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Morphological and Molecular Identification of the Pathogen Associated with Pink Disease of Cocoa (*Theobroma cacao* L.) in the Nawa Region of Côte d'Ivoire

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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

Pink cocoa disease, long considered a minor disease in ivorian cocoa farming, has become a major problem for cocoa production, especially in the Nawa region (Soubre), one of the main cocoa-producing areas in Côte d'Ivoire. The disease is characterized by the appearance of spots on infected branches, with fruiting bodies color white to salmon, and red to brownish lesion under the bark of the attacked trunk or branch. When attacks are severe, they cause cankers, leading to defoliation and death of the tree. In Côte d'Ivoire, no studies have yet been carried out on this disease. This study was therefore carried out in this region in order to identify the pathogen. Thirteen (13) farms in three localities of the region were surveyed, and 03 samples of infected organs were collected from each infected plot. Nine isolates were characterized on the basis of morphological characteristics (mycelial growth, color and size of conidia) and molecular characteristics (PCR-sequencing tests) using universal primers (ITS1 and ITS4). The results revealed morphotypes characterized by striated, radiated, sparse or cottony facies, with an average growth rate of 10.70 ± 3.20 mm/d. Alignment of the ITS rDNA sequences of these isolates against *Erythricium salmonicolor* sequences available in GenBank identified them as *Erythricium salmonicolor*, with a similarity rate ranging from 92 to 100%. In addition, phylogenetic analysis of *E. salmonicolor* strains revealed low genetic diversity. This study therefore provides a basis for further research into the pink disease of cocoa in Côte d'Ivoire.

Keywords: Isolate; Erythricium salmonicolor; caracterisation.

1. INTRODUCTION

With an average annual production of 2,228,422 tonnes in 2021/2022, i.e. around 40% of world supply, Côte d'Ivoire is the world's leading cocoa producer. Cocoa farming covers an area of over 2,000,000 hectares (6% of the national territory) and involves a farming population of over 800,000. Cocoa generates over 30 % of export earnings and contributes over 15 % to Gross Domestic Product. Despite this performance, the sustainability of cocoa cultivation in Côte d'Ivoire is compromised by several abiotic and biotic constraints [1]. Abiotic constraints mainly concern the decline in soil fertility under cocoa due to overexploitation, climate change and the ageing of the orchard [2]. Biotic constraints concern parasitic pressures due to the action of bioaggressors such as viruses, in particular the Swollen-shoot virus. Insect pests such as mirids are the most important for cocoa [3], as are rodents, borers and diseases, one of the most dreaded of which is *Phytophthora* spp. Since the introduction of cocoa (*Theobroma cacao*, L.) to Côte d'Ivoire in the late 19th century, the main fungal disease has been black pod caused by *Phytophthora palmivora* and *P. megakarya* [4]. In addition black pod, the pink disease caused by the fungus *Erythricium* sp, has also been known on cocoa for many years [5-6], but has only been studied as a pathogen of rubber [7-8]. It had already been described in Ceylon in 1873 [9] and the pathogen has been found on many plant species and over 141 genus (Smith, 1985). Host plants of economic importance include rubber, tea, coffee, cocoa, grapefruit, orange, nutmeg, mango and apple. In New Guinea and Malaysia, pink disease is associated with cover crops and shade trees [10-11]. The disease was first reported in Ghana by Wharton [12]. In 1999, the disease proved severe, affecting different cocoa varieties in experimental plots at Bunso in the eastern region of Ghana [13]. During routine surveys to monitor the spread and distribution of the black pod pathogen, *Phythophthora megakarya* and *P. palmivora* on cacao in Côte d'Ivoire, pink disease was found on cacao farms in Soubre department, one of important cocoa-producing areas. However, a better understanding of this disease would open up rapid control possibilities and help to considerably reduce damage in Côte d'Ivoire's cocoa orchards.

2. MATERIALS AND METHODS

2.1 Study Area

Erythricium isolates were obtained from cocoa trees surveyed in 6 plots in the Soubre department of Côte d'Ivoire (Fig. 1). Sampling was carried out during the rainy season in May and June on cocoa trees showing symptoms of pink disease.). Soubre's sub-equatorial climate is characterized by a dry season (December-March) and 2 rainy seasons (April-June and September-November). Average temperatures oscillate between 26°C and 28°C, and can reach

up to 30°C during the dry season. Average rainfall is between 1300 and 1600 mm/year.

2.2 Plant and Fungal Material

The plant material used for this study consisted of cocoa stems showing symptoms of the disease (Fig. 2). The fungal material consisted of 09 isolates of *Erythricium* isolates obtained from samples of cocoa stems showing symptoms of pink cocoa disease collected during a survey of various plots in Soubre department (Table 1).

