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DIVERSITY OF *FUSARIUM OXYSPORUM* ISOLATES RESPONSIBLE FOR GRANDE NAINE BANANA TREE'SLEAF CHLOROSIS (CAVENDISH SUBGROUP) IN CÔTE D'IVOIRE

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ABSTRACT

In banana tree plantations, leaf yellowing was noticed by producers. With the aim of making the etiology of banana tree chlorosis so as to contribute to the improvement of banana tree yield, the distribution of the disease in plantations was observed. The phytosanitary status of infected banana tree plantations was assessed by calculating the prevalence of leaf chlorosis. The characteristic symptoms of banana treeleaf chlorosis were described. Sampling was done in order to isolate the causative agent. An identification of fungal isolates associated with symptoms of banana tree leaf chlorosis was made. The isolates responsible for leaf chlorosis were made by a pathogenicity test on vivoplantsin greenhouse. The results showed that sick banana trees were randomly distributed in plantations. A high prevalence of the disease was noticed with variations depending on the plantations surveyed. The symptoms noticed were leaf chlorosis, leaf yellowing and vascular tissue necrosis. Fungi of the different genera were isolated. Thirteen *Fusariumoxysporum* morphotypes were identified, of which three *Fusariumoxysporum* f sp. *Cubense* morphotypes induced the characteristic symptoms of leafchlorosis on the vivoplants used. Banana plantations could be infected by *Fusariumoxysporum* f. sp. *cubense*isolates responsible for leaf chlorosis in Côte d'Ivoire.

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INTRODUCTION

Banana tree is a plant grown in more than 120 countries and nearly over 10 million hectares worldwide (FAO, 2006). Banana tree can be grown throughout the year for its fruit, which plays an important role in global food security (Lassoudière, 2007). It is a cash crop, a source of employment and income for people (Arias *et al*., 2003). In terms of gross value of production, banana ranks fourth worldwide behind rice, wheat and maize. The global annual banana yield was 110 000 000 tons of fruit/year (FAO, 2006). The African continent yielded 15 million tons of dessert bananas in 2012 over a surface area of 1.4 million hectares (FAO, 2012). Côte d'Ivoire is the second largest African producer of dessert bananas after Cameroon (FIRCA, 2014). Over a surface area of 5 500 ha, Côte d'Ivoire yielded 330 460 tons in 2014 (FAO, 2015) and ranked third in terms of food crops after yam and cassava.

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The banana sector in Côte d'Ivoire accounts for 8% of agricultural GDP, 2% of national GDP and offers direct employment to nearly 8 000 to 10 000 people (MINAGRI, 2009). The main areas of production of dessert banana are mainly located around the big cities of south-eastern Côte d'Ivoire. 85% of banana yield is composed of plantain consumed locally but also exported to the sub-region countries. The 15% of total yield composed of dessert banana is exported to European Union countries. However, banana tree cultivation is subject to various biotic and abiotic constraints which often lead to significant yield losses. In terms of biotic constraints, several diseases, especially those of fungal origin, are more and more noticed in intensive and extensive cultivation. These diseases cause yield losses ranging from 20 to 70% (Ploetz *et al*., 2003), especially leaf chlorosis, which is one of the most serious diseases of banana tree. This disease might be due to the action of several pathogenic fungi among which *Fusariumoxysporum* is one of the most frequent and most damaging (Nelson *et al*., 1981). *Fusariumoxysporum* causes yield losses of up to 60% in banana plantations worldwide (Ploetz, 2015). It is a

cosmopolitan fungus which lives in soil, air or even on plant debris. Its ability to adapt to several living environments favors the multiplication of a variety of pathogenic species on different host plants. Moreover, several races of *Fusariumoxysporum* have been identified on several banana tree cultivars sensitive to leaf chlorosis (Armstrong *et al*. 1981). The first step in effective disease control is the identification of the causative plant pathogen agent. In banana tree, disease-control methods exist against fungal diseases. However, none of them has been effective against banana tree Fusarium wilt. The objective of this study is to contribute to the search for an effective control method against banana tree chlorosis in Côte d'Ivoire by identifying the causative pathogenic agent.

