Fruit, Vegetable and Cereal Science and Biotechnology ©2010 Global Science Books

Occurrence of *Pythium aphanidermatum* Root and Collar Rot of Papaya (*Carica papaya* L.) in Côte d'Ivoire

Clovis N. B. Koffi[1] • Hortense A. Diallo[1*] • Justin Y. Kouadio[1] • Paula Kelly[2] • Alan G. Buddie[2] • Lukasz M. Tymo[2]

[1] Université d'Abobo-Adjamé, UFR-SN, Laboratoire de Biologie et Amélioration des Productions Végétales, 02 BP 801 Abidjan 02, Côte d'Ivoire
[2] CABI Europe-UK, Bakeham Lane, Egham, Surrey, TW20 9TY, United Kingdom

Corresponding author: * attakyhortense@yahoo.com

ABSTRACT

In June 2007, a new disease causing significant losses was observed in papaya orchards in Côte d'Ivoire. The disease was characterized by root rot and/or collar rot leading to complete wilt of the plant followed by its death. Although fungi such as *Fusarium* and *Rhizoctonia* were isolated from diseased plants, pathogenicity tests showed that *Pythium aphanidermatum* was the causal agent of the disease. Disease incidence of 68.25% was observed when *P. aphanidermatum* was inoculated alone in pathogenicity testing. However, on untreated soil coming from the field, an incidence of 42.85% was obtained. A representative strain (subsequently designated IMI396181) re-isolated from infected plant material (from the pathogenicity testing) was identified by sequence analysis of the internal transcribed spacer (ITS) regions of the ribosomal RNA gene cluster (GenBank Accession No. EU861392) confirmed the taxonomy of the pathogen. To our knowledge, this is the first report of *P. aphanidermatum* infecting papaya in Côte d'Ivoire.

Keywords: ITS sequences, molecular characterisation, pathogenicity test

INTRODUCTION

Papaya (*Carica papaya* L.) is a plant with multiple uses. The fruit is rich in proteins and vitamins A and C (SPC 1980). Papain produced in the fruit has many industrial applications, including the manufacture of cosmetics, the degumming of natural silk and food-based applications involving the clarification of beer and the tenderizing of meat products (Ko 1982). Papaya leaves and seeds are also used in traditional medicine for the treatment of boils, warts and freckles (Teixeira da Silva *et al*. 2007). In Côte d'Ivoire, papaya is the third most commonly exported fruit after banana and pineapple. Between 200 to 300 tons are produced for the export market per year (Lanuzel 2004). Recently, however, production has fallen in comparison to fruit export volumes in previous years. This setback is directly linked with the devastating effect of *Papaya ringspot virus* (PRSV) which is one of the major biotic constraints (Diallo *et al.* 2008), in addition to mite attacks and other diseases.

In June 2007, there was a serious outbreak of a disease in Côte d'Ivoire. The disease was characterized by a yellowing of the leaves followed by wilting of the plants. Then, the entire plant collapsed. Collar rot and/or root rots were associated with the diseased plants. Similar infections have been described in other regions. Root rots of papaya due to *Phytophthora nicotianae* van Breda de Haan, *Phytophthora palmivora* (Butler) Butler and *Pythium aphanidermatum* (Edson.) Fitz Pat., have been reported as the most important cause of root rot of papaya in Hawaii (Parris 1941; Trujillo and Hine 1965; Ko 1971), Costa Rica (Mora and Morales 1980) and Mexico (Guadalupe 1981). The present work focuses on the morphological and molecular characterization of the causal agent and the disease incidence.

MATERIALS AND METHODS

Symptom observation and field diagnosis

Field diagnosis of the disease was based on visual observation of the symptoms on papaya plants in an orchard located in Toumodi. Observations were made on leaves, stems and roots. Symptoms observed were described and the infected plant parts were collected for laboratory work. The distribution of the disease plants in the field was also noted.

Plant materials and seed source

The plant material used in this study included the papaya variety 'Golden'. Infected plant parts (roots and collar) of this variety were collected from the field for isolation of the potential pathogenic agents. For the pathogenicity tests, three-week old seedlings were used. Seeds of variety 'Golden' obtained from a commercial field were sown in autoclaved plantation soil contained in 11 cm diameter pots; four seeds per pot.