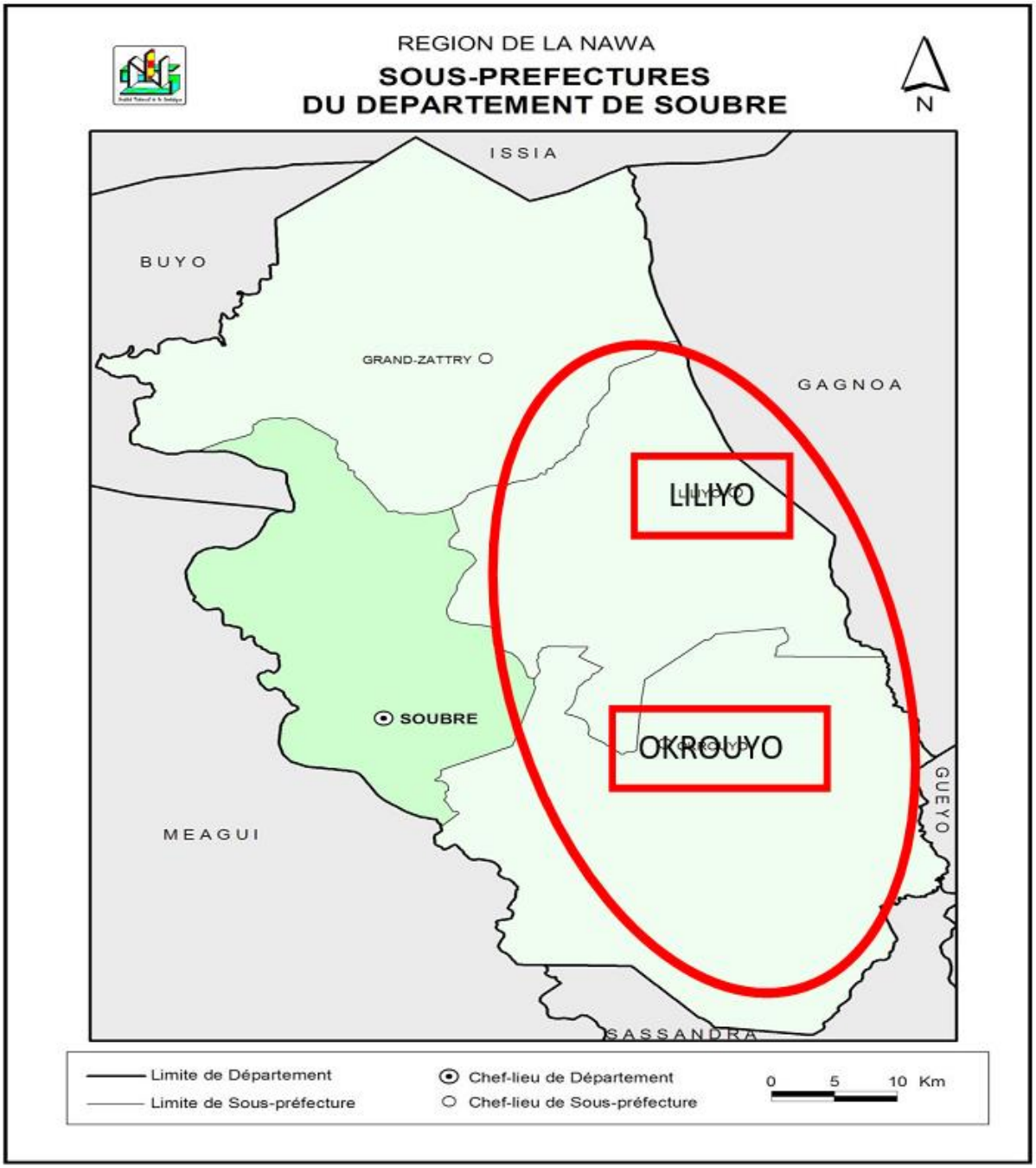

Fig. 1. Map of Soubre department (INS, 2015)

