

Sensitivity of *Colletotrichum* species responsible for banana anthracnose disease to some fungicides used in postharvest treatments in Côte d'Ivoire

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Abstract— Anthracnose is a major postharvest disease of banana fruit in Côte d'Ivoire. *Colletotrichum musae* is usually associated with banana anthracnose disease. Persistent symptoms of anthracnose despite the post-harvest treatment requires accurate characterization of pathogens for effective control. The present study was conducted to identify the species of *Colletotrichum* responsible for banana anthracnose and test their sensitivity to fungicides. The morphological study and molecular identification of isolated species associated with anthracnose symptoms had identified *Colletotrichum gloeosporioides* and *Colletotrichum musae*. Pathogenicity tests with representative isolates were conducted on symptomless banana fruits. All tested isolates caused anthracnose lesions on banana fruit, however *C. musae* was significantly more aggressive than *C. gloeosporioides*. Sensitivity tests including imazalil, azoxystrobin and boscalid showed a sensitivity of *C. musae* and *C. gloeosporioides* to imazalil. However, a resistance of both species to azoxystrobin and boscalid was recorded. This study highlighted the presence of resistant strains of *Colletotrichum* responsible for anthracnose in Côte d'Ivoire.

Keywords— Banana, Anthracnose, *C. musae*, *C. gloeosporioides*, Sensitivity

I. INTRODUCTION

Banana is an economically important crop in tropical and subtropical regions. Côte d'Ivoire is one of the main African producers of banana dessert. The bulk of Ivorian production is destined for the European market with an estimated export volume of 330460 tons in 2014 (Faostat, 2016).

However, fungal diseases are responsible for large losses in the banana production chains, especially during postharvest period. Banana anthracnose is considered as one of the most important diseases of banana (Jeffries *et al.* 1990). Infection on the banana usually starts during the development of the fruit but remains quiescent until the

fruit ripens; symptoms often manifest during storage. Symptoms appear as brown or black spots which enlarge depressed with ripening (Ranasinghe *et al.* 2005). Anthracnose deteriorates quality of bananas and causes economic losses for producers and traders (Ara *et al.* 2012).

In Côte d'Ivoire, anthracnose was found to be recurrent on bananas despite postharvest treatments. *Colletotrichum* species are agents associated with anthracnose on many tropical and subtropical fruits (Bailey *et al.* 1992). however, only *C. musae* was identified as responsible for banana anthracnose (Stover *et al.*, 1987).

Chemical control methods are the most used strategies against the fungi responsible for banana postharvest diseases (Gang *et al.*, 2015). Chemical control of banana anthracnose includes the use of active ingredients such as benzimidazoles, Strobilurines, and dicarboxamides (Young *et al.*, 2010). However, persistence of banana anthracnose has been noted in recent decades despite antifungal treatment applied by producers in Côte d'Ivoire. Resistant strains to these fungicides have emerged. Indeed, the recurrence of banana anthracnose could be due to intensive monoculture in industrial plantations which could favor the development of new pathogens strains.

The objective of this work was to identify the species of *Colletotrichum* responsible of banana anthracnose and evaluate their sensitivity to fungicides currently used in postharvest treatment.

II. MATERIAL AND METHODS

2.1. Sampling and isolation of *Colletotrichum* spp.

Boxed banana (Cavendish Subgroup, cv. Grande Naine) treated with fungicide were randomly selected at different banana production sites in Côte d'Ivoire (Abengourou, Abgenville, Aboisso Azaguié, Dabou, Grand Bassam and Tiassalé). Banana boxes were placed in storage at the laboratory at room temperature (27 ± 1 ° C) for 21 days and observed daily. During this period, symptoms

developed on the epicarp were observed and described. Banana fruits showing anthracnose symptoms were used for fungi isolation according to the method of Davet and Rouxel (1997). Pure cultures were obtained by single spore isolation carried out using the procedure described by Choi *et al* (1999), with modifications.