MATERIALS AND METHODS

Infection Status and description of leaf chlorosis in banana tree plantations

The selected plantations were visited in order to identify the disease distribution mode. In infected plants, symptom description was made on organs at the external and internal levels from transverse and longitudinal sections made in the organs. The average infection level of plantations was assessed by disease prevalence according to formula 1.

$$Pm\ (\%) = \frac{NPi}{NTP} \times 100 \quad (1)$$

Pm: Disease prevalence; NPi : Number of infected plants; NTP : Total Number of Plants

A classification of infection levels was made according to the Trapero-Casas (1983) scale: 0 (nil); 0.1 to 5% (low); 5.1 to 20% (moderately high); 20.1 to 50% (high); > 50% (very high).

Collecting and getting plant material

Sampling was done in three plantations on organs of three banana trees that had young yellow leaves and three others which were apparently healthy per plantation. On each randomly selected banana tree, two portions of stipe at extremities and a median of about 30 cm were taken. Three roots and 5 g of banana tree rhizosphere soil were also collected. The samples taken were separately placed in sterile plastic bags and sent to the laboratory for analyses. 60 banana treevivoplantsof the Grande Naine variety aged 2 months were obtained by the PIF (Seedlings from Stem Fragments) method.

Isolation and morphological identification of *Fusarium* sp. isolates associated to banana tree leaf chlorosis

The fungi found on the infected banana tree samples were isolated on PDA medium in primary culture. Those of stipe portions and roots were isolated according to the Rapilly method (1968). As for vascular tissue ones, they were isolated according to the Ploetzmethod (1990). Rhizosphere soil fungi were isolated according to the Warcupmethod (1950). Five culture dishes on the PDA medium were prepared per sample types used. After inoculation, all the sealed dishes were incubated at laboratory temperature (25 °C ± 2) and observed daily until the appearance of mycelial colonies. The developed mycelial colonies were separately subcultured until homogeneous and individualized fungal colonies were obtained. The isolated fungi were identified with the identification keys of Barnett *et al*. (1972) and Botton *et al*. (1990).

Counting of isolated fungi

The percentages of isolated fungi were assessed according to the Walder formula (1996).

$$FI\ (\%) = \frac{NI}{NTI} \times 100 \quad (2)$$

FI (%): Isolation Frequency ; NI : Number of Isolation ; NTI : Total Number of Isolation

After the general identification of all the isolated fungi, only the isolates of *Fusariumoxysporum*, a fungus likely to cause banana tree leaf chlorosis according to Saravanan *et al*. (2003), were object of morphological, pathogenic and molecular characterizations. As a result, after obtaining *Fusarium* sp. isolates previously identified following primary cultures, a monospore culture was prepared according to the Ho and Komethod (1997) so as to have pure colonies of each isolate.

Cultural and morphological features of *Fusarium* sp. Isolates

The macroscopic and microscopic features of the different isolates were observed after seven days on PDA medium in Petri dishes. A fungal inoculum of 6 mm in diameter from a seven-day-old colony was seeded in the center of Petri dishes containing solidified PDA medium at the rate of five dishes per isolate and incubated under laboratory conditions (25-27 °C) until total coverage of the culture medium in one of the dishes. Daily measurements were made along two perpendicular axes drawn on the back of each culture dish. The average growth diameter of the colony of each isolate was calculated according to the following formula:

$$L = \frac{D - Di}{2} \times 100 \quad (3)$$

L: mycelial growth; D: diameterof the colony; Di: diameter of the explant.

Molecular characterization of *Fusarium* sp.isolates

After monospore cultures, primary isolates that have revealed more than two other isolates, one of the latter will be selected randomly. In contrast, only one isolate will be selected among those stemming from primary ones that have revealed only one. The genomic DNA of *Fusarium* isolates selected as above was extracted according to the Murray and Thomson method (1980). The universal primer pair ITS1 and ITS4: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used for fungal identification according to the of White *et al*. method (1990). Then, the specific primer pair Foc1 (5'-CAGGGGATGTATGAGGAGGCT-3') and Foc 2 (5'-GTGACAGCGTCGTCTAGTTCC-3') was used for identification of the *Fusariumoxysporum* f. sp. *Cubense*species according to the Lin *et al*. method (2009).

