

Full Length Research Paper

Isolation and molecular Identification of fungi associated with the spoilage of farms fruit in Southern Côte d'Ivoire

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The spoilage of pawpaw (*Carica papaya* L.), pepper (*Capsicum* sp.) and tomato (*Lycopersicon esculentum*) from three selected farms in Agboville area, south of Côte d'Ivoire were investigated. 50 samples of each type of fruit (pawpaw, pepper and tomato) showing spoilage signs were examined for the presence of fungal pathogens inducing spoilage. Eight fungal species, *Mucor velutinosus*, *Meyerozyma guilliermondii*, *Colletotrichum higginsianum*, *Rhizopus oryzae*, *Mucor circinelloides* f. *circinelloides*, *Fusarium oxysporum*, *Rhizopus stolonifer* and *Geotrichum* sp. identified by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), were found associated with the deterioration of the fruit. All fungi found in the fruit were common to pawpaw (*C. papaya*) and tomato (*L. esculentum*) except *M. guilliermondii* which was only found in pawpaw (*C. papaya*). *F. oxysporum*, *R. stolonifer* and *R. oryzae* were associated with the spoilage of pepper (*Capsicum* sp.). *F. oxysporum* had the highest rate of occurrence among the isolated fungi (30%) followed by *R. oryzae* with 15% occurrence. *M. velutinosus*, *Geotrichum* sp., *M. circinelloides* f. *circinelloides*, *R. stolonifer* and *C. higginsianum* had all 10% occurrence. *M. guilliermondii* had the lowest rate at 5%. The result reveals that the presence of fungi before harvesting may be damageable to the fruit, so the use of natural control agents to ensure an effectiveness of the production, the marketing, product quality food safety appears to be very important.

Key words: Tomato (*L. esculentum*), pawpaw (*Carica papaya*), pepper (*Capsicum* sp.), polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE).

INTRODUCTION

Fruit represent an important part of food produce in African tropical countries and they play a significant role

in human diet through the supply of vitamins and minerals to the organism. Tropical fruit in Côte d'Ivoire

include crops such as pawpaw, tomato and pepper, which are consumed fresh, cooked or processed to products such as juice and jam.

In 2010, world production of fruit and vegetables amounted to 1,639 million tons, 67, 13, 10, 9 and 1% respectively for Asia, America, Europe, Africa and Oceania. World trade in fresh fruit and vegetables displayed, meanwhile, 97 million tons (FAOSTAT, 2010).

Among the various fruit grown in Côte d'Ivoire, pawpaw (*C. papaya*, var. Solo8) is one of the most important tropical fruit exported every year toward the European Union. The pawpaw fruit contain minerals such as Na, K, Ca, Mg, P, Fe, Cu, Zn and Mn. It is also a source of carotenoids, vitamins C, thiamin, riboflavin, vitamin B6 and vitamin K (Adetuyi et al., 2008). The hot pepper (*Capsicum annuum*) is also considered to be the most known and nutritive legume. It is rich in nutrients and is used for its flavor, as a spice, and as colorant to food due to its color properties (Mueller et al., 2010; Kouassi and Koffi-Nevry, 2012). The hot peppers are abundant and savory during the months of August and September and are consumed during all year around. The tomato (*Lycopersicon esculentum*) is a climacteric fruit, with very high nutritional qualities due to its composition which includes dry matter, soluble solids, amino acids, pigments, simple sugar, organic acids, citric acid and others volatile compounds and more than 400 aromatic compounds contributing to its taste, flavor, and savor (Thybo et al., 2006). However, the quality of tropical fruit is commonly affected by postharvest diseases such as fruit rot, which are mostly caused by improper handling and storage during transportation and marketing. The fruit are affected by a large range of micro-organisms such as pathogenic fungi, which cause their degradation as indicated by the changing in the taste, the odor, the appearance or the texture, resulting in less appealing and toxic fruit. These microorganisms can contaminate the seed prior to sowing, during plant growth in the farms, harvesting, post-harvest handling, or while these fruit are stored and during distribution (Barth et al., 2009). About 20 to 25% of the harvested fruit are rotten by these agents during the post-harvest handling even in the developed countries, negatively influencing the economic value of the fruit (Droby, 2006). The amount of global food losses and waste annually represents about 40-50% of roots, fruit and vegetables (FAO, 2014). Fungal activities can also lead to contamination with mycotoxins, and could represent a health risk to the consumers. Such risks could be reviewed in studies on papaya fungal and viral postharvest diseases accomplished in Côte d'Ivoire (Diallo et al., 2007; Koffi-Nevry et al., 2011).