2.2. Morphological and cultural characterization

Each isolate from pure cultures was plated onto PDA and incubated at room temperature ($27 \pm 1^\circ \text{C}$). After 7 days, colony size, shape, margin and colour were recorded. Colony diameter of every culture was recorded daily for 7 days. Growth rate was calculated as the 7-day average of mean daily growth (mm per day). Three cultures of each isolate were investigated and experiments were conducted twice. For examination of conidial morphology, all isolates were subcultured as mentioned above. Cultures were washed with sterile water and drops of the suspension were placed on microscope slides. Length and width were measured for 50 conidia per isolate. Conidial shape (cylindrical or falcate) was also recorded.

2.3. Pathogenicity Test

Pathogenicity tests were performed with a representative set of isolates, from all morphological groups, using non-infected matured green unripe banana fruits (Cavendish Subgroup, cv. Grande Naine). Banana fruits were disinfested by immersing them in 1 % NaOCl solution for 1 min, washed twice with sterile distilled water. The fruits were blotted dry with a sterile paper tissue and inoculated using the wound/ drop method (Lim *et al.*, 2002; Kanchana-udomkan *et al.*, 2004). The wound/drop method involved pin-pricking the surface of the fruit to a 1 mm depth and then placing 10 μl of conidial suspension (1×10^6 spores ml/1) over the wound. Nine fruits were tested per isolate and experiments were conducted twice. The inoculated fruit, along with appropriate controls (fruit inoculated with sterile distilled water) were placed in storage into sterile plastic containers and incubated at laboratory temperature ($27 \pm 1^\circ \text{C}$). Symptoms were recorded 5 days after inoculation (d.a.i.) and re-isolation, according to Koch's postulates, was made from all resulting lesions. The lesion diameter was measured 5 days after inoculation and the mean diameter of the lesions was calculated.

2.4. Molecular characterization

2.4.1. DNA extraction

Total genomic DNA was extracted from fungal mycelium grown on potato dextrose agar (PDA) following the protocol of Murray and Thomson. (1980).

2.4.2. PCR and sequencing

PCR amplification of the rDNA ITS region was done using the universal primers ITS1 and ITS4 (White *et al.*, 1990) in a thermocycler T 100 (Bio Rad). The following amplification program was used: initial denaturation (5 min at 94°C), followed by 35 cycles each comprising a denaturation step (30 s at 94°C), a step of annealing (30 s at 55°C) and an elongation step (30 s at 72°C) and a final elongation for 10 min at 72°C . The PCR products were migrated at a voltage of 100 V for 30 min by electrophoresis in an agarose gel at 1% in a $0.5 \times \text{TBE}$ buffer (Tris-Borate 90 mM, 1 mM EDTA) and visualized in a visual reader of EBOX-VX5 brand. The PCR products were purified observed amplicons sequenced Sporometrics, 219 Dufferin Street in Canada.

2.5. *In vitro* susceptibility of *Colletotrichum* species to fungicides

Three commercial fungicides Bankit, Cumora, Sulima (Table 1) were evaluated *in vitro* using the poison food technique (Adams and Wong, 1991). Fungicide suspension of 1 ppm, 10 ppm, 50 ppm, 100 ppm, 500 ppm, 1000 ppm and 1500 ppm were prepared by dissolving requisite quantities of each fungicide in autoclaved cooled PDA just before pouring into Petri dishes. Fifteen ml of fungicide amended media was poured into each 9 cm sterilized Petri dishes. Three replicates were performed for each concentration of each fungicide. Medium without fungicide served as a control. After solidification of the medium, each dish was inoculated with a mycelial disc (5-mm diameter) taken from the periphery of actively growing colonies on PDA. The Petri dishes were incubated in dark at $27 \pm 1^\circ \text{C}$ until the control colony reached the margins of the Petri dish. The measurement of the diameter of the mycelial growth of the fungus were recorded on a daily basis, beginning with 24 hours after inoculation. Percent inhibition of growth of *Colletotrichum* species was recorded using the following formula (1):

$$I (\%) = \frac{dc - dt}{dc} \times 100 \quad (1)$$

where, **dc** is the average fungal colony diameter measured in control plate, with no treatment, and **dt** is the average fungal colony diameter measured in treated dishes