Fungal isolation and identification

The collar and roots of diseased plants previously washed with tap water were cut into small pieces and disinfected for 1 min with 8 °Chlorometric (Gay-Lussac chlorometric degree) bleach (containing 25.36 g of active chlorine per liter) diluted at 10%. Acidified potato dextrose agar (PDA) was used for plating infected plant pieces. The PDA medium was prepared by boiling 200 g of peeled potato for 30 min in 1 L of water. After filtration through layers of cheesecloth, 20 g of glucose and 20 of agar were added. Water was added to make 1 L of solution which was then autoclaved at a pressure of 1 bar for 30 min. After cooling, 1 g of citric acid was added. The plates were incubated for 7 days, in the dark, at temperatures varying between 27 and 29°C. The isolation frequency of each fungus was calculated. For each fungus observed, pure cultures were obtained on PDA. A portion of a three-day old culture of each fungus isolate was placed in the centre of the Petri dish containing PDA. At the bottom of the plate, two perpendicu-

Received: 19 September, 2009. Accepted: 2 March, 2010.

Original Research Paper

lar lines passing through the centre were previously drawn. Radial growth was measured until the plates were totally covered. The cultures were grown for 8 days to allow for spore formation. An average of 100 characteristic spores was measured. The identification keys used were those of Lanier *et al*. (1969), Ravissé and Bocass (1978), Barnett and Barry (1972).

Inoculation technique

The inoculum used for the infection of the seedlings was prepared for each fungus isolate using the sand mixed with corn flour technique of Haware and Nene (1982). A small piece of each fungus growing on PDA was transferred to the CMS (corn meal sand) medium in 9 cm Petri dishes. The fungal cultures were incubated for 14 days in the dark, at the temperature of 28 ± 1°C.

After the incubation period, the CMS medium covered with each fungus mycelium was transferred unto sterilized soil in 11 cm diameter pots. One quarter of the Petri dish content was used for each pot. The pots were incubated for 10 days before transplantation of the 20-day old papaya seedlings.

Pathogenicity testing

The pathogenicity tests were carried out with two separate fungal taxa. The choice of fungus was made according to two criteria: (i) the abundance (number of time isolated from the infected plant pieces plated), (ii) data gathered from the literature regarding fungi previously associated with the observed symptoms. As a result, three isolates representing two genera of fungus were used for the test: *Pythium* and *Fusarium.* The treatments were as follows:

T0: Sterilized plantation soil mixed with CMS medium without any fungus;
T1: Non-sterilized plantation soil mixed with CMS medium without any fungus;
T2: Sterilized plantation soil inoculated with *Pythium* sp.;
T3: Sterilized plantation soil inoculated with *Fusarium solani*;
T4: Sterilized plantation soil inoculated with '*Fusarium* sp.1.'

The plantation soil was sterilized twice at 121 °C in an autoclave at a pressure of 1 bar for 30 min. Twenty papaya seedlings (2 per pots) were used for each treatment. The experiment was repeated three times.

Disease development was followed. Symptom evaluation was carried out four weeks after transplanting of seedlings. The 0 to 3 rating scale of the symptoms proposed by Vakalounakis and Fragkiadakis (1999) was used. Disease incidence (%) was calculated using the formula proposed by Song *et al*. (2004): (∑Values × Number of infected seedlings) × 100 / (Highest value × total number of the seedlings).

Confirmatory tests

After the inoculation tests, fungi were re-isolated from the infected plants. These fungi were compared under the microscope with the fungi used for the initial inoculation.

Molecular characterization to confirm fungi taxonomy

One isolate of the fungi, identified on the basis of morphological characteristics (Vander Plaats-Niterink 1981), was molecularly characterized. The characterization was carried out at the Global Plant Clinic (GPC) and the Microbial Identification Service at CABI, UK.

DNA extraction

Pythium cultures already on PDA were subcultured onto malt agar distilled water (MADW) plates and incubated at 25°C for 3-5 days in order to assess purity. Total genomic DNA was obtained using a complex DNA release solution (microLYSIS®-PLUS, Microzone Ltd., UK) in accordance with the manufacturer's instructions with a modification in the first step of the Thermal Cycler lysis profile (30 min at 65°C, 2 min at 96°C, 4 min at 65°C, 1 min at 96°C, 1 min at 65°C, 30 s at 96°C, hold at 20°C).

