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RESEARCH ARTICLE

CONTROL OF FUNGAL ISOLATES RESPONSIBLE FOR POSTHARVEST CROWN ROT OF BANANA (*MUSA* SP. CAVENDISH SUB GROUP, CV. GRANDE NAINE) BY THREE FUNGICIDES IN CÔTE D'IVOIRE

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ABSTRACT

The health status of treated banana bunches in three boxes was assessed 48 hours and 21 days of storage for two consecutive years. The colonies grown on bananas after the conservation periods were isolated, identified and counted by fungal genus. Similarly, developed symptoms on bananas have been described. The prevalence of each observed postharvest disease was evaluated. The prevalence of crown rot ranged from 4 to 98% while the necrosis of the epicarp and the decay of the distal end ranged respectively from 0 to 70 and 0 to 61%. A pathogenicity test of fungi associated with the most prevalent disease was performed. Molecular identification of those responsible fungi of the most prevalent disease was done and phylogeny of these isolates was established. White mould was observed on the surface of banana end fingers after 48 hours without noticeable symptoms. However necrosis of the epicarp and crown rots and the distal end of bananas were observed after 21 days of conservation. Banana crown rot was the most prevalent postharvest disease. The pathogenicity test and molecular analysis showed that *Phellinus noxius* and *Botryodiplodia theobromae* were identified as the causative agents of banana crown rot disease in Côte d'Ivoire.

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INTRODUCTION

Côte d'Ivoire is a producer of the dessert banana Cavendish Subgroup, cv. Grande Naine. Banana productions in Côte d'Ivoire from 2016 to 2014 were about to 290 000 and 330 946 tons (FAO, 2016). In the same period, exports rose in the order of 280 000 to 332 500 tons (Commodafrica, 2017). In Côte d'Ivoire, the most banana production is exported to Europe, North America, Japan, Russia and the countries of the West African sub-region. The productions increased in recent years have raised the Côte d'Ivoire at the forefront of African bananas dessert producers and is a major input source of foreign exchange for the country. It represents 8% of the agricultural GDP and 2% of GDP (Commodafrica, 2017). The banana cultivation has grown from subsistence farming to intensive industrial culture for the exportation. The crops have intensified to meet increased consumer demand. However, this method of cultivation has led to the weakening of soil fertility without any conservation measure, pest attacks and greater vulnerability to banana diseases. Various diseases affecting the banana tree during its cultivation and production after harvest such as anthracnose, finger end rot and crown rot. Of these banana infections, the most serious is the crown rot disease (Triest, 2016).

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The evolution of the disease systematically causes early ripening of bananas during the period of export. To control bananas postharvest infections, producers use various antifungal molecules in postharvest treatment before packaging. Despite the application of these fungicides at the doses indicated by manufacturers, a recurrence of postharvest disease is observed on treated banana. Inefficient of chemical fungicides in the control of banana postharvest diseases could be justified by the lack of knowledge of the identity of the pathogen. Côte d'Ivoire is planning to reach a yield of 500 000 tons of bananas in 2020 (Commodafrica, 2017) that can not be possible without an effective control method of the causative agents for the main banana postharvest disease. The objective of this work is to improve the production of bananas for export.

MATERIALS AND METHODS

Postharvest treatment of banana before the packaging in station

Bunches of bananas shipped in station were dehanded in the washing tanks. After washing, banana were trimmed into smaller hands and placed in baskets and then transported to the antifungal treatment cabin. Bananas hands arranged in baskets were treated separately with Boscalid, Azoxystrobin and

Imazalil at concentrations specified by the manufacturer as showed in Table 1. The antifungal treatment of banana hands was only done on the surface of crown and the epicarp. Treated bananas were then packaged in transparent polyethylene and placed in aerated box.

Table 1. List of pesticides used for antifungal treatment in the banana dessert stations

Active ingredients	Dose treatment
Azoxystrobin (250 g/l)	1.2 ml/l
Boscalid 500 g/l)	0.5 ml/l
Imazalil (750 g/kg)	0.8 g/l

Samplings of boxed bananas

Sampling was done every six months for two years in production areas. The boxes of bananas aged 18 weeks treated with different fungicides were collected randomly in the pallet station from seven main production areas. Three boxes of bananas containing about 18 bunches of 3-5 fingers were collected in each production station with three stations per area.