A: White felting on infected branches

B: Brown alteration under the bark of infected trunks

Fig. 2. Symptoms of pink cocoa disease

2.3 Methods

2.3.1 Sample collection and isolation the organism associated to pink disease

Samples of disease-infected branches were taken at random from all cocoa farms where pink disease had been reported. These samples were taken from branches covered with a whitish to salmon-colored mycelial mat, using pruning shears or a machete. The infected stems tissues were then placed in plastic bags bearing the sampling references (location, date, etc.) and returned in a cooler to the laboratory for isolation of the microorganisms. Using a sterile scalpel, fragments of around 0.30 cm^2 were cut at the growth margin of the diseased tissue, sterilized in 95% ethanol for one minute, rinsed three times in sterile distilled water and dried on sterile blotting paper. The sterilized fragments were then placed on 1.5% agar-water culture medium in 90 mm diameter Petri dishes. Incubation took place in an oven at 26°C for 5 days. After incubation, the colonies obtained were purified by two successive subcultures on small pea agar medium. Purification consisted in removing a fragment of mycelium from each colony from the growth front of 1.5% agar water cultures, using a sterile cookie cutter, and transferring it to Petri dishes containing agar pea medium [4].

2.3.2 Morphological characterization of *Eythricium* isolates

The morphological study was carried out on three culture media; Pea-Agar (93 g of pea steamed and 15 g Agar powder), Carrot-Agar (200 g of slice carrot and 15 g Agar powder) and Potato Dextrose Agar (PDA– 4 g of Potato extract, 20 g of Dextrose and 15 g Agar powder). For each isolate, a 5 mm-diameter fragment of mycelium was taken with a cookie cutter at the growth front and deposited in the center of each 90 mm-diameter Petri dish in four (4) replicates for each culture. Cultures were incubated in the dark for 5 days. Morphocultural characteristics of the isolates were determined by mycelial growth, color and texture. Determination of mycelial growth was facilitated by plotting two perpendicular lines on the reverse side of each Petri dish, representing the abscissa and ordinate axes (X and Y) respectively, and passing through the center of the mycelial explant. Mycelial growth was measured daily using a transparent ruler graduated along both axes (X and Y) until each Petri dish was fully occupied. The values thus obtained were translated into mean values for each given culture medium, according to Dianda's formula [14]: $D = (d1 + d2)/2$; where D: average diameter of the isolate in a dish, d1 and d2: measurements of the two perpendicular lines. For microscopic characterization of isolates, size, color, presence or absence of septa and conidial density were assessed after 07 days of incubation. To estimate conidial concentration, the mycelium of each *Erythricium* isolate, aged 07 days on each of three 2% agar culture media, was scraped superficially with a sterile spatula. The resulting mycelial mass was flooded with 10 ml sterile distilled water to detach the conidia. The number of conidia per milliliter of suspension for each

isolate was estimated using a light microscope equipped with a micrometer (GX40) on Malassez cell. The average number of conidia was determined according to the following formula: N = 1 25∑ (ni) x 10^5; with N: average number of conidia per square; ni: number of conidia in the square; 25: number of squares taken into account and 10^5: the factor that reduces the volume of a square (0.01 mm^3) to the milliliter of solution.

Table 1. Geographical origin of *Erytrhicium* sp isolates

Isolate number	Isolate code	Locality
1	AM'4.4.1	LILIYO
2	AM'5.1	LILIYO
3	AM'1.1	LILIYO
4	AM 1.2.1	SOUBRE
5	AM'5.5	LILIYO
6	AM'5.2.1	LILIYO
7	AM 5.2	SOUBRE
8	YA 4.1.1	OKROUYO
9	YA 1.2.2	OKROUYO

2.4 Molecular Characterization of *Erythricium* Isolates

2.4.1 Culture and DNA extraction of *Erythricium* isolates

Erythricium isolates, from which DNA was to be extracted, were seeded on V8 broth (20% V8 juice, 3 g of CaCO3 and 15 g Agar powder) culture medium in 90 mm diameter Petri dishes and stored in an oven at 26°C for 7 days. At the end of this incubation period, the mycelium of each isolate was harvested with a sterile spatula and lightly dehydrated on blotting paper. The resulting harvest (150 mg) was transferred to sterile 2-ml Eppendorf microtubes. For DNA extraction, 150 mg of mycelium from each sample was washed with sterile water and collected by centrifugation at 12,000 rpm for 10 min, then ground in liquid nitrogen using a Geno / Grinder ball mill (Spex Sample Prep) at 1,700 rpm for 45 s. The powdered crushed material was then dispersed in 1 mL extraction buffer previously heated in a water bath at 65°C for 30 min at 10 min intervals. Each tube was mixed by inversion and vortexed for 3 s to prevent aggregation, then incubated in a water bath for 30 min at 65°C. On leaving the water bath, the tubes were vortexed for a further 10 s, then cooled to room temperature. DNA was extracted with 500µl of a 24:1 chloroform-isoamyl alcohol mixture at 4°C, followed by inversion shaking and centrifugation at room temperature at 12,000 rpm for 10 min. The aqueous phase (supernatant) was transferred to new 1.5 mL tubes labelled with the number of each sample. The DNA was then precipitated in 1 mL isopropanol at -4°C and stored at -20°C overnight to separate the DNA from any impurities. The DNA pellet was repripped from the tubes using a pasteur pipette and transferred to new labelled Eppendorfs tubes. The DNA was then washed with 500 µL of 70% ethanol to remove salts and polysacharides. The DNA was then taken up in 100 µL TE [10 mM Tris HCL, 1 mM EDTA (pH 8.0)] and centrifuged at 12,000 rpm for 10 min. Next, 1 µL RNase (10mg/ mL) was added to the mixture to remove all traces of RNA. Finally, DNA was diluted to the tenth (1/10) to optimize PCR. DNA samples were checked for quantity and quality by visualization after electrophoresis on 1% agarose gel in the presence of a 1 kb size marker. The final DNA concentration was reduced to 1 ng/µL.