Table.1: Characteristics of fungicides used in banana postharvest treatment

actives ingredients	commercial name	Groups	Concentration
<u>Azoxystrobin</u>	<u>Bankit</u>	<u>Strobilurin</u>	250 g/l
Imazalil	<u>Sulima</u>	<u>Imidazol</u>	750 g/l
Boscalid	<u>Cumora</u>	<u>Carboxamid</u>	500 g/l

2.6. Statistical analysis

Analysis of variance (ANOVA) to a classification criterion was performed to compare mean growth of isolates, mean length and width of conidia. The average lesion diameter and susceptibility of fungi to fungicides were also compared. The means were separated using Least Significance Different Test at $p < 0.05$. The analyzes were performed using IBM SPSS Statistics 20 software.

III. RESULTS

3.1. Morphological and cultural characterization

Common morphological characteristics of colonies allowed the grouping of the isolates into two morphological groups (Table 2). Isolates of Group 1 had colonies with reddish slight pink moderate aerial mycelia. The colonies produced by isolates of Group 2 had white colonies with moderate aerial mycelia and copious cinnamon conidial masses (Fig 1). The mean growth rate of the various groups was calculated. There was no significant difference ($P = 0.74$) in growth rate among isolates of two groups. Isolates of group 1 had growth rates ranging from 4.36 to 4.8 mm day⁻¹. Isolates from group 2 had growth rates ranging from 4.9 to 5.2 mm day⁻¹. Isolates of group 1 had significantly longer conidia than those of group 2. Isolates of group 1 produced hyaline, cylindrical conidia with acute apex. The average length and width of the conidia were 11.25 μm and 2.5 μm , respectively. The group 2 conidia were all cylindrical with both ends rounded. The average length and width of the conidia were 23 μm and 5.5 μm , respectively. Isolated belonging to group 1 were confirmed to be *C. gloeosporioides* and isolated of the group 2 were *C.*

musae according to morphological and cultural characteristics.

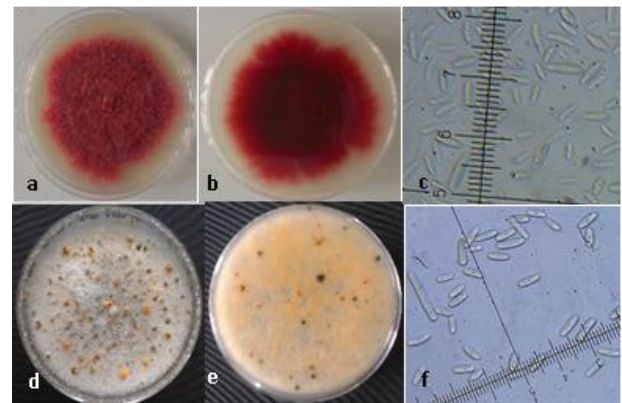


Fig.1: Morphological characteristics of *Colletotrichum gloeosporioides* and *C. musae* isolated from anthracnose of banana. *C. gloeosporioides* (a-c): (a) upper surface, (b) lower surface, (c) conidia, *C. musae* (d-f): (d) upper surface, (e) lower surface, (f) conidia.

3.2. Molecular identification of *Colletotrichum* species

The ITS region, including the 5.8S gene of all isolates was successfully amplified and sequenced. The size of the amplification product obtained was estimated to be 450 bp (Fig 2). *Colletotrichum* species belonging to group 1 was identified as *Colletotrichum gloeosporioides* under accession number MG515233.1. *Colletotrichum* species belonging to the morphological group 2 were identified under the accession number MG515228.1 as *Colletotrichum musae*.