Health status of banana bunches after antifungal treatments

The health status of banana bunches treated in three boxes was assessed 48 hours after stored in favorable conditions for the development of any viable fungal structures. The colonies grown on bananas after this conservation periodwere isolated according to the method of Davet and Rouxel (1998) on sterile Petri plates containing PDA medium. The inoculated Petri plates were incubated and observed daily in laboratory conditions (25 ± 1°C). Developed fungal colonies were re inoculated on new PDA until obtained homogeneous and individualized colonies. Fungal colonies were then identified using identification keys of Botton *et al.* (1990); Kiffer *et al.* (1997) and Champion (1997).

Evaluation of postharvest diseases of banana after antifungal treatments

Two banana boxes were incubated in favorable conditions to the development of postharvest diseases in the laboratory for 21 days. After this incubation period, the number of crown and fingers infected by postharvest diseases was counted by banana boxes. The total number of bunches and fingers in each carton was also counted. The prevalence of postharvest diseases was estimated using the following formula:

P = NIBDi/NIB X 100
NIBDi: Number of Infected banana by the disease i; NBI: Number of Infected Banana; P: Prevalence (21 days)

Isolation and identification of fungi associated with postharvest diseases of banana

Fungi associated with different symptoms of postharvest diseases of banana were isolated by the method of Davet and Rouxel (1998) on the PDA medium in Petri plates with three boxes per explants and symptoms. The developed fungal colonies were re-inoculated on new PDA media until obtained homogeneous and individualized colonies. The morphotypes of each fungal were identified. The fungi isolated after 48 hours and 21 days were grouped according to the conservation periods of fungicides used in a summary table.

Pathogenicity of fungal isolates responsible for the most prevalent postharvest disease

The pathogenicity of the isolates associated with the most prevalent disease was tested on symptomless banana. The pathogenicity test was conducted by inoculation of bananas with the fungal isolates in order to identify those responsible for the symptoms of the most prevalent disease. To do this, 30 bananas were disinfected with sodium hypochlorite diluted to 10% and wiped with sterile blotting paper. Conidial suspensions of 10^6 conidia per mL were prepared for each fungal isolate. A 50 µl droplet of conidial suspension for each fungi was then deposited midway through the exposed surface of the crown and covered with a sterile plastic wrap. Five banana fingers per isolates were used for the test. Fifteen other banana fingers were inoculated with sterile distilled water and covered with plastic wrap as previously done for the control. Inoculated bananas were incubated under controlled conditions in sterile plastic boxes for each fungal isolate and observed daily for 21 days. A Koch's postulate was done to establish the relationship between each fungi and disease developed on the inoculated bananas. Three repetitions were performed.

Phylogenetic analysis of fungal isolates responsible for the most prevalent postharvest disease

Fungal DNA extraction

A single-spore culture of each fungal isolate responsible for the symptoms of the most prevalent disease was done on the PDA medium according to the method of Henn *et al*. (1994). The extraction of DNA isolates was made according to the method of Doyle and Doyle (1990). The DNA pellet at the bottom of each tube was suspended in 50 µl of sterile pure water and stored at -20 °C for further work.

DNA amplification by PCR and sequencing

The pair of universal primer ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') was used for amplification of the DNA of the isolates according to the method of White *et al.* (1990). Thirty microliters of amplified fungal DNA samples were sequenced at the University of Toronto in Canada.

Phylogenetic analysis

Sequencing of fungal DNA was performed by Sporometrics (Toronto Lab in Canada). The taxonomic position of the fungal isolates was obtained after Blast DNA sequences with those of the NCBI gene bank (Http: //www.ncbi.nlm.nih.gov/blast). Similarity percentages are then determined between the sequences obtained in this study and the closest sequences listed in Gen Bank. The entire sequence is then aligned using the program ClustalW (Thompson *et al*., 1994). The Neighbor-Joining algorithm (Saitou and Nei, 1987) allowed to represent the phylogenetic affiliation of all sequences with the software MEGA 4 (Tamura *et al*., 2007).

RESULTS

Health status of banana fingers after phytosanitary treatments: The fingertips of bananas in the three boxes collected by area showed white fungal colonies after 48 hours

of storage visible through the transparent polyethylene (Figure 1). However, no symptoms were observed on the still green banana fingers.