Polymerase chain reaction (PCR) and sequencing of rDNA regions

Partial ribosomal RNA gene cluster (part of 18S small subunit RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, part of 28S large subunit ribosomal RNA gene) was amplified by PCR, using primers ITS6 (5′-GAA GGT GAA GTC GTA ACA AGG-3′; Cooke and Duncan 1997) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′; White *et al*. 1990). PCR was carried out in a final volume of 20 µL containing 3 ρmoles of each primer, 1 µL of the DNA template, (8 µL of ultra pure water) and 10 µL of MegaMix~Royal (Microzone Ltd., UK) containing optimised mixture of *Taq* polymerase, anti-*Taq* polymerase monoclonal antibodies in 2X Reaction Buffer (6 mM $MgCl_2$) with 400 µM dNTPs, Stabiliser and Blue loading dye. Amplification conditions were: an initial denaturation step of 95°C for 5 min followed by 30 cycles of 30 s at 95°C, 30 s at 52°C, 45 s at 72°C, followed by 10 min at 72°C. PCR products were assessed for quality by 1.5% (w/v) agarose gel electrophoresis at 5 Vcm^{-1} in 0.5X TBE buffer (45 mM Tris; 45 mM boric acid; 1.25 mM EDTA, pH 7.5) containing 5 µL of SafeView Nucleic Acid Stain (NBS Biologicals Ltd., UK) per 100 ml buffer. UV gel image was then captured by using the UVP ImageStore 5000 (Ultra-Violet Products Ltd., Cambridge, UK). PCR products were purified with the microCLEAN PCR Purification Kit (Microzone Ltd., UK) following the manufacturer's instructions. The purified PCR products were sequenced by using Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, UK) using 10 ρmoles of primer ITS4 (as above). Sequencing conditions were: an initial denaturation step of 96°C for 1 min followed by 25 cycles of 20 s at 96°C, 10 s at 50°C, 4 min at 60°C (rate 1°C/s). Excess unincorporated dye was removed with DyeEx 2.0 (Qiagen Ltd., UK) resin spin columns, according to the manufacturer's instructions, and the sequencing reaction products were suspended in HiDi™ Formamide (Applied Biosystems, UK). The resultant samples were separated on a capillary array sequencer 3130 Genetic Analyzer (Applied Biosystems, UK).

DNA analysis

Sequence trace files were initially examined using the Sequencing Analysis Software v5.2 Patch 2 (Applied Biosystems, UK), assessed for quality using the software package Chromas v2.23 (Technelysium, Australia) and exported as text files. The sequence of the fragment obtained from IMI396181) was screened against the holdings of GenBank and EMBL using the BLAST (Altschul *et al*. 1990, 1997) and FASTA (Pearson and Lipman 1988) algorithms. Following successful identification the sequence from IMI396181 was submitted to GenBank and given the GenBank Accession N°: EU861392.

Statistical analysis

A test of comparison of two proportions by Walpole and Myers (1978) was carried out at $\alpha = 0.05$ using the formula:

$$Z = (p_1-p_2)/\sqrt{(pq[(1/n_1)+(1/n_2)])}$$

p_1 (proportion) $= x_1/n_1$,

p_2 (proportion) $= x_2/n_2$ and

$p = (x_1+x_2) / (n_1+n_2)$.

where n_2: sample size, n_2: sample size, x_2: number of successes, x_2: number of successes.

RESULTS

Description of the disease symptoms in the field

During the rainy season of June 2007, severe attacks characterized by soft rot at the collar and on the roots of papaya trees were observed in a commercial orchard. The diseased trees were randomly distributed in the field. The incidence

Table 1 Fungi isolated and their corresponding proportion of propagules.

Fungus	Proportion of total propagules recovered (%)
Fusarium solani	40
F. sp. 1	8
F. sp. 2	5
F. chlamydosporum	2
Rhizoctonia sp.	20
Pythium sp.	10
Others	5

Fig. 1 Symptoms observed on papaya plants infected with *P. aphanidermatum* and *Fusarium solani* four weeks after inoculation. (**A**) Uninoculated controls (left), wilted plants inoculated with *P. aphanidermatum* (right). (**B**) Plants inoculated with *F. solani* (left), wilted plants on untreated plantation soil (right). (**C**) Stunted seedlings on untreated plantation soil (left) and on soil inoculated with *P. aphanidermatum* (right). (**D**) Mycelium of *P. aphanidermatum* around crown of papaya seedling.

of the disease was estimated at between 10 and 15% in the infested zones. The disease symptoms were observed on the seedlings in the nursery and also on the trees in production. The pathogenic agent causing root and collar rot of the papaya tree in Côte d'Ivoire, infected the plants at all stages of development.

Inventory of fungi associated with root and collar rot

An inventory of the fungi associated with root and collar rot of papaya gave a total of eight isolates. According to the morphological characteristics of the spores, these isolates were classified into four groups (**Table 1**). The isolates whose microscopic characteristics reveal a hyaline mycelium, segmented and ramified with hyaline septate conidia, curved and tapered at the ends, constituted the first group. These features corresponded to the genus *Fusarium* (Link ex Gray). The second group showed features common to members of the genus *Rhizoctonia* (de Candolle) and were characterized by dark mycelium, septate with right angled ramifications and no spores. Further observations highlighted fungi with non-septate mycelium and having many oospores. The conidiophores were limited by the formation of the spore. These fungi resembled members of the *Pythiacae* family. The last group called "other fungi" represented fungi whose classification was not possible using the available identification keys. They were characterized by segmented mycelium, ramified without conidia (**Table 1**). In the *Fusarium* group, four species were observed. They were: *Fusarium solani*, *Fusarium chlamydosporum* and two, incompletely characterised taxa herein referred to as '*Fusarium* sp. 1' and '*Fusarium* sp. 2'. Fungi belonging to the genus *Fusarium* were the most abundant, representing 55% of all fungi isolates obtained. *F.* solani represented 72% of the total *Fusarium* strains isolated. Only a single representative of each of the genera, *Pythium* and *Rhizoctonia*, was isolated. However, *Rhizoctonia* was more abundant than *Pythium*. Finally, unidentified fungi accounted for 5% of the total propagules recovered.