2.4.2 PCR amplification and DNA sequencing of the ITS region

For molecular identification, PCR amplification of the ITS region of 09 *Erythricium* isolates was carried out using two universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC 3') as described by White [15]. PCR was performed in a GeneAmp PCR System 9700 thermal cycler. In 30 successive cycles, 2 µL of DNA was amplified in 10 µL reaction volume consisting of 5.05 µL of deionized water, 1 µL of PCR buffer, 0.8 µL of DNTP at 5 mM each, 0.3 µL of Mgcl2 at 25 mM, 0.4 µL at 20 pmol each of primers ITS1 and ITS4, 0.05 µL of Taq polymerase at 0.5 units and then a drop of mineral oil (SIGMA) was placed on top of each reaction mixture before the amplification cycle. Thermal cycling conditions for ITS amplification were initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, hybridization at 55°C for 45 s, elongation at 72°C for 2 min and final elongation at 72°C for 6 min. Successful amplification was verified by electrophoresis on 1.5% (w/v) agarose gel containing 0.5 mg/mL ethidium bromide and visualized on a computer screen connected to a Molecular Image ChimiDoc XRS+ biordad. PCR products were then sent to the Eurofins MWG Operon DNA Sequencing Department (Germany), where they were sequenced in both directions using primers ITS1 and ITS4.

2.5 Phylogenetic Analysis

Phylogenetic analysis was carried out to confirm the molecular identification of *Erythricium* isolates based on PCR and ITS sequences. The taxonomic position of the sequenced *Erythricium* isolates was determined by comparing the sequences obtained with those already listed in the gene bank of the National Center for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi), using the "BLAST search" option. The server's taxonomic browser was used to find the affiliation of the *Erythricium* isolates. Similarity rates were then determined between the sequences studied and the closest GenBank sequences. Two of these sequences are considered similar only when they have homology percentage greater than 96 %. Sequences were aligned using MEGA 11 software. Alignments were carefully checked and corrected manually. The resulting matrix gave the complete and exact consensus sequence for each isolate, which was then manually checked and optimized to obtain the best alignment. The resulting sequences were compared to establish the level of genetic diversity between them. The sequences were then deposited with GenBank under access numbers KR818094 to KR818212.

2.6 Statistical Analysis

STATISTICA software version 7.1 was used for statistical analysis. To assess disease incidence, analysis of variance was used to compare the average rate of pods affected by black rot. Two-factors analysis of variance (isolate and culture medium) were used to compare *Erythricium* isolates according to their morphological (morpho-cultural and microscopic) characteristics. The analysis focused on mycelial growth, conidial size and conidial concentration of the isolates. The ANOVA test (Newman-Keuls) was used to determine different homogeneity groups at the 5 % probability threshold.

3. RESULTS

3.1 Macroscopic characteristics of *Erythricium* isolates

The morpho-cultural study of the various isolates on the three media (Pea-Agar medium, Potato Dextrose Agar (PDA) medium and Carrot-Agar (CA) medium) revealed three morphotypes illustrated in Fig. 3. These morphotypes were characterized by a striated and radiated, sparse or cottony facies with a regular and uniform growth front. Generally speaking, the daily growth rate of the isolates varied from 5.52 to 16.57 mm/day, with an average of 10.70 ± 3.20 mm/d (Fig. 4). Analysis of variance for growth rates showed a significant difference between *Erythricium* isolates. However, no significant difference was observed between culture media for mean daily mycelial growth rates of *Erythricium* isolates. Thus, whatever the culture medium, the highest average daily mycelial growth was observed in isolate YA 1.2 (16.57 mm/d) compared with isolate AM'5.5, which showed the lowest mycelial growth (5.52 mm/d).