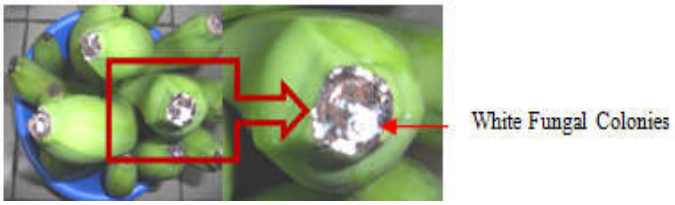

Figure 1. White fungal colonies at the end of banana fingers treated after 48 hours of storage

Symptoms of postharvest diseases of banana

Bananas released into conservation laboratory temperature developed symptoms of postharvest diseases (Figure 2). The epicarp bananas showed randomly arranged necrosis. Necrotic tasks of varying sizes with or without small orange dots clustered in the middle. The epicarp necrotic fingers are sometimes ripe or green. The crown sometimes unripe banana bunches was rotten to the stalk. Fingers on bouquets easily detached by holding the crown. The tips of the fingers were sometimes rotten. The rotten part was covered with flaky mycelia colonies.

Figure 2. Bananas infected with the disease postharvest after 21 days of storage at the laboratory

Prevalence of postharvest diseases of banana

Banana fingers have developed symptoms of postharvest diseases with varying prevalence in the same area as an area to another during the two campaign years (Figures 3 AG). The crown rot developed on bananas produced in seven areas after 21 days of conservation, which varied from 55 to 98; 15-65; 4 to 65; 15-80; 10-67; 52-74 and 20 to 75%. Necrosis of the epicarp was observed with prevalence that ranged from 10 to 61; 4 to 50; 5 to 20; 2 to 30; 0 to 4; From 20 to 44 and 6 to 45%. However, the prevalence of finger end rot ranged from 5 to 60; 1 to 20; 2 to 50; 10-55; 0 to 45; 12-70 and 12 to 70%. The prevalence of crown rot ranged from 4 to 98% while the necrosis of the epicarp and the finger end rot ranged respectively 0 to 70 and 0 to 61%. Finger end rot and necrosis of the epicarp are less present on bananas released into conservation. The crown rot was the most prevalent postharvest disease developed on bananas treated with various fungicides after 21 days of storage.

Isolation and morphological identification of fungi associated with symptoms of banana postharvest diseases

Fungal isolates associated with different symptoms of banana postharvest diseases were identified as *Botryodiplodia*, *Fusarium*, *Colletotrichum*, *Musicillium* and an unidentified genus (Isolate x). A wide variety of isolates generic genre *Botryodiplodia*, *Fusarium*, *Colletotrichum*, *Musicillium* and isolate x has been associated with the decay of the finger end rot. *Botryodiplodia* sp is olates. *Colletotrichum* sp. Isolates and x were obtained on bananas infected with crown rot, necrosis of the epicarp has revealed mostly *Colletotrichum* sp isolates. Several isolates were associated with crown rot, the most prevalent banana postharvest disease in conservation.

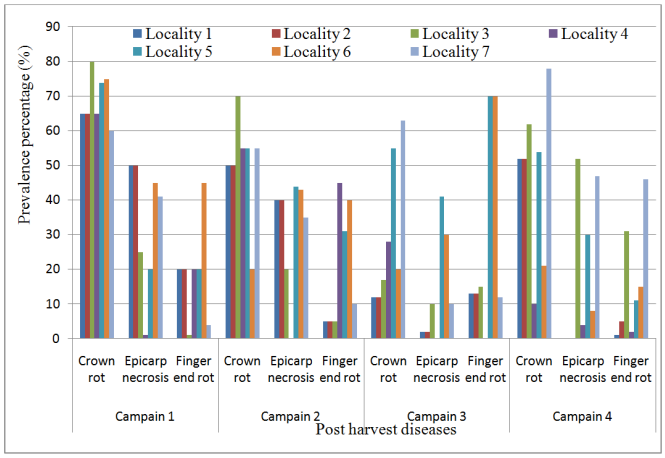

Figure 3. Prevalence of banana postharvest disease after antifungal treatments depending on the infected organ