Identification of *Fusarium* isolates responsible for leaf chlorosis symptoms

The identification of *Fusarium* sp. isolates responsible for banana tree leaf chlorosis was made by a pathogenicity test.

Fusarium sp. isolates whose PCR products were amplified with the primer pair Foc1 and Foc 2 were used for the pathogenicity test. An inoculum of 40 ml suspension and 10^6 spores/mlconcentration of each isolate was prepared in a sterile 250 ml Erlenmeyer flask.

Botryodyplodia, Curvularia, Fusarium, Mucor, Penicillium, Trichoderma and *Verticilium* genera. Rhizosphere soils and roots were more infested than stipes. Fungi of the *Fusarium* genus were more isolated (Table 1).

Table . Number of isolates per fungal genus isolated depending on infected banana tree samples

Number of isolates per fungal genus depending on samples				
Fungalgenera	Rhizospheresoil	Roots	Stipes	Isolation percentages
Aspergillus	2	2	0	9.30
Alternaria	2	1	0	6.97
Botryodyplodia	3	1	3	13.95
Curvularia	1	0	0	2.32
Fusarium	4	5	6	34.88
Mucor	1	0	0	2.32
Penicillium	2	1	0	6.97
Trichoderma	2	2	1	11.62
Verticilium	2	1	2	11.62
Total	19	13	11	100

A CMS medium consisting of 5 g of sterile sand and 35 g of maize flour was autoclaved at 120 °C for 30 min at a pressure of 1 bar and allowed to cool at room temperature in a jar. Forest soil was collected and sterilized three times at 24 hour intervals at 120 °C for 30 min at a pressure of 1 bar in an autoclave. Then, the previously prepared inoculum was inoculated in CMS medium and incubated in the dark at room temperature for 14 days so as to obtain a CMS medium infested with each *Fusarium* sp. isolate.150 g of CMS medium infested with each isolate was mixed separately with 350 g of previously sterilized forest soil. This mixture (CMS plus infested soil) was then distributed in 500 ml polyethylene culture pots as a soil for culturing banana vivoplants. The inoculation of banana vivoplants by *Fusarium* sp. isolates was done according to the (Meddah *et al*., 2011) inoculation method. The vivoplants were gron in pots on the soils infested with each *Fusarium* sp. isolate at a rate of five pots per isolate. Five vivo plants seedlings were also grown under the same conditions as above on non-infested soils to serve as control. Trial and control cultures were put in greenhouse and observed daily so as to notice leaf chlorosis and death of vivoplants. At the end of the pathogenicity test, Koch's postulate was verified in order to establish the host-parasite relationship.

RESULTS

Infection Status of banana tree plantations

Banana trees with yellow leaves in vegetation were distributed randomly in the infected plantations. The prevalence of banana treeleaf chlorosis ranged from 14.42 to 48.59%, that is, 28.23% on average for the three plantations. According to the Trapero-casas scale, disease prevalence was high in the plantations visited. The organs of infected banana trees showed external and internal symptoms. Outside, the majority of leaves were yellow (Figure 1A) and wilted (Figure 1B) and sometimes necrotic (Figure 1C). The stipes showed necrosis spots (Figure 1D). Internal symptoms were noticed inside the stipes in cross sections (Figure 2A). In the roots, brown necrosis spots were observed in the longitudinal sections (Figure 2C). However, none of these symptoms were observed in apparently healthy banana tree organs.

Fungal isolates from the rhizosphere soil and associated with banana tree leaf chlorosis

The isolations revealed 43 isolates in the infected banana tree samples belonging to the *Aspergillus, Alternaria,*

Figure 1. Syndrome of banana tree vascular infection A: External symptoms. B: internal symptoms

Fusarium sp. isolates stemming from monospore culture

The monospore culture of the 15 *Fusarium* sp. isolates stemming from primary cultures revealed the presence of 34 other *Fusarium* isolates divided into 13 morphological groups. The colonies of the morphological groups obtained were white, ivory, pink or purple with cottony, flaky aspects (Figure 2).