3.2 Microscopic Characteristics of *Erythricium* Isolates

Microscopic characteristics of *Erythricium* isolates were described on all three media (PA, PDA and CA). The microscopic characteristics of the isolates revealed, for all isolates, a hyaline-colored, thick, septate mycelium with intertwined branching at the thin ends (Fig. 5). Conidia were oval in shape and hyaline in color (Fig. 6). Fig. 7 shows the average conidial densities of

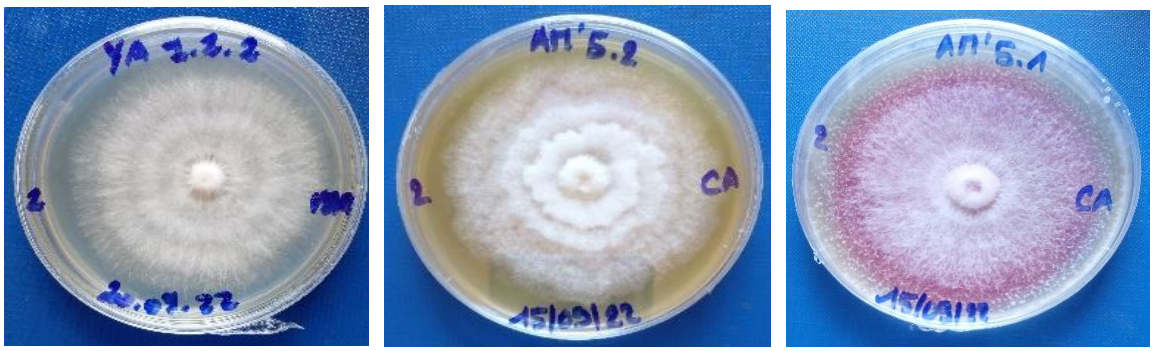

Fig. 3. Mycelial growth of *Erythricium* sp isolates on PA, CA, PDA media

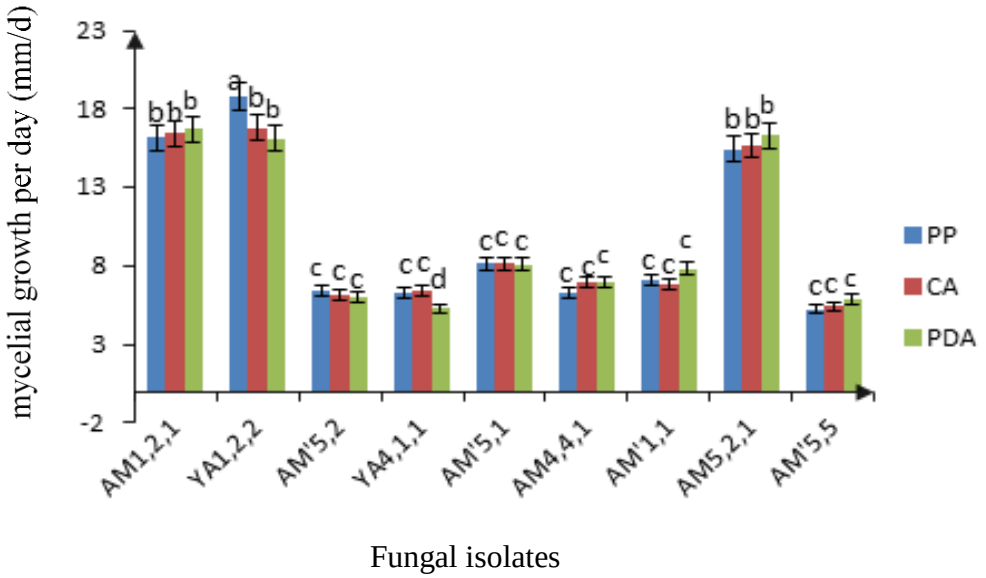

Fig. 4. Average growth rate of *Erythricium* isolates on PP, CA, PDA media. Bands followed by the same letter are not significantly different according to the Newman-Keuls test at the 5% significance level

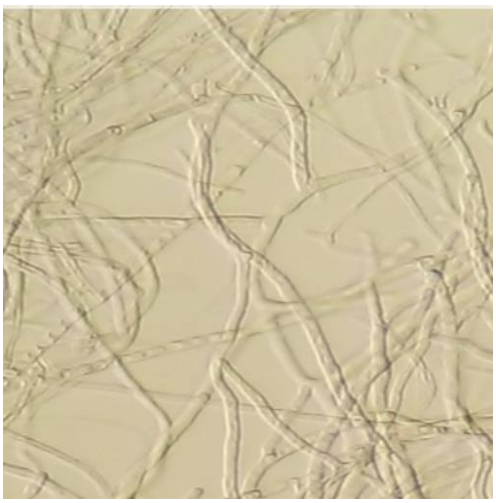

Fig. 5. Microscopic aspects of mycelium (G X 40)