However, little has been done to prevent fungi spoilage of the fruit in farms of Côte d'Ivoire. These recent years, the frequency of the fungal attack on the fruit has requires a special attention of current researches in order to isolate and identify fungi associated to these various deteriorations on farm fruit. The purpose of the present study was to identify the fungal strains profiles associated to the rotting of pawpaw, tomato and hot pepper in different farms of Côte d'Ivoire.

MATERIALS AND METHODS

Sampling

Three types of fruit (hot pepper, pawpaw, tomato) found with symptoms of fungal infection were sampled in three different farms in the area of Azaguié and Tomassé in the morning (8.00 AM to 12.00PM). The fruit were harvested at random in 2011 in the region of Agneby-Tiassa. It is located between 5° 38'00" North and 4° 5'00" west on the road to Agboville about 35 kilometers from Abidjan (Côte d'Ivoire). The sequences obtained were deposited in GenBank. For this study, 50 samples of pawpaw were sampled in a farm at Tomassé, 50 samples of tomatoes and 50 samples of hot pepper were sampled in two different farms at Azaguié. The farm fruit presenting symptoms were harvested on the plants, placed in sterilized stomacher bag and stored in a cooler at 8°C.

Isolation of fungi

After receiving the samples, physical observation of the diseases were described. Each of the fruit was washed with (1/10) sodium hypochlorite solution. A little infected portion of each fruit was cut and inoculated. The various portions cut describing rots were placed directly onto sterile Sabouraud chloramphenicol medium plates (Figure 1). All plates were incubated at 25 °C for 72 h. Each fungal strain is inoculated on three different Sabouraud chloramphenicol medium plates for isolation and purification. The isolated strains were macroscopically characterized by a visual observation, based on the description of the morphology and color of the colonies.

Molecular identification of fungi

The strains isolated were identified by a Denaturing Gradient Gel Electrophoresis -PCR (DGGE-PCR) method characterized by the extraction of the total fungal DNA, the amplification of the DNA by the PCR, and the DGGE separation (Li et al., 2008; El Sheikha et al., 2011). For the PCR amplification, the D1/D2 region of the 28S rDNA from the fungi was amplified by two GC-rich primer set U1f (5'- CGC CCG CCG CGC GCG GCG GCGGG GCG GGG GTG AAA TTG TTG AAAGGG AA - 3') and U2r (5'- GAC TCC TTG GTC CGT GTT - 3'). For the DGGE method, a GC-clamp of 30 nucleotides was added to the U1f primer at the 5'-end in order to ensure that the resulting DNA fragments will partially remain (Sheffield et al., 1989). Then the denaturing gradient gel