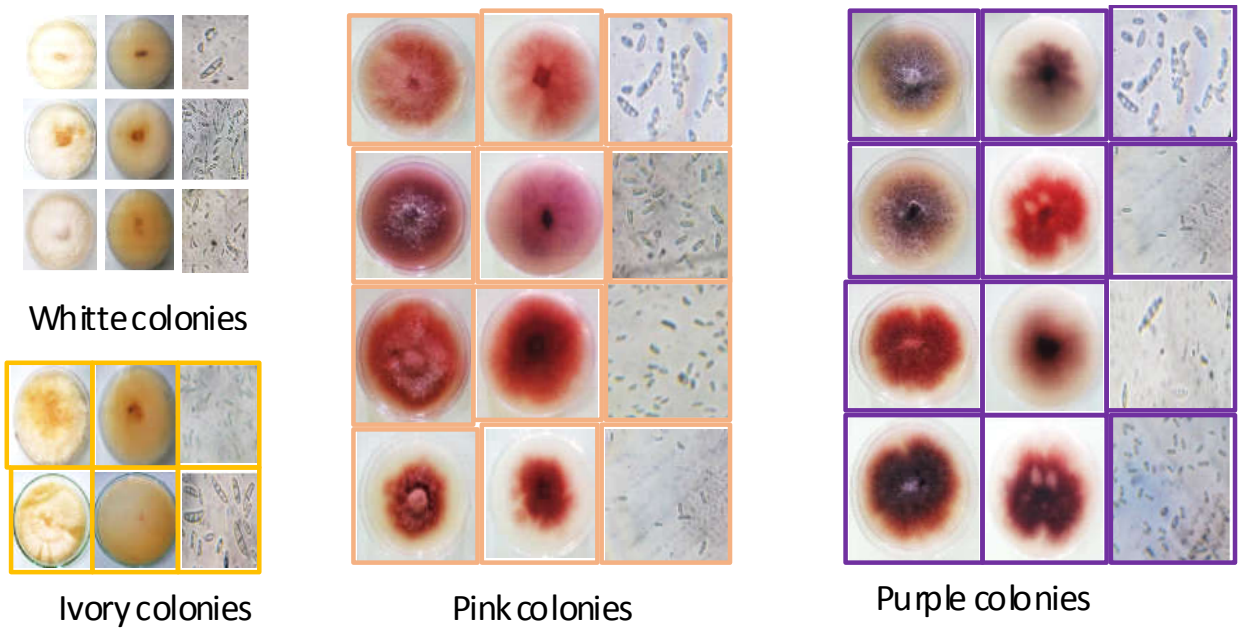

Figure 2. Colors and appearance of Fusarium sp. isolates stemming from samples of banana trees with chlorotic leaves