Fig. 6. Microscopic aspects of conidia (G X 40)

Erythricium isolates. Analysis of Fig. 7 indicated a variation in conidial concentrations of isolates ranging from 0.2. 10^5 conidia/ml to 27.8. 10^5 conidia/ml depending on the culture medium. Average conidial densities varied from 0.2. 10^5 to 27.8. 10^5 conidia/ml on Pea Agar (PA) medium, from 0.2. 10^5 to 9 conidia/ml on Potato Dextrose Agar (PDA) medium and from 0.2. 10^5 to 19.8. 10^5 conidia/ml on (CA) medium. Analysis of variance showed a significant difference between isolates and culture media (P <0,001), reflecting an interaction between culture medium and conidial concentration. Thus, the highest average conidial concentration (4.73.10^5 conidia /ml) was observed on Pea-Agar medium (PA), while the lowest average conidial concentration (1.95. 10^5 conidia /ml) was obtained on PDA medium. Furthermore, isolate YA 4.1.1 showed the highest mean conidial concentration (10.46 conidia /ml), while the lowest conidial concentration was obtained with isolate YA 1.2.2 (0.33. 10^5 conidia / ml).

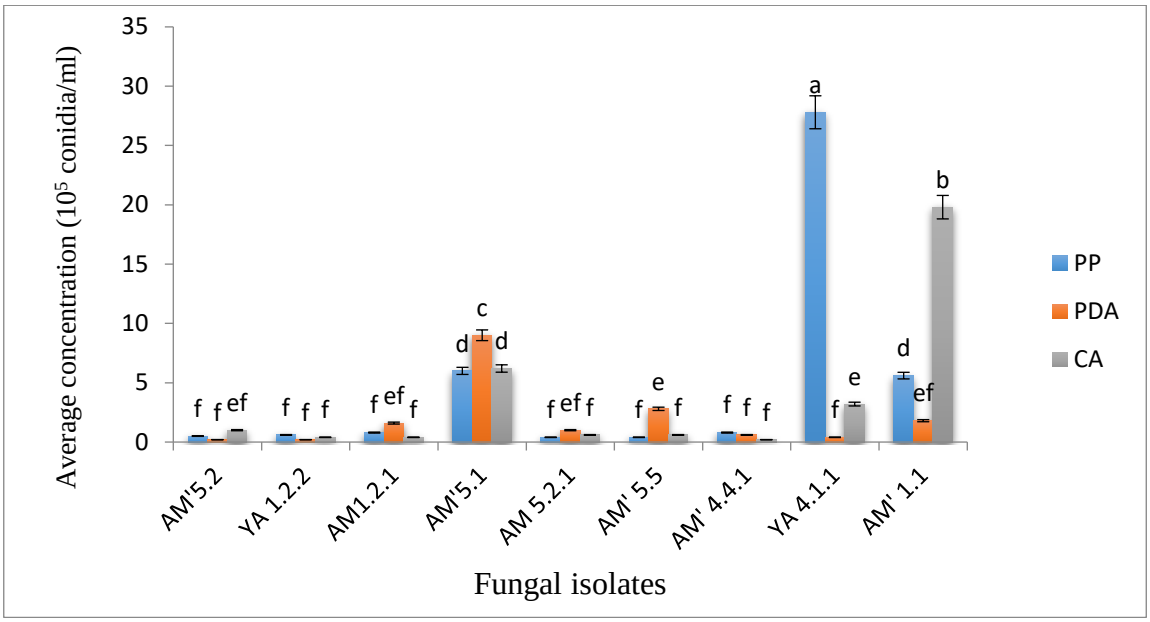

Fig. 7. Conidial density of *Erythricium* sp isolates

Bands followed by the same letter are not significantly different according to the Newman-Keuls test at the 5% significance level.