Table 2 Effect of different soil treatments on papaya seedlings development.

State of the plants	Soil treatment[x]				
	T0	T1	T2	T3	T4[y]
Dead	0	3	9	0	0
Wilted	0	8	7	0	0
Apparently healthy	20	9	4	20	20
Disease incidence (%)[z]	0 c	45 b	71.25 a	0 c	0 c

[x]Twenty plants were inoculated for each treatment and evaluated four weeks later.
[y]T0: Sterile plantation soil added to the CMS medium without any fungus; T1: Non sterilized plantation soil and the CMS medium without any fungus; T2: Sterilized plantation soil inoculated with *Pythium*; T3: Sterilized plantation soil inoculated with *Fusarium solani*; T4: Sterilized plantation soil inoculated with *Fusarium* sp.1
[z]Numbers followed by the same letters on the same line are not significantly different according to Walpole and Myers (α = 0.05).

Results of pathogenicity testing

1. Effect of the inoculation with the different fungi on the above-ground plant parts

Various reactions were recorded on young papaya seedlings four weeks after the inoculation tests with each of the three different fungi (*Pythium*, *Fusarium solani* and '*Fusarium* sp. 1'). Depending on the treatments used, seedlings either displayed some symptoms or remained apparently healthy. The seedlings submitted to treatments T3, T4 and the negative control (T0) did not present any of the symptoms observed in the papaya plantation. With these seedlings, growth was normal. On the contrary, with treatments T1 (naturally infested plantation soil) and T2 (sterilized soil inoculated with *Pythium*), wilted plants were observed.

Symptoms were observed on the infected seedlings 4 to 7 days after transplanting. The leaves were slightly yellow, then the plant became completely wilted a few days later (**Figs. 1-3**). These plants were also stunted. On occasion, cottony mycelium appeared on stem at the collar level of the infected plant (**Fig. 4**). All the wilted plants generally died two to three days later. During this phase, root and collar rots were observed on the plants (**Fig. 2**).

Disease incidence reached 71.25 and 45% on soil artificially inoculated with *Pythium* (T2) and on untreated plantation soil (T1), respectively. The statistical analysis indicated a significant difference in disease incidence between treatments T1 and T2. No significant difference was observed between these treatments concerning the number of wilted plants. As for the number of dead plants, this was significantly higher with treatment T2 than with treatment T1 (**Table 2**).

2. Effect of the inoculations with different fungi on the root system

Observation of the roots during the infection process showed partial or total destruction of the root system of the infected seedlings. These symptoms were characterized by a soft rot of the plant part attacked. The infection started with attacks of the secondary roots. At first, the infection sites were lightly brown. Then, as the infection progressed, the rot covered all the secondary roots. The primary root became infected simultaneously with, or after, the rot of the secondary roots (**Fig. 2**).

Fig. 2 Collar and roots systems of papaya seedlings grown in soil infested naturally or artificially with different fungi. (**A**) crown rot of papaya caused by *P. aphanidermatum*. (**B**) Papaya root rot caused by *P. aphanidermatum* (left) and healthy roots of uninoculated control (right). (**C**) Papaya root rot caused by *P. aphanidermatum* (left) and healthy roots from soil inoculated with *Fusarium* species (right). (**D**) Papaya root rot caused by *P. aphanidermatum* after artificial inoculation (left) and root rot caused on non sterilised plantation soil (right).

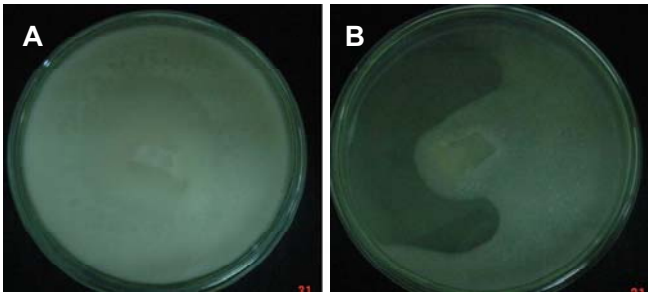

Fig. 3 Variation of *P. aphanidermatum* thallus at different periods. (**A**) Aerial and cottony mycelium three to four days after culturing on PDA. (**B**) Subsidence of aerial thallus after two to three weeks.