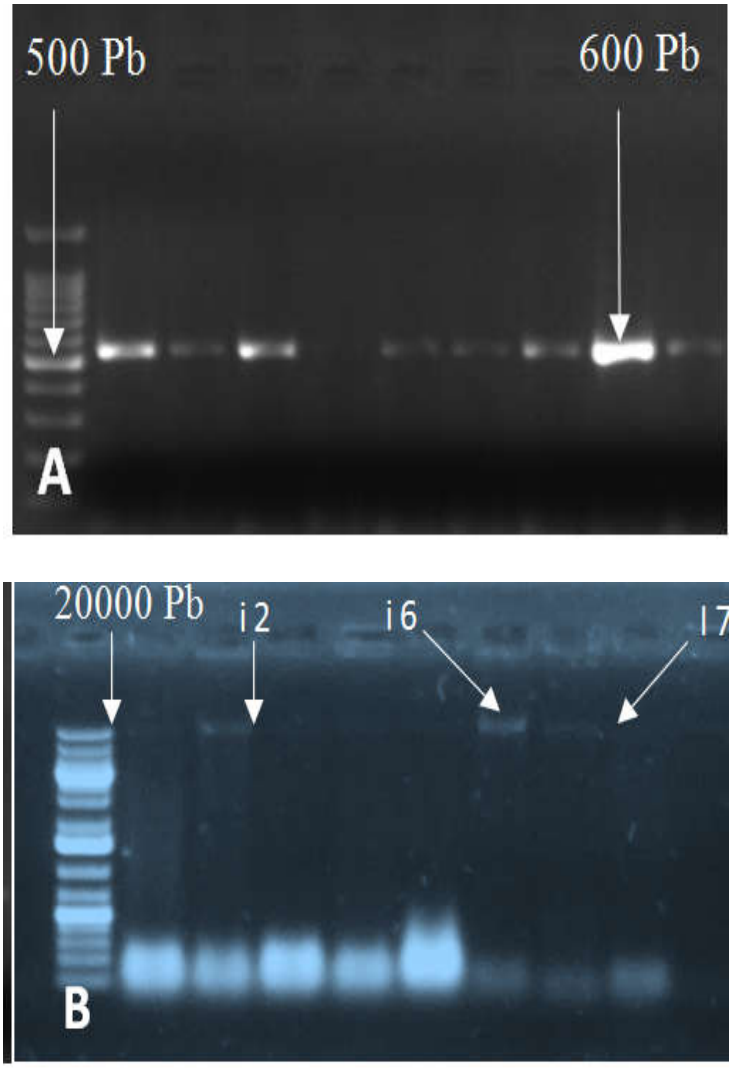

Figure 3: Electrophoresis gels of DNA PCR products of *Fusarium* isolates on 1.5% agarose gel. A: PCR products amplified with primer pair IT1/ITS4. B: PCR products amplified with the Foc1/Foc2 primer pair of *Fusarium* isolates DNA on 1.5% agarose gel

Molecular features of Fusarium sp.isolates

The DNA of nine isolates stemming from monospore culture was characterized. DNA amplification of the nine isolates with the ITS1/ITS4 primer pair generated a single 600 bp band (Figure 3A). Then, the amplification of the DNA of the nine isolates with the Foc1/Foc2 primer pair, only three isolates generated a band of 20 000 in the electrophoresis gel (Figure 3B)

Pathogenicity of *Fusariumoxysporum* f. sp. *cubense*isolates on banana treevivoplants

All *Fusariumoxysporum* f. sp. *cubense* isolates identified and tested caused yellowing, necrosis, leaf drying and death of the plants 21 days after inoculation of the vivoplants. In contrast, no symptoms were observed in control plants (Figure 4).
The host-parasite relationship was established by verification of Koch's postulate. The tested *Fusariumoxysporum* f. sp. *cubense* isolates caused the same symptoms observed in plantation on experimentally inoculated plants.

Figure 4. Banana tree plants after 21 days of inoculation with *Fusariumoxysporum f. sp. cubensemorphotypes*

DISCUSSION

The banana tree plantations visited were highly infected with leaf chlorosis. The high prevalence of leaf chlorosis in plantations. The varieties Corne 1 and Grande Naine grown by producers could be very sensitive to the causative agent of banana tree leaf chlorosis (Shall *et al* 2009, Raju *et al*., 2008). Infected banana trees expressed more the disease in the aerial organs especially the leaves and the stipe. Foliar discoloration might be due to chlorophyll degradation or dysfunction of chloroplast synthesis. According to Endry (1987), the causes of chlorophyll degradation are life-cycle changes, turn-over or continual replacement in chloroplasts and premature death caused by diseases and digestion by organisms. The causative agent might multiply in the vascular system and cause necrosis as well as the early senescence of chloroplasts observed. In Australia, works conducted by Moore *et al*. (1995) showed banana tree leaf chlorosis following Fusarium wilt infection. However, they also observed internal symptoms on the infected organs. In this work, the internal symptoms observed in infected banana trees were a necrotic root and vascular system. The causative agent of the disease could be a telluric vascular endo-parasite.