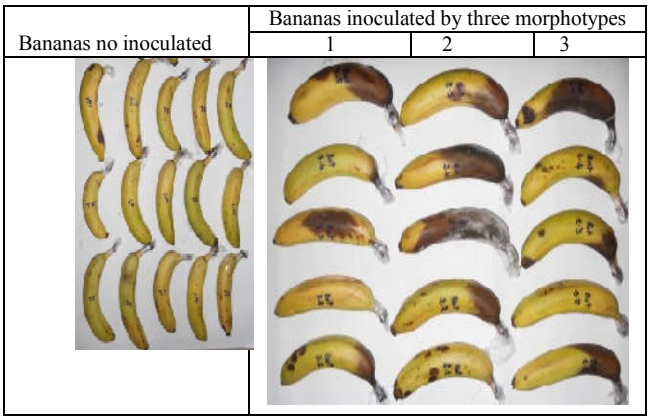

Figure 4. Crown rot bananas infected with the isolates associated with infection

Morphological and pathogenicity of isolates associated with banana postharvest rot: The single-spore culture isolates colonies associated with crown rot of banana showed three morphotypes. Morphotype 1 showed a dark gray mycelial colony more bristling at the center with lined contour. Morphotype 2 x presented a clear brown flaky mycelial colony little downy. As for the morphotype 3, mycelial colony was gray with scattered beads on the surface and denser in the center. Bananas inoculated with isolates representing each morphotype showed crown rot (Figure 4). Bananas inoculated with the morphological type 1 isolate developed the disease of crown rot at a 60% infection rate while those inoculated with the isolate morphotype 2 and 3 were infected to 80%. However, bananas inoculated isolate morphotype 2 (Isolate x) developed the crown rot with greater severity unlike other. Non-inoculated bananas have shown no symptoms of infection. The three isolates representing each morphological type associated with crown rot of banana induced infection on bananas artificially inoculated.

Phylogeny of three isolates responsible for crown rot of bananas: The molecular analysis results and the three morphotypes isolates DNA sequencing showed that the crown rot of banana was caused by two species of fungi,

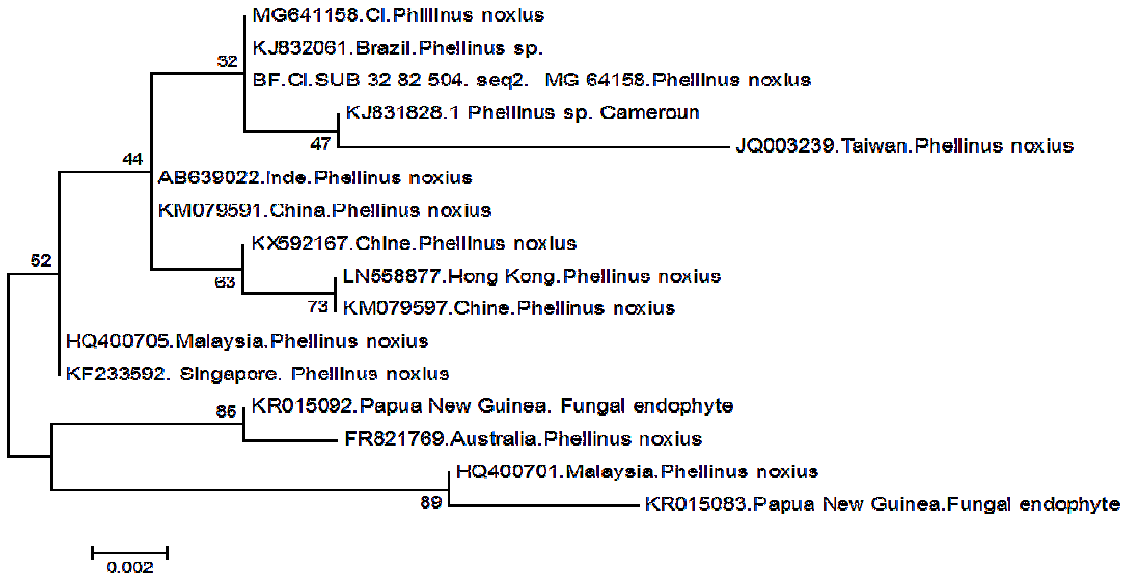

Figure 5. Phylogenetic tree isolate *Phellinus noxius* responsible for crown rot of bananas in Côte d'Ivoire

Phellinus noxius and *Botrodiplodia theobromae* (Figure 5). Morphotypes 1 and 3 were *Botryodiplodia theobromae*. However, the morphological type 2 (Isolate x) has been identified as *Phellinus noxius*. The phylogenetic tree showed that the strain of *P. noxius* responsible for crown rot of banana in Côte d'Ivoire (32 BF.CI.SUB 504.seq2 82) was identical to that of Brazil, but different Cameroon and those of other continents.