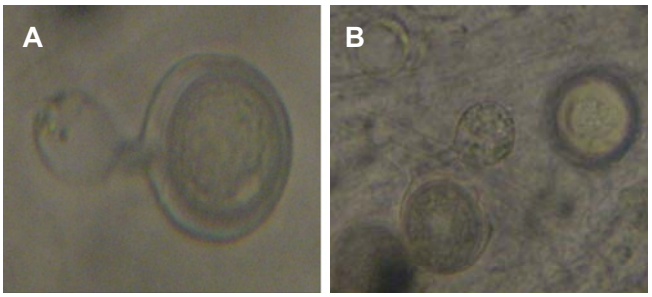

Fig. 4 Spores of *P. aphanidermatum*. (**A**) Germinating oospore. (**B**) Sporangium and oospore (× 400).

Disease development after inoculation with *Pythium*

The symptoms began with a soft watery lesion at the soil line of the papaya seedlings, at the collar level. This change appeared one week after inoculation for some seedlings and two to three weeks for others. The collar then turned slightly brownish and shiny. The leaves at this time were not very much affected; they turned lightly yellow later. After this stage, the thinning and browning of the collar increased. All the leaves turned yellow and the whole plants wilted, ultimately collapsing and dying. At root level, the disease began with browning at the tips of the primary and secondary roots. This rot progressed quickly through the entire root system. This stage corresponded to the general wilt observed on the above ground plant parts.

Pythium strains simultaneously attacked either the roots and the collar, or one or the other of these plant parts. When the attack occurred only on the roots, the plant, at times, exhibited stunting (**Fig. 1C**).

Morphological characteristics of the *Pythium* species isolated

Colonies of *Pythium*, produced cottony white aerial mycelium on PDA (**Fig. 3**). The mycelium, hyaline, ramified and nonseptate, was 8-16 µm wide. The size of the sporangia varied between 20 and 28 µm, while that of the oospores varied from 20 to 36 µm, sometimes reaching 40 µm (**Fig. 4**). The growth rate of *Pythium* species isolated on the papaya trees in Côte d'Ivoire was estimated at 2.25 cm per day (data not shown). The aerial thallus subsided after the mycelium reached the limits of the Petri dish. This phenomenon generally started a week after culture (**Fig. 3**).

Molecular characterization and identification of *Pythium aphanidermatum*

The *Pythium* isolate obtained from the papaya trees was identified as *P. aphanidermatum* by CABI on the basis of both morphological and molecular characteristics.

The identity of *P. aphanidermatum* isolated from the papaya tree was confirmed through the partial sequences of the ITS-1 and ITS-2 regions and the complete sequences of the 5.8S of the ribosomal DNA amplified using the primers ITS6f and ITS4r. These primers have been used widely with fungi. Primer ITS4r is considered to be a universal fungal primer for the end of the ITS region (White *et al*. 1990) whilst primer ITS6 was designed specifically for use in Chromistan or Stramenipilean "fungi" to which the genus *Pythium* belongs (Cooke and Duncan 1997). The 659 bp sequence of isolate IMI-396181 (GenBank Accession N° EU861392) displayed 100% identity with the sequences of *P. aphanidermatum* present in GenBank isolate 35 A. Following the successful identification of IMI-396181, its ITS sequence was submitted to GenBank and given the GenBank Accession N°: EU861392 (**Fig. 5**).

DISCUSSION

The pathogenic agent, responsible for the papaya root and collar rot in Côte d'Ivoire, attacked the plant at all growth and development stages. Ravissé and Boccas (1969) reported similar observations on avocado plants infected with *Pythium aphanidermatum* in Congo. The symptoms appear on the aerial part of the plant but also as on the root system. The development of these symptoms resembles that of papaya root rot caused by *Phytophthora palmivora* (Ko 1982). In that study, collar rot was not observed. On the basis of morphological characteristics, four groups of fungi were distinguished with a prevalence of *Fusarium*, *Rhizoctonia* and *Pythium.* Similar results were obtained by Manoch (2003) and confirm that *Fusarium*, *Rhizoctonia* and *Pythium* are the fungi genera generally found on the surface layers of tropical soils. Similarly, the strong preponderance of *Fusarium* recorded in our inventory confirms the results of Koffi (2005) which indicated that this genus was observed in forest soil profiles in Côte d'Ivoire.