The symptoms observed could be attributed to the activity of plant pathogenic bacteria or fungi (Ploetz *et al.*, 2003). In the case of a bacterial infection, there would have been exudate production in the cross sections of the stipe (Liberato & Gasparotto, 2006). No exudate production was noticed in stipes in cross section. Plant pathogenic fungi might be the potential causative agents of banana tree leaf chlorosis (Su *et al*., 1986). In the case of fungal infection, the medium favors an infestation of the agent before it infects its host. The isolations made on the different samples of infected banana trees showed a great morphological diversity of *Fusarium* sp. isolates. These results were also noted by Meddah *et al*., (2010). The banana tree organs and the rhizosphere soil of infected plants could be favorable conditions for the multiplication and infection of *Fusarium* sp. isolates. In fact, Côte d'Ivoire is located in the tropical zone where it is both hot with sometimes heavy rains. These climatic conditions favor the development of fungal agents, especially those of the soil. Navas-cortes *et al*. (2000); Landa *et al*. (2006) and Sharma and Merehlbauer (2007) showed that temperature variations were a favorable factor for banana tree infection by fungi.
Moreover, the works of Kra *et al*. (2011) showed that Grande Nainebanana treeis a variety sensitive to Fusarium wilt, a disease caused by *Fusarium oxysporum* in Côte d'Ivoire. The banana tree infection observed could be the action of *Fusarium* sp. isolates stemming from collected samples (Kra *et al*., 2011). The presence of an agent on an infected host does not justify its involvement in the development of the disease. In contrast, the molecular identification of an agent responsible for a specific infection on a host or the pathogenicity test confirms the involvement of such agent in the development of the infection. The results of molecular analyses helped identify three *Fusariumoxysporum* f. sp. *cubense (Foc)* isolates. Among the *Fusarium* sp. isolates stemming from infected banana tree samples, there are banana tree pathogens and non-pathogens (Nel*et al*., 2005).

The pathogenicity test performed with the three *Foc*i solates showed the same symptoms on all vivoplants as those observed in infected plantations followed by their death after 21 days. The three *Foc* isolates caused leaf chlorosis of Grand Nainevivoplants by a succession of pathogenic activities. These results were also noted by Meddah *et al*. (2011) who observed leaf chlorosis of banana trees inoculated with identified strains of *Fusarium oxysporum* f. sp. *cubense* in Morocco. The works of Di-Pietro *et al*. (2003) and Jimenez-Gasco *et al*. (2004) showed that *Fusarium oxysporum* is a cosmopolitan fungus of telluric origin, which has a very high genetic and ecological diversity. The conditions were favorable to the infestation of a variety of *Foc*i solates in culture soils on the one hand. On the other hand, the sensitivity of banana tree variety allowed colonization and infection of the root system materialized by observed root necrosis. From the infected roots, *Foc* produces conidia which could be driven to the stipe by the ascending raw sap. Once in the stipe, the infection of vascular tissues is materialized by the presence of necroses. The dysfunction of the root system and obstruction of vascular tissue could prevent the rise of mineral elements, water and raw sap to the leaves which in turn would become yellow.

The *Foc* isolates identified might therefore be responsible for Grande Naine banana tree schlorosis observed in Côte d'Ivoire. Banana tree cultivation in Côte d'Ivoire is a source of income, for the state and people, which could be threatened by leaf chlorosis. The disease is expressed by several symptoms in plantation on plants of any age with a high prevalence. The cultivation soils of infected banana trees is favorable to the development of many plant pathogenic fungi, of which

Fusariumoxysporum might be the most significant and the cause of the infection. Molecular identification of three *Fusariumoxysporum* f. sp. *cubense* isolates is the first observation of this plant pathogenic agent among those previously responsible for banana tree infections in Côte d'Ivoire. The Grande Nainevariety cultivated by producers in rural and industrial plantations especially for export, is sensitive to *Fusarium oxysporum* f. sp. *cubense*. The identification of several *Fusariumoxysporum* f. sp. *cubense* isolates among the plant pathogenic fungi of banana tree in Côte d'Ivoire therefore has a scientific and economic interest.

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