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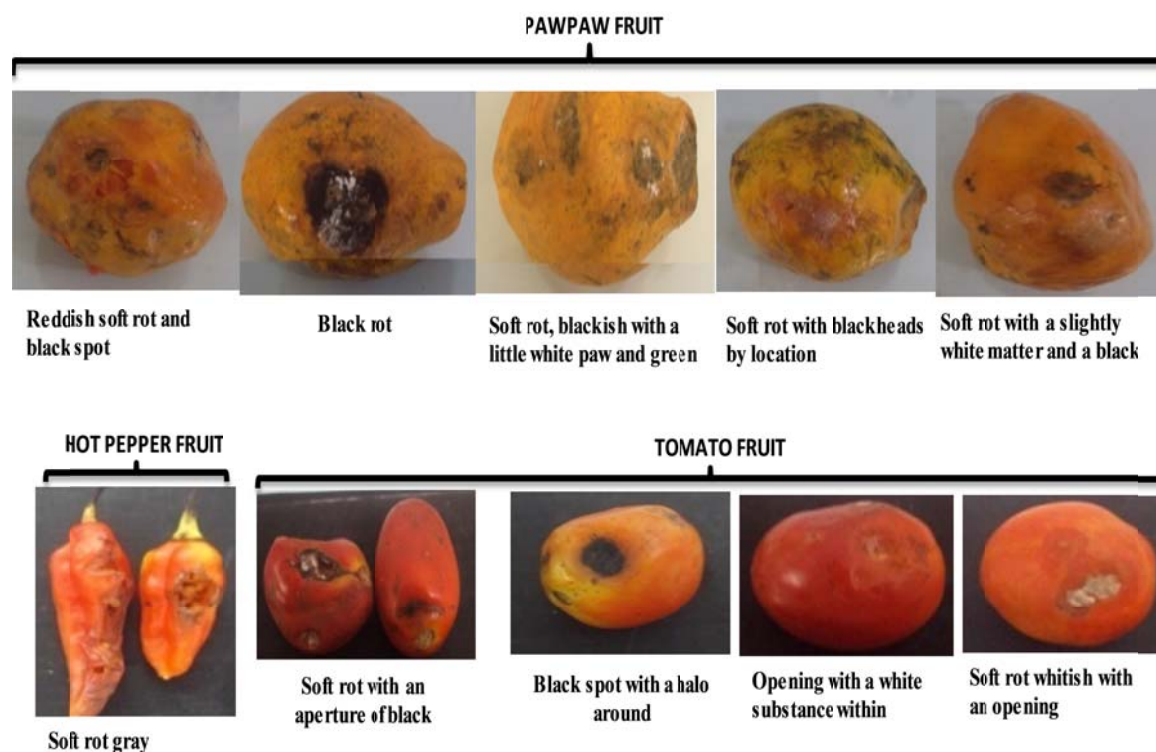


Figure 1. Description of fungi disease symptoms on fruits.

Table 1. Macroscopic description of the strains

N° strains	Macroscopic description of the strains
1	Whitish mycelium with invasive character as duvet.
2	Colony with cottony globular aspect that becomes brown-black when it is matured. There are also creeping branches, markedly elevated and away from the substrate.
3	Colony cottony, white-yellow color with invasiveness. Colonies are fast growing, flat, dusty the waxy, white cream on the surface.
4	A strain having an aspect in form cottony white, creamy white then turning purple with a purple background color.
5	The strain gave invasive gray colonies, presenting also a globular aspect at the beginning and becomes dark at maturity.
6	A cottony colony mycelium sparse becoming gray with maturity with a yellow-whitish center.
7	A cottony strain, whitish at the beginning and becoming brownish gray to blackish gray after the third day.
8	A colony of cottony aspect and an orange color with a background colorless after 3 days of growth.

electrophoresis (DGGE) was done after ensuring that the PCR product from acrylamide gel (8%) (37, 5/1, v/v/v, Promega, France) contains a chemical denaturing gradient (40 to 70 % urea) and formamide. The DGGE was performed according to the method used by Kouakou et al. (2012). For the sequencing of the DNA fragment from DGGE bands, the bands were cut from the DGGE gel with a sterilized scalpel and eluted in 100 ml of TE buffer at 4°C for 24 h. The DNA is then re-amplified in the same condition using a primer without GC-clamp before the products were sent for sequencing at GATC Biotech (Germany). The 28S rDNA sequences obtained were compared with those available in GenBank database, at the National Center for Biotechnology Information (NCBI) website using the program BLAST in order to determine the nearest known sequences (Table 2).