The study of the involvement of the different fungi in the disease development revealed an absence of symptoms following the inoculation with the different isolates of *Fusarium*. These results show that *Fusarium solani* and '*Fusarium* sp. 1' were not responsible for the symptoms observed in the papaya plantations. The wilting of the papaya seedlings and rots observed at the collar level following inoculations with *Pythium aphanidermatum* showed that this fungus was involved in the development of the symptoms previously described. The consistent isolation of *P. aphanidermatum* from affected plants, the development of

```
EU861392 1    CCCATTTACCTAATACTGATCTATACTCCAAAAACGAAAGTTTATGGTTTTAATCTATAA
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EU245039 146  CCCATTTACCTAATACTGATCTATACTCCAAAAACGAAAGTTTATGGTTTTAATCTATAA

EU861392 61   CAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGAT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EU245039 206  CAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGAT

EU861392 121  ACGTAATGCGAATTGCAGAATTCAGTGAGTCATCGAAATTTTGAACGCACATTGCACTTT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EU245039 266  ACGTAATGCGAATTGCAGAATTCAGTGAGTCATCGAAATTTTGAACGCACATTGCACTTT

EU861392 181  CGGGTTATGCCTGGAAGTATGCCTGTATCAGTGTCCGTACATCAAACTTGCCTTTCTTTT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EU245039 326  CGGGTTATGCCTGGAAGTATGCCTGTATCAGTGTCCGTACATCAAACTTGCCTTTCTTTT

EU861392 241  TCTGTGTAGTCAGGGAGAGAGATGGCAGAATGTGAGGTGTCTCGCTGGCTCCCTTTTCGG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EU245039 386  TCTGTGTAGTCAGGGAGAGAGATGGCAGAATGTGAGGTGTCTCGCTGGCTCCCTTTTCGG

EU861392 301  AGGAGAAGACGCGAGTCCCTTTAAATGTACGTTCGCTCTTTCTTGTGTCTAAGATGAAGT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EU245039 446  AGGAGAAGACGCGAGTCCCTTTAAATGTACGTTCGCTCTTTCTTGTGTCTAAGATGAAGT

EU861392 361  GTGATTCTCGAATCGCGGTGATCTGTTTGGATCGCTTTGCGCATTTGGGCGACTTCGGTT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EU245039 506  GTGATTCTCGAATCGCGGTGATCTGTTTGGATCGCTTTGCGCATTTGGGCGACTTCGGTT

EU861392 421  AGGACATTAAAGGAAGCAACCTCTATTGGCGGTATGTTAGGCTTCGGCCCGACGTTGCAG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EU245039 566  AGGACATTAAAGGAAGCAACCTCTATTGGCGGTATGTTAGGCTTCGGCCCGACGTTGCAG

EU861392 481  CTGACAGAGTGTGGTTTTCTGTTCTTTCCTTGAGGTGTACCTGAATTGTGTGAGGCAATG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EU245039 626  CTGACAGAGTGTGGTTTTCTGTTCTTTCCTTGAGGTGTACCTGAATTGTGTGAGGCAATG

EU861392 541  GTCTGGGCAAATGGTTGCTGTGTAGTAGGGTTTTGCTGCTCTTGGACGCCCTGTTTTCGG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EU245039 686  GTCTGGGCAAATGGTTGCTGTGTAGTAGGGTTTTGCTGCTCTTGGACGCCCTGTTTTCGG

EU861392 601  ATAGGGTAAAGGAGGCAACACCAATTTGGGACTGTTTGCAATTTATTGTGAACAACTTT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EU245039 746  ATAGGGTAAAGGAGGCAACACCAATTTGGGACTGTTTGCAATTTATTGTGAACAACTTT
```

Fig. 5 Comparison of nucleotide sequence of rDNA regions of a published sequence of *Pythium aphanidermatum* (Genbank accession N° EU245039) and the IMI396181 isolate of *Pythium aphanidermatum* (Genbank accession N° EU861392) showing 100% identity between the sequences across the aligned sequence length.

collar rot symptoms on inoculated plants and re-isolation of the pathogen from these inoculated plants confirm this pathogen's status as the primary cause of papaya collar and root rots. Symptoms similar to those caused by *P. aphanidermatum* have been recorded in countries such as Nigeria, Thailand and the United States of America (Ko 1982; Oluman and Oladiran 1993; Vawdrey and Westerhuis 2007). However, studies on the identification of the pathogenic agents responsible for these diseases highlighted the involvement of *P. aphanidermatum* in the diseases recorded in Nigeria and in Thailand and that of *Phytophthora palmivora* in those reported in the United States and Australia (Ko 1982; Oluman and Oladiran 1993; Vawdrey and Westerhuis 2007). Taking into account the diversity of the pathogenic agents causing collar rot, plant health surveys in the papaya production zones in Côte d'Ivoire are necessary to confirm the absence of other parasites besides *P. aphanidermatum* that have been reported from the other countries.