Amplification of the ITS regions of all fungal isolates generated a 750 bp fragment, characteristic of *E. salmonicolor* (Fig. 8). Alignment results of the ITS rDNA sequences of these isolates showed 92-100% similarity with the ITS rDNA sequences of various *E. salmonicolor* isolates. By analyzing the results of the ncbi server's taxonomic browser and using the "blast search" option, it was possible to find the affiliation of the *Erythricium* isolates by comparison with the closest *Erythricium salmonicolor* sequences available in GenBank. The sequences most similar to these isolates were identified as *Erythricium salmonicolor*, with similarity rates ranging from 92% to 100%. Phylogenetic analysis by rDNA sequencing revealed genetic variation among *Erythricium salmonicolor* isolates, revealing 3 genetic groups (Fig. 9). The isolates (okro1 and okro2) representing group 2 and from Okrouyo sub-prefecture are more than 99% similar. Only the Soub isolate represented group 3, with a similarity rate of 99.73% compared with isolates from groups 1 and 2 (Table 2).

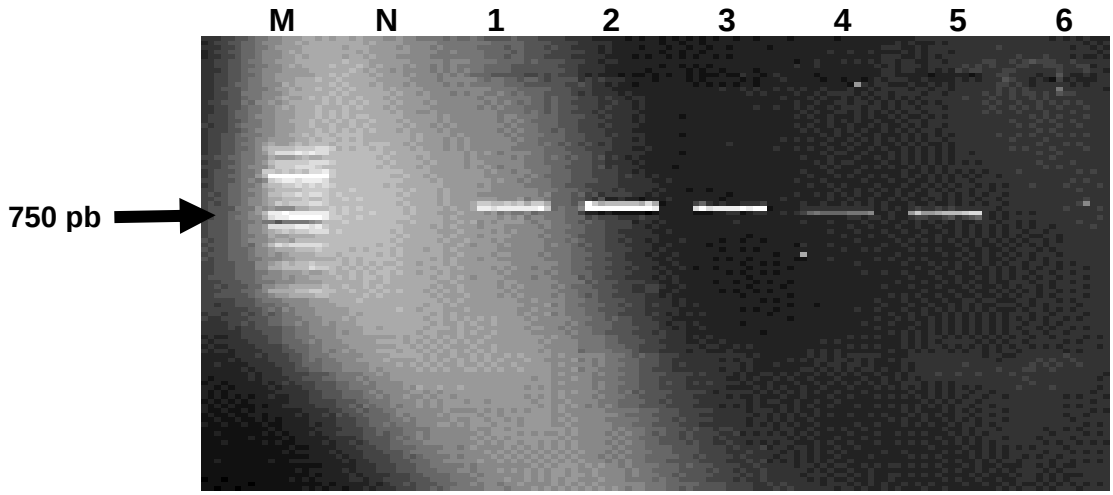

Fig. 8. DNA extracts from *Erythricium* sp amplified with primers ITS1 and ITS4
M: Ladder; N: Sterile water; Lanes 1-6: DNA extracts of Erythricium isolates

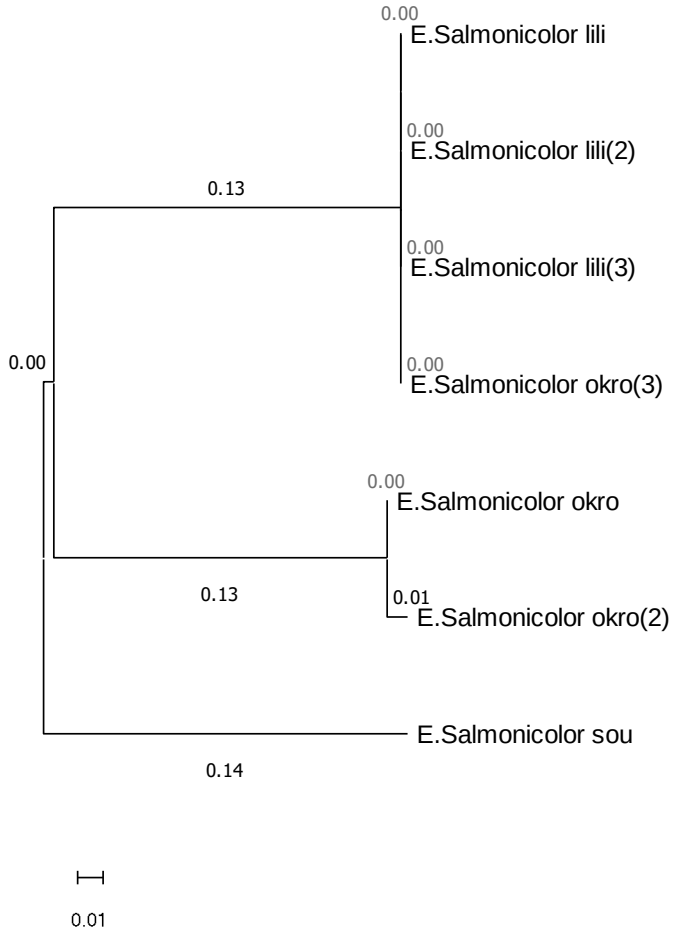

Fig. 9. Phylogenetic tree, illustrating the relationship between isolates of *Erythricium*