RESULTS AND DISCUSSION

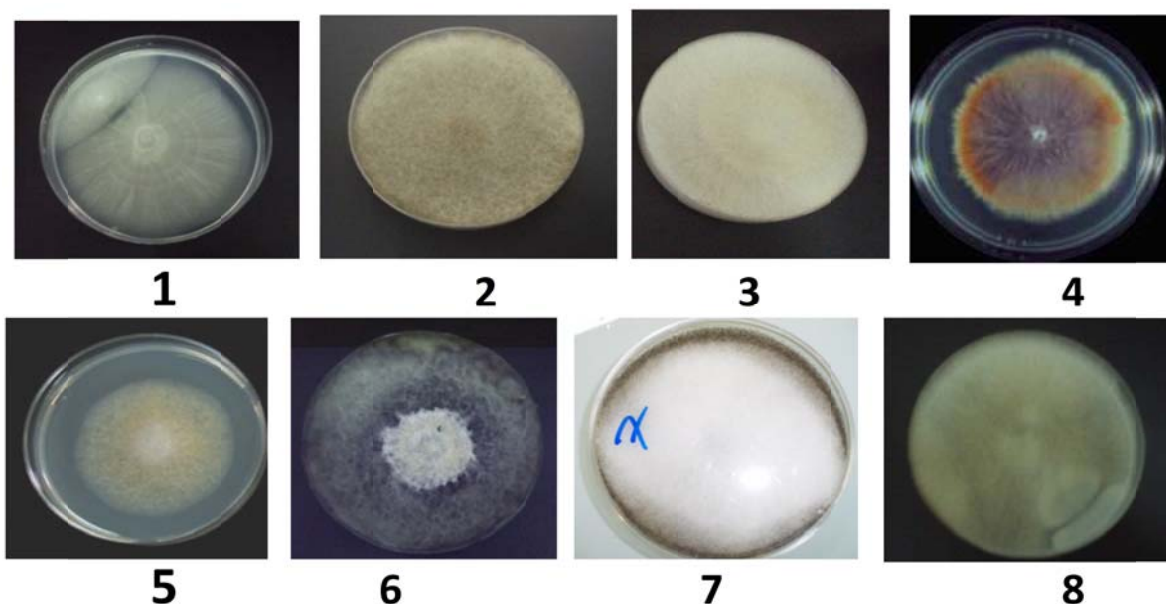
Descriptions of the fungal symptoms on fruits

Symptoms of microbial contamination occurred in the form of necrosis of soft rots apparently reddish, blackish, whitish, greenish or grayish color with or without openings and also the presence of round spots as shown in Figure 1 and Table 1. These symptoms are characteristics of fungal diseases similar to those described by Cannon et al. (2012) on exported tropical fruit.

Table 2. Summary of strains identified for each fruit.

Strains	Identity (%)	Genbank accession number	Fruits		
			Pawpaw	Tomato	Pepper
<i>Mucor velutinosus</i>	98	JN874486	+	+	-
<i>Meyerozyma guilliermondii</i>	98	JX423568	+	-	-
<i>Colletotrichum higginsianum</i>	86	CACQ02008496	+	+	-
<i>Geotrichum sp</i>	97	AB741076	+	+	-
<i>Mucor circinelloides f. circinelloides</i>	97	JN939203	+	+	-
<i>Fusarium oxysporum</i>	95	JX081386	+	+	+
<i>Rhizopus stolonifer</i>	100	JN938904	+	+	+
<i>Rhizopus oryzae</i>	99	JN938902	+	+	+

+, Strain present; -, strain absent.

**Figure 2.** Macroscopic description of the strains

Isolation and macroscopic identification of fungal strains

From the infected fruit, 24 strains of fungi were isolated. The analysis of the morphology, after isolation and purification, permitted obtaining eight (08) morphological groups strains different from each other by their appearance, density, color, size, and the mycelium as described in Figure 2.

Molecular identification of the fungal strains

Strains were identified using the molecular method of PCR-DGGE. The quality of the DNA was confirmed by

PCR amplification of the fungal conserved 28S rDNA region using GC U1F⁻ - CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GTG AAA TTG TTG AAA GGG AA - 3[']), U2r (5[']- GAC TCC TTG GTC CGT GTT - 3[']) primers with control DNA from pure strains as are presented (Figure 3).