With regard to the seedlings placed in culture on naturally infested soil (T1), the symptoms observed were identical to those obtained by artificial inoculation with *P. aphanidermatum*. Moreover, confirmatory tests made by re-inoculation of the fungi isolated from treatment T1 led to the same symptoms (wilting and rots). That result shows that the fungi responsible for the disease observed in the plantations came from the soil. Indeed, *P. aphanidermatum* is a polyphagous fungus which attacks several plant species including tomato, cotton, amaranths, melon, cucumber and pepper (Lanier 1978; Mitchell 1978; Al-Sa' di 2008). In Côte d'Ivoire, the fungus has been reported from plants other than papaya (CMI 1978). These biological characteristics increase the aptitude for survival in nature and consequently reduce the effectiveness of the control methods against this fungus.

Concerning the incidence of the collar rot, the statistical analysis showed a significant difference between soil artificially inoculated with *P. aphanidermatum* (71.25%) and untreated soil coming from the infection site (45%). This difference could be explained on the one hand by the difference in the inoculum densities of the two media and on the other hand by the probable existence of a microflora antagonistic to *P. aphanidermatum*. Indeed, fungi such as

Trichoderma viride have been found to antagonize a number of soil fungi (Dommergues and Mangenot 1970). That specific fungus or others could be present in the nonsterilized plantation soil.

The enrichment of the inoculum in the corn-sand medium favoured the rapid development of the propagules. Additionally, in the natural environment, the relatively low water content due to evaporation can favour cyst formation. In that case, a latent period is necessary for the fungus to infect the host plant. In this experiment, the inoculum was prepared freshly and incubated before the inoculation. Moreover, the death rates recorded for infected seedlings were of the same order of magnitude as the incidence. The relative differences in mortality between treatment T2 (45%) and T1 (15%) could thus be explained by the same phenomenon. Also, work carried out by Stern *et al*. (1977) showed the influence of the soil potential matrix variation, texture and amendment (biogeochemical variations) on disease caused by *Phytophthora cinnamomi*. These factors undoubtedly influenced the attack of the pathogen in the naturally infected soil (T1).

The characterization study undertaken on the pathogenic agent revealed from the morphological point of view that *P. aphanidermatum*, is a fast growing fungus, with a white thallus with a hyaline, nonseptate, mycelium. The propagules are round and/or oval of about 40 µm in diameter. The oospore does not fill the oogonium and that is a characteristic of *Pythium*. These morphological characteristics observed are similar to those described by Ravissé and Boccas (1969), Bruehl (1987) and Agrios (1997). The analysis of the partial ribosomal RNA gene cluster (part of 18S small subunit RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, part of 28S large subunit ribosomal RNA gene) sequence of isolate IMI-396181 (Genbank Accession N° EU861392) showed 100% identity to *Pythium aphanidermatum* isolate 35A (Genbank Accession N° EU245039), confirming the taxonomy of *Pythium aphanidermatum*. There is therefore a necessity to find adequate and effective control measures.

CONCLUSION

The research work carried out made possible the diagnosis of the disease observed in the papaya plantations visited. Symptoms consisted of root and collar rot of the papaya leading to wilting and the eventual death of the papaya tree. The disease is caused by *Pythium aphanidermatum*. The pathogenicity of the fungus was demonstrated by appropriate testing. In addition, the molecular characterization confirmed the identity of the fungus responsible. To our knowledge, this is the first report of papaya root and collar rot caused by *Pythium aphanidermatum* in Côte d'Ivoire. Future research should focus on the impact of mixed infections involving two or more fungi as well as on the development of adequate control measures.

REFERENCES

Agrios GN (1997) *Plant Pathology* (4th Edn), Academic Press, San Diego, 404 pp

Al-Sa'di AM, **Drenth A, Deadman M, de Cock A, Aitken EA** (2008) Molecular characterization and pathogenicity of *Pythium* species associated with damping-off in greenhouse cucumber (*Cucumis sativus*) in Oman. *Plant Pathology* **56**, 140-149

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403-410

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation protein database search programs. *Nucleic Acids Research* **25**, 3389-3402

Alvarez AM, Nelson MG (1982) Control of *Phytophthora palmivora* in papaya orchard with weekly sprays of chlorothanil. *Plant Disease* **66**, 37-39

Barnett HL, Barry HH (1972) *Illustrated Genera of Imperfect Fungi* (3rd Edn), Burgess Publishing, Minneapolis, USA 241 pp

CMI (1978) *Distribution Maps of Plant Diseases* (Map No. 309), CAB International, Wallingford, UK

Cooke DEL, Duncan JM (1997) Phylogenetic analysis of *Phytophthora* species based on ITS1 and ITS2 sequences of the ribosomal RNA gene repeat. *Mycological Research* **100**, 667-677

Diallo HA, Monger W, Kouassi N, Yoro TD, Jones P (2008) Occurrence of *Papaya ringspot virus* infecting papaya in Ivory Coast. *Plant Viruses* **2 (1)**, 52-57

