42	MT644188.1	*P. palmivora*	786	100
CIV225.43	MT113313.1	*P. palmivora*	810	100
CIV225.44	MG865534.1	*P. megakarya*	818	99,74
CIV225.45	MG865534.1	*P. megakarya*	818	100
CIV225.46	MT644188.1	*P. palmivora*	786	100
CIV225.47	MG865534.1	*P. megakarya*	818	99,60
CIV225.48	MT644188.1	*P. palmivora*	786	99,87
CIV225.49	MG865534.1	*P. megakarya*	818	100
CIV225.50	MT644188.1	*P. palmivora*	786	100
MT644188_*P. palmivora*	MT644188.1	*P. palmivora*	786	100
MT113313_*P. palmivora*	MT113313.1	*P. palmivora*	810	100
MT052675_*P. palmivora*	MT052675.1	*P. palmivora*	880	100
MZ541882_*P. megakarya*	MZ541882.1	*P. megakarya*	852	100
MG865534_*P. megakarya*	MG865534.1	*P. megakarya*	818	100
KR818149_*P. megakarya*	KR818149.1	*P. megakarya*	782	100

The percentage of nucleotide identity with *Phytophthora megakarya* ranged from 87.52% (CIV225.1) to 100% (CIV225.6, CIV225.7, CIV225.10, CIV225.15, CIV225.20, CIV225.21, CIV225.28, CIV225.39, CIV225.45). In contrast, the percentage of nucleotide identity with *Phytophthora palmivora* ranged from 99% (CIV225.25, CIV225.34) to 100% (CIV225.4; CIV225.9; CIV225.11; CIV225. 13; CIV225.16; CIV225.18; CIV225.19; CIV225.22; CIV225.24; CIV225.27; CIV225.30; CIV225.35; CIV225.36; CIV225.38; CIV225.41; CIV225.42; CIV225.43; CIV225.46; CIV225.50). In addition, the 50 sequences were aligned and analyzed using MEGA 11 software to show the genetic diversity among the isolates studied. The dendrogram of Fig. 2 show the phylogenetic relationships between *Phytophthora* spp., isolates collected from natural black pod infected cocoa plantations in Côte d'Ivoire. The dendrogram was also constructed to determine the genetic diversity of the isolates studied. Analysis of the dendrogram revealed two phylogenetic groups within the population of isolates identified. These groups are; group 1, comprising the *Phytophtora palmivora* species, and group 2, represented by the *Phytophtora megakarya* species (Fig. 2).

Fig. 2. Dendrogram showing the phylogenetic relationships between *Phytophthora* sp. isolates collected in cocoa orchards infected by black pod disease in Côte d'Ivoire.

Discussion

Migration results showed polymorphism of restriction fragments for both Alu I and Hae III restriction enzymes. The Alu I endonuclease produced 4 restriction fragments with sizes of 372 bp, 217 bp, 171 bp and 113 bp for A1 sex sign isolates. On the other hand, it produced three restriction fragments with sizes of 521 bp, 169 bp and 143 bp for A2 sex-sign isolates. As for the Hae III endonuclease, it generated 2 restriction fragments of 410 bp and 490 bp for isolates with A1 sexual sign and a fragment with an estimated size of 900 bp for A2 sexual sign. These results corroborate those of various studies on the identification of *Phytophthora* and *Phytium* species (Rafin *et al.*, 1995; Ali *et al.*, 2016; Coulibaly *et al.*, 2018). In fact, according to Appiah (2004), several *Phytophthora* species could be clearly distinguished by PCR amplification of the ITS region followed by restriction analysis with the enzymes HaeIII, HinfI, PvuII and AluI. Sequencing of rRNA ITS regions appears to be the most effective and widely used current method for distinguishing Phytophthora species, especially *P. palmivora* and *P. megakarya*, which are responsible for brown pod rot in cocoa (Appiah, 2004).

Indeed, this method responds to the fact that in *Phytophthora* spp., a length polymorphism is observed between ITS regions. Several studies have successfully applied the PCR-RFLP method of ITS regions in the identification of species of the genus *Phytophthora* (case of the ITS region of *P. capsici* with a length of 219 bp versus 233 bp for *P. fragariae*). Similar results were obtained with the restriction enzymes Alu I, MspI, RsaI and TaqI, which allowed the identification of 31 out of 152 species of the genus *Phytophthora* (Martin and Tooley, 2004). According to their results, the Alu I restriction enzyme allowed the identification of more species in this study, especially *P. megakarya* and *P. palmivora*. Alignment of the ITS DNA sequences of 50 isolates of *Phytophthora* spp., in the NCBI Genbank database identified the species *P. palmivora* and *P. megakarya*. This confirms the presence and emergence of *P. megakarya* in cocoa plantations of Côte d'Ivoire.

The presence of *P. megakarya* species was first reported in Côte d'Ivoire in 2003 (Risterucci *et al.*, 2003). Several studies have shown that *P. megakarya* species are more aggressive. This species tended to replace *P. palmivora* in the cocoa growing areas of Cameroon, Nigeria, Ghana and Côte d'Ivoire (Djiekpor *et al.*, 1982; Dakwa, 1987; Nyassé *et al.*, 1999; Koné, 1999; Risterucci *et al.*, 2003).

According to Opokou (2000), the emergence of *P. megakarya* in cocoa led to an increase in losses due to black pod disease. The appearance of *P. megakarya* in the Ivorian orchard is thought to have originated in Ghana (Appiah *et al.*, 2004), where the species has been well established for several years. Other studies have also shown that the diversity center of *P. megakarya* is Cameroon, and that the species has spread from Cameroon to other Central and West African countries where the largest cocoa producers are located (Nyassé *et al.*, 1999; Mfegue, 2012). The spread of *P. megakarya* species is facilitated by the exchange of infected plant material, contaminated soil and equipment (ten Hoopen *et al.*, 2012; Ndoungué Djeumekop, 2020). Other natural factors such as rain, wind, insects and certain alternative host plants of the pathogen can also facilitate the spread of *P. megakarya* (Ristaino and Gumpertz, 2000; Husson *et al.*, 2006; Themann *et al.*, 2002; Akrofi *et al.*, 2015; Rizali *et al.*, 2017).

Conclusion

Black pod disease has a negative impact on cocoa production in Côte d'Ivoire. The emergence of new *Phytophthora* strains, such as *Phytophthora megakarya*, has led to high disease incidence in the main cocoa producing regions of Côte d'Ivoire. The results of molecular characterization of the DNA sequences of *Phytophthora* isolates collected from cocoa orchards in Côte d'Ivoire confirmed the presence of two species, *Phytophthora palmivora* and *Phytophthora megakarya*. This highlights the need for new control strategies against cocoa black pod disease in Côte d'Ivoire. For example, the doses of fungicides used in phytosanitary treatments must be updated, and climatic data must be regularly monitored to warn farmers of periods of high temperature and humidity, which favour the development of *P. megakarya* and hence the high incidence of black pod disease.

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