PCR products provided unique, unambiguous and intense bands between 298 and 220 bp, which corresponded to the expected size of 260 bp for fungi. The negative control was performed with the reaction mixture without the addition of DNA extract. The absence of a band for the negative control showed that there was no contamination of the PCR reaction mixture. The bands of the DGGE profile obtained were finally sequenced for strains identification. Identification of strains revealed (08)

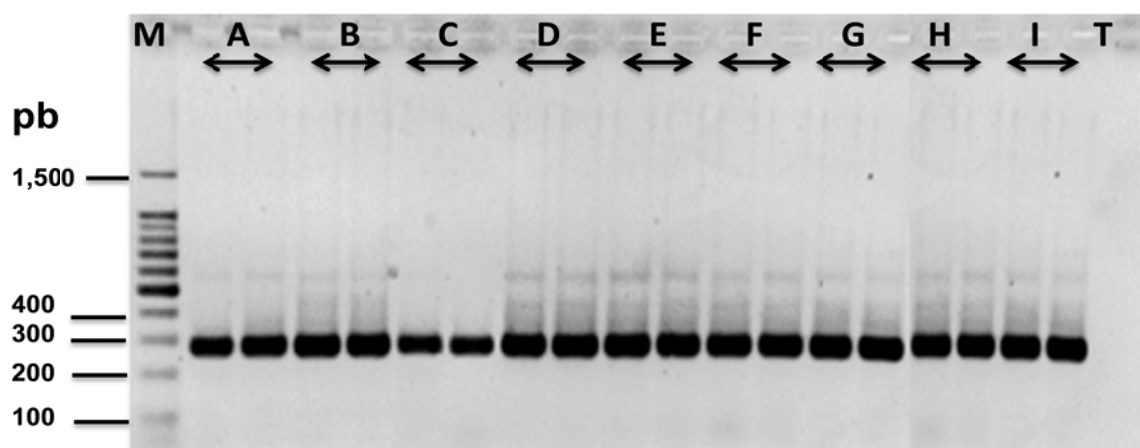
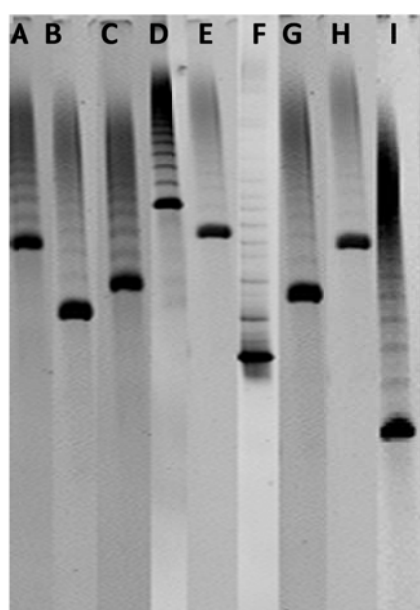


Figure 3. Gel of PCR of fungi DNA verification. T, negative control; M, Marker; bp, base pair; ABCDEFGHI, different fungi



- A:** *Mucor velutinosus*
B: *Meyerozyma guilliermondii*
C: *Rhizopus oryzae*
D: *Geotrichum sp.*
E: *Mucor circinelloides f. circinelloides*
F: *Fusarium oxysporum*
G: *Rhizopus stolonifer*
H: *Mucor circinelloides f. circinelloides*
I: *Colletotrichum higginsianum*

Figure 4. DGGE Gel and identified fungi strains.

distinct species such as: *M. velutinosus*, *M. guilliermondii*, *C. higginsianum*, *R. oryzae*, *M. circinelloides f. circinelloides*, *F. oxysporum*, *R. stolonifer*, and *Geotrichum sp.* (Figure 4).

These results show that these fruit and vegetables (pawpaw, tomato and chili) collected in different farms are true hosts of these fungal agents. So they are responsible for diseases affecting pawpaw fruit on the farms. This result is in conformity with those of Chukwuka et al. (2010) who recently reported that *Fusarium sp.*, *Mucor sp.* and other germs were responsible for the rot of

pawpaw in the Nigeria farms. *M. guilliermondii* was only isolated from pawpaw sample from Tomassé farms indicating that the strains vary according to the farm. Fungi isolated from tomato (*L. esculentus*) included *F. oxysporum*, *M. velutinosus*, *C. higginsianum*, *R. oryzae*, *M. circinelloides f. circinelloides*, *R. stolonifer* and *Geotrichum sp.* Almost all of the species found on papaya were also present on tomatoes except for *M. guilliermondii* (Table 2).