Dommergues Y, Mangenot F (1970) *Ecologie Microbienne du Sol*, Masson, Paris, 795 pp

Guadalupe GIJ (1981) Papaya cultivation in the collima region. *Circula IAB (Mexico)* **60**, 60

Haware MP, Nene YL (1982) Races of *Fusarium oxysporum* f. sp. *ciceri*. *Plant Disease* **66**, 809-810

Ko WH (1971) Biological control of seedling of pawpaw caused by *Phytophthora nicotianae*. *Phytopathology* **61**, 780-782

Ko WH (1982) Biological control of *Phytophthora* root rot of papaya with virgin soil. *Plant Disease* **66**, 446-448

Koffi NBC (2005) *Flore Fongique Comparée des Sols Evoluant sous Trois Couverts Végétaux*, Mémoire de DEA, Université d'Abobo-Adjamé, Côte d'Ivoire, 80 pp

Lanier L, **Joly P, Bondoux P, Bellemère A** (1978) *Mycologie et Pathologie Forestières I*, Masson, Paris, 486 pp

Lannuzel V (2004) Le développement de la papaye en Côte d'Ivoire: Promotion et exportation. *Promexa Info* **10**, 10

Loeillet D (2006) Plaintes contre le nouveau système tarifaire des taxes de l'UE sur les fruits tropicaux. *FruiTop* **130**, 3-5

Manoch L, **Jaroenthai K, Busarakam K, Jeamjitt O, Dethoup T, Kokaew J** (2003) Diversity and distribution of microfungi from different habitats of surface soil in Thailand. *Symposium* 12, paper 2332, Kaetsart University, Bangkok

Mitchell DJ (1978) Relationships of inoculum levels of several soilborne species of *Phytophthora* and *Pythium* to infection of several hosts. *Phytopathology* **68**, 1754-1759

Mora HD, Morales BF (1980) Etiology of papaya root rot in Costa Rica. *Agronomia Costarricense* **4**, 191-193

Oluma H, Oladiran A (1993) *Pythium aphanidermatum* root rot of papaw (*Carica papaya* L.) in Nigeria. *Mycopathologia* **123**, 111-115

Parris GK (1941) *Phytophthora nicotianae* on papaya in Hawaii. *Phytopathology* **31**, 314-320

Pearson WR, Lipman DJ (1988) Improved tools for biological sequence comparison. *Proceedings of the National Academy of Sciences USA* **85**, 2444-2448

Ravissé A, Boccas B (1969) *Première liste annotée des Pythiacées parasites au Congo* (TOME VII, Fascicule 1), Cahier de la MABOKE, Paris, 69 pp

Song W, Zhou L, Yang C, Cao X, Zhang L, Liu X (2004) Tomato *Fusarium* wilt and its chemical control strategies in hydroponic system. *Crop Protection* **23**, 243-247

SPC (South Pacific Commission) (1980) Papaw: A food for people. *South Pacific Commission*, Leaflet N° 2

Sterne RE, Zentmyer GA, Kaufmann MR (1977) The influence of matrix potential, soil texture and soil amendment on root disease caused by *Phytophthora cinnamomi*. *Phytopathology* **67**, 1495-1500

Teixeira da Silva JA, Rashid Z, Nhut DT, Sivakumar D, Souza Jr. MT, Tennant PF (2007) Papaya (*Carica papaya* L.) biology and biotechnology. *Tree and Forestry Science and Biotechnology* **1**, 47-73

Trujillo H, Hine R (1965) The role of papaya residues in papaya root rot caused by *Pythium aphanidermatum* and *Phytophthora nicotianae*. *Phytopathology* **55**, 1293-1298

Vakalounakis DJ, **Fragkiadakis GA** (1999) Genetic diversity of *Fusarium oxysporum* isolates from cucumber: differentiation by pathogenicity, vegetative compatibility and RAPD fingerprinting. *Phytopathology* **89**, 161-168

van der Plaats-Niterink AJ (1981) Monograph of the genus *Pythium*. *Centraalbureau voor Schimmelcultures (Netherlands): Studies in Mycology* **21**, 242

Vawdrey LL, Westerhuis D (2007) Field and glasshouse evaluation of metalaxyl, potassium phosphonate, acibenzolar and tea tree oil in managing *Phytophthora* root rot of papaya in far northern Queensland, Australia. *Australasian Plant Pathology* **10**, 123-130

Walpole RE, Myers RH (1972) *Probability and Statistics for Engineers and Scientists* (2nd Edn), Macmillan Publishing, New York, USA, 580 pp

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for Phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, While TJ (Ed) *PCR Protocols: A Guide to Methods and Applications*, Academic Press, New York, USA, pp 315-322