Table.2: Summary of morphological data for *Colletotrichum* species

species	Colony color	Conidia (mean)			Growth rate (mm/day)
		Length (μm)	Width (μm)	Shape	
<i>C. gloeosporioides</i>	reddish slight pink	11.25	2.5	cylindrical with acute apex.	4.36-4.8
<i>C. musae</i>	white	23	5.5	cylindrical with both ends rounded	4.9-5.2

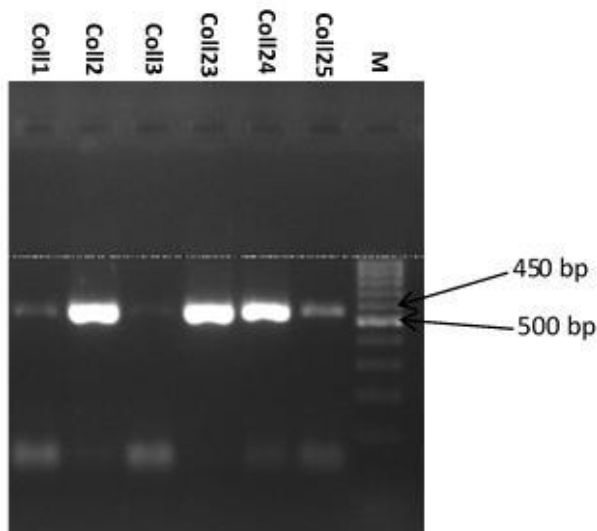


Fig.2: PCR amplification of ITS region of ribosomal DNA of *Colletotrichum* isolates with primer pairs ITS1 and ITS4

3.3. Pathogenicity testing

Characteristic symptoms of anthracnose were developed by all the isolates of *Colletotrichum* on banana fruits after wound inoculation. Generally, the lesions appeared 3 days post inoculation and expanded rapidly over 3–5 days. Lesions were black necrotic, circular and sunken, and these lesions showed white mycelia growth and produced orange colored conidial masses later. (Fig 3). Infections stimulate ripening of fruits, and lesions enlarge with ripening. Koch's postulates were confirmed by re-isolation of the fungi causing the lesions and identification as *C. musae* and *C. gloeosporioides*. Diameter of the anthracnose lesions varied among *Colletotrichum* isolates significantly ($P < 0.05$). The highest diameter of lesion was recorded with *C. musae* (4.03 cm). *C. gloeosporioides* gave the lowest lesion diameter (3.01 cm).



Fig.3: Symptoms of anthracnose disease on banana fruits after wound inoculation: (a): dry dark brown to black lesions with irregular margin and tan-to-orange-colored spores typical of *Colletotrichum musae* infection; (b): large black lesion with white mycelia typical of *C. gloeosporioides* infection ; (c): control

3.4. In vitro susceptibility of *Colletotrichum* species to fungicides

The susceptibility of *Colletotrichum* species to the various fungicides was presented through the mycelial growth inhibition rates in Table 3. There was a significant difference ($P < 0.05$) in susceptibility of *Colletotrichum* species to fungicide. For all fungicides tested, mycelial growth inhibition rate increased at the highest concentrations. Imazalil induced a complete inhibition of the mycelial growth of each species at 100 ppm. Imazalil was significantly superior to all the other fungicides followed by Azoxystrobin at all the test concentrations. *C. musae* and *C. gloeosporioides* were tolerant to Imazalil at concentrations ranging from 100 to 1500ppm. However both *Colletotrichum* species showed resistance to Azoxystrobin and Boscalid at all the test concentrations.

Table.3: Effect of different fungicides on mycelial growth (cm) of *Colletotrichum* species. by poisoned food technique after 7 days

species	fungicide	Percentage inhibition(%)						
		Concentrations (ppm)						
		1	10	50	100	500	1000	1500
<i>C. musae</i>	Imazalil	38 ^b	58 ^b	60 ^b	100 ^a	100 ^a	100 ^a	100 ^a
	Boscalid	0	0	0	0	0	20 ^c	25 ^c
	Azoxystrobin	0	0	20 ^c	24 ^b	35 ^b	35 ^b	36 ^b
<i>C. gloeosporioides</i>	Imazalil	65 ^a	68 ^a	70 ^a	100 ^a	100 ^a	100 ^a	100 ^a
	Boscalid	0	0	0	0	0	25 ^c	30 ^c
	Azoxystrobin	13 ^c	22 ^c	25 ^c	28 ^b	35 ^b	37 ^b	40 ^b