Table 2. Sequence by pair similarity rates for *Erythricium salmonicolor* isolates

	E.Salmonicolor_lili_1	**E.Salmonicolor_lili_2**	**E.Salmonicolor_lili_3**	**E.Salmonicolor_okro_1**	**E.Salmonicolor_okro_2**	**E.Salmonicolor_okro_3**	**E.Salmonicolor_sou**
E.Salmonicolor_lili_1	-						
E.Salmonicolor_lili_2	100	-					
E.Salmonicolor_lili_3	100	100	-				
E.Salmonicolor_okro_1	99,74	99,74	99,74	-			
E.Salmonicolor_okro_2	99,72	99,72	99,72	100	-		
E.Salmonicolor_okro_3	100	100	100	99,74	99,73	-	
E.Salmonicolor_sou	99,73	99,73	99,73	99,74	99,73	99,73	-

4. DISCUSSION

Morphology is one of the first steps in characterizing *Erythricium* fungus. The results obtained showed variability between isolates and culture media. The Newman-Keuls multiple comparison test ($P<0.05$), applied to the three culture media (PA, PDA, CA), enabled us to identify different homogeneous groups of *Erythricium* isolates. Indeed, the isolates studied showed different morphocultural facies according to the culture media, with the exception of isolates YA 1.2.2; AM' 4.4.1, each of which showed a homogeneous group on PA, PDA and CA media of average spore concentration. This could be explained by the adaptation of these isolates to the physico-chemical conditions of the medium. These results are in line with those of Fernanda et al. [16], who studied the genetic variability and vegetative compatibility of *Erythricium* isolates. According to these authors, the high genetic variability and coexistence of different fungal strains genotypically from the same region may predict great difficulty in controlling this disease. The morphotypes of the isolates studied were characterized by a striated and radiated, sparse or cottony facies with a regular, uniform growth front. All isolates also showed spider-web-like mycelial growth. Similar results were obtained by Old et al. [17], who concluded that the spider web stage was one of the specific symptomatic stages of this disease, as also observed in the field. Also, in terms of mycelial growth and conidial density of *Erythricium* isolates, the PDA medium recorded the lowest mycelial growth and spore density compared with the other three culture media. These results are linked to the nutrient sources (carbohydrates, amino acids and vitamins) used in the PDA medium, which could have an effect on mycelial growth and conidial density of *Erythricium* isolates. These results are in line with those of a previous study by Akrofi, Rosa and Ishmael Amoako-Attah et al. [18-19-20], who reported that sporulation was not observed in colony growth on PDA. For these authors, the absence of sporulation was linked to the nutrients used to varying degrees. Their study showed that pink disease isolates with better mycelial growth and conidial density made better use of polysaccharides such as disaccharides and monosaccharides, aspartic acid and folic acid. In order to make the morphological characteristics, often considered by some authors to be unreliable because they may overlap with those of other species in the genus, more robust molecular tests were needed. Thus, ITS rDNA sequencing carried out on isolates following PCR with the universal primers ITS1 and ITS4 confirmed the identity of this species of fungus associated with pink disease in this cocoa-growing region with a similarity score of over 97%, compared with the ITS rDNA sequences of various strains of *Erythricium salmonicolor* in the ncbi gene bank. Similar studies have contributed to the characterization of this pathogen as responsible for pink disease in Ghana and Brazil [21-22]. The resulting dendrogram showed low genetic variability between isolates, grouping them into three groups with genetic similarity ranging from 99 to 100%. The high degree of similarity between isolates suggests a lack of specificity between *Erythricium salmonicolor* isolates and their localities of origin. A similar study by authors [23-24] described low genetic variability between *Erythricium salmonicolor* strains in the same region. According to Colauto et al. [25], such a similarity coefficient is likely to indicate common source of origin.

5. CONCLUSION

The aim of the present study was to identify the pathogen associated with the pink cocoa disease-like symptoms observed in the Soubré department. Analysis of the results, based on the morphological and molecular characteristics of the isolates, revealed that the species *Erythricium salmonicolor* was identified as the pathogen responsible for the symptoms associated with pink disease. Phylogenetic analysis of the *Erythricium salmonicolor* strains revealed low genetic diversity, suggesting the possibility of rapid control of the disease. This discovery therefore represents a serious threat to cocoa production in the area. However, further work is needed to determine its incidence and distribution throughout the cocoa orchard.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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