These results show that pawpaw and tomato might be infected by the same germs, causing diseases in the

Table 3. Frequency of occurrence of strains on fruits

Strain	Number	Frequency of occurrence (%)
<i>Mucorvelutinosus</i>	2	10
<i>Meyerozyma guilliermondii</i>	1	5
<i>Colletotrichum higginsianum</i>	2	10
<i>Geotrichum sp</i>	2	10
<i>Mucor circinelloides f. circinelloides</i>	2	10
<i>Fusarium oxysporum</i>	6	30
<i>Rhizopus stolonifer</i>	2	10
<i>Rhizopus oryzae</i>	3	15
Total	20	100

farm. The presence of these germs on these studied fruit and vegetables would be favored by the climatic conditions of the country because these organisms have the ability to grow at 37°C as highlighted by Sugui et al. (2011). Among the isolated germs, *F. oxysporum*, *R. stolonifer* and *Mucor* sp. were pathogenic to tomato as confirmed by studies of Chuku et al. (2008) and Akinmusire (2011), who have taxed these germs to be responsible for soft rotting of tomato. *F. oxysporum* was also held responsible for rotting pawpaw, tomato, banana, and guava (Latiffah et al., 2012). Furthermore works of Kleemann et al. (2008) associated *C. higginsianum* with anthracnose disease affecting these fruit. Other studies on fungi have shown that *F. oxysporum*, *R. stolonifer*, *Mucor* sp., and *Geotrichum* sp. were associated with soft rot and sour of tomato and pawpaw (Chuku et al., 2008; Nnebue et al., 2013). *R. oryzae* would be responsible for the disease causing rotting of these fruit as in the case of sunflower due to favorable climatic condition such as the temperature (Yildiz and Baysal, 2006).

Concerning the pepper, it revealed the presence of *F. oxysporum*, *R. stolonifer* and *R. oryzae* pathogens of this fruit. However, Hammami et al. (2014) found species of *Aspergillus tamarii*, and *Aspergillus Flavus* in Egyptian chili powder.

The germs isolated from the disease-affected zones of the fruit (pawpaw, pepper and tomato) are pathogenesis of these fruit at different levels as reported by Akintobi et al. (2011). According to the works of these authors, *R. stolonifer* has a high degree of pathogenicity compared to *Fusarium* sp. Moreover *M. guilliermondii* was isolated only on the pawpaw in that study. Although it was isolated on pawpaw rots, it is not necessary a pathogen of the fruit. Recent studies showed that *M. guilliermondii* inhibits the growth of fungi during the storage of maize in Cameroon, according to Su-lin et al. (2012). However, *M. velutinosus* isolated on the pawpaw and the tomato was also pathogens agent for human because they were responsible for skin infection as reported by Sugui et al.

(2011) suggesting that they represent a threat to the health of farmers and consumers. *F. oxysporum*, *R. stolonifer* and *R. oryzae* were common pathogens strains of the three types of fruit in this study. But for the frequency of occurrence of the isolated fungi associated to the fruit deterioration, *F. oxysporum* was the most frequently isolated (30%) on the three types of fruit. It was followed by *R. oryzae* with an infection rate of 15% while *M. guilliermondii* was the less observed (5%) (Table 3).

Conclusion

Eight (8) spoilage fungi such as *M. velutinosus*, *M. guilliermondii*, *C. higginsianum*, *R. oryzae*, *M. circinelloides f. circinelloides*, *F. oxysporum*, *R. stolonifer*, and *Geotrichum* sp. were found responsible for the rotting and the diseases of local pawpaw, tomato, and chili fruit in the farms of south of Côte d'Ivoire with *F. oxysporum* being the major contaminant. This result implies tough realities for the farmers in guaranteeing safe products for consumption and for trading. The high rate of some fungi contamination requires the adoption of appropriate control measures against farms infection. Therefore, the deterioration of fruit can be controlled in the farms by using natural antifungal extracts from plants and by the correct handling during the harvesting in order to avoid cuts and reduce losses.

Conflict of Interests

The authors have not declared any conflict of interests.

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