Means having a common letter in the same column are not significantly different at $P = 0.05$ according to Least Significant Difference Test

IV. DISCUSSION

A diversity of *Colletotrichum* species, including *C. musae* and *C. gloeosporioides* were isolated on bananas showing anthracnose symptoms. These results provide evidence that, in anthracnose pathosystems, the same host is often infected by different *Colletotrichum* species. Freeman *et al* (1998) showed that anthracnose of mango was caused by several species of *Colletotrichum*, including *C. acutatum*, *C. fragariae* and *C. gloeosporioides*. Than *et al* (2008) also characterized four species of *Colletotrichum* causes Capsicum spp anthracnose in Thailand. In the present study, the isolation of *C. gloeosporioides* on bananas showed anthracnose symptoms is justified by the potential of this species to infect a wide range of tropical fruits. *C. gloeosporioides* is the causative agent of anthracnose of many economically important hosts such as apple, strawberry and avocado (Afanador-Kafuri *et al.*, 2003). Moreover Latiffah *et al* (2014) showed that *C. gloeosporioides* and *C. musae* were associated with anthracnose of banana, and *C. gloeosporioides* was more prevalent than *C. musae* in Malaysia.

The identification of the isolates based on morphological characteristics showed a variation among *Colletotrichum* species. *C. gloeosporioides* isolates exhibited characteristics already described by several authors (Photita *et al.*, 2005; Cannon *et al.*, 2008) allowing their identification. Moreover morphological characters of *C. musae* described in this study are similar to the characters observed by Photita *et al.* (2005) and Thangamani *et al.* (2011).

Pathogenicity tests with the *Colletotrichum* species isolated, showed that all were able to infect and cause symptoms in wounded banana fruit, proving that both species were causal agents of anthracnose infection on banana. This study is the first report in Côte d'Ivoire to highlight the pathogenicity of *C. gloeosporioides* on banana. The results of Latiffah *et al* (2008) also showed the involvement of *C. gloeosporioides* in banana anthracnose in Malaysia. The fact that *C. gloeosporioides* was a pathogen of banana confirmed numerous reports about the cross-infection potential among different species of *Colletotrichum* on a multitude of hosts (Freeman *et al.*, 1998). A difference in virulence was found depending on the inoculated *Colletotrichum* species. The highest virulence of *C. musae* could be explained by the affinity of this species to banana. *C. musae* has been reported to be the most common causal anthracnose of many banana cultivars (Jinyoung Lim *et al.*, 2002, Priyadarshanie *et al.*, 2015).

Variability in fungicidal sensitivity among the *Colletotrichum* species was observed. A greater sensitivity of *C. gloeosporioides* to Imazalil was observed compared to *C. musae* which showed moderate

sensitivity. The inhibition of ergosterol biosynthesis induced by Imazalil would be more pronounced on *C. gloeosporioides*. This effectiveness of Imazalil was also shown by Andrivon (1997) on *Colletotrichum coccodes*. However, with Azoxystrobin and Boscalid, marked resistance of both species was noted. The strains of *C. musae* and *C. gloeosporioides* identified in this study were not be sensitive to these actives ingredients. Resistance to azoxystrobin and boscalid fungicides by fungal pathogens of banana fruits in Côte d'Ivoire may be attributed to continuous and indiscriminate use of these fungicides without rotation or alternating with other fungicides for the control of preharvest and postharvest banana diseases. According to Hobbelen *et al.* (2013), resistance phenomenon occurs after continued use of a fungicide on the target agent. A recent study in Senegal indicated that treatment with azoxystrobin showed no efficacy against post-harvest diseases of banana (Diedhiou *et al*, 2014).

V. CONCLUSION

The results of this study are relevant because, by demonstrating the diversity, virulence and sensitivity to fungicides of *Colletotrichum* species infecting banana fruits. These results will facilitate the development and implementation of disease management practices, thereby allowing producers to reduce the economic losses in banana production caused by anthracnose.

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