DISCUSSION

Green bananas treated with various fungicides without symptoms showed white fungal colonies at the fingertips after 48 hours of storage. Fungal multiplication structures on fingertips from plantations would not be susceptible to of fungicides applied to postharvest bananas. The mode of action of antifungal molecules can be summarized in inhibition of the essential functions of target fungi (Bonnemain, 2003). However, storage structures are non-living and senescent. This is a limiting factor in the mechanism of action of fungicide hence the development of fungal colonies at the end of banana fingers. These banana fingers ends could also be a reservoir of conservations structures of fungi that might develop in favorable conditions. The flowers are constantly moist and sweet could be favorable to the development of heterotrophic fungi in the air, but also carried by insects while foraging. A senescence of flowers, reproductive organs such as pistils are attached to the fingertips and are colonized with fungal structures. The conditions of high humidity and hypoxia observed into bananas boxes could favor the lifting of quiescence and lead to the development of viable structures represented by the observed fungal colonies (Lecellier, 2013). The flowers are constantly moist and sweet could be favorable to the development of heterotrophic fungi in the air, but also carried by insects while foraging. Bananas released into conservation have developed symptoms of postharvest diseases on the crown, the epicarpand on the fingertips. Bananas storage conditions favor the development of pathogenic fungi. The banana ripens producing ethylene which promotes hydrolysis of sugars, carbon sources for the development of fungi (Lassoudière, 2012).

Fungi on banana conservation induced necrosis of the epicarp, finger end rot and crown rot with a high prevalence of the latter. The crown could be a way of easy access and also contain nutrients for fungi unlike the epicarp and the finger tips. The wounds made during the making of bouquets openings that facilitate the penetration and infection of fungi of crown rot (Blancard, 2009). Isolates of the genus *Fusarium, Colletotrichum, Musicillium Botryodiplodia* and have been associated with symptoms of various postharvest diseases of banana. However, most isolates *Botryodiplodia* sp. have been associated with the crown rot. *Botryodiplodia* sp conidia could multiply faster on the crown storage conditions of the banana. Indeed, conidial germination and establishment of a mycelial colony by a mitotic division are deployed infestation powers fungi favorable conditions (Lecellier, 2013) before infecting the host. The three morphotypes identified *Botryodiplodia* associated with crown rot have been responsible. The crown rot is caused by several isolates of *Botryodiplodia* sp. The high prevalence of crown rot could be justified by the diversity of isolates involved in the infection. Each isolate may have a specific pathogenic but with the same effect marked by crown rot bouquets.

The molecular analysis results and sequencing of the DNA of the three morphotypes have shown that morphotypes 1 and 3 were *Botryodiplodia theobromae*. However, the morphological type 2 was identified as *Phellinus noxius*. To date, this is the first time that this fungus responsible for rot in forests and *Hevea brasiliensis* (Geiger, 1986) has been identified as responsible for crown rot of bananas in postharvest in Côte d'Ivoire. Pathogenic activities of different isolates of *B. theobromae* and *P. noxius* could explain the extent of crown rot disease observed on bananas. The failure of antifungal post-harvest treatments by producers could be justified by the inadequacy of the fungicide to the fungus causing the infection.

Conclusion

The fungi responsible for post-harvest banana diseases come from plantations. A variety of fungal genera is involved in banana infections. The failures of fungal treatments in the fight

against fungi responsible for banana infections could be affected by the diversity of postharvest fungi that evolve on bananas. Effective control of post-harvest banana diseases should take into account the diversity of fungi that evolve in banana plantations. Excluding isolated fungi, this is the first time that *phellinus noxius* has been identified as responsible for crown rots of bananas.

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