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Cultural, Morphological and Pathogenic Variability in *Colletotrichum gloeosporioides* f. sp. *Manihotis* Isolates from Cassava (*Manihot esculenta*) in Nigeria

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Abstract: Variability in fungal features in 30 isolates of *C. gloeosporioides* f. sp. *manihotis* Henn (Penz). Sacc (causal organism of cassava anthracnose disease CAD), from seven cassava growing regions of Nigeria (Oyo, Ogun, Ondo, Osun, Plateau, Kwara and Cross River State), where the disease is prevalent, were investigated on the basis of culture, morphology and pathogenicity. Culture and morphology showed variations in mycelial pigmentation, growth media colour, radial growth pattern, presence or absence of setae and acervuli production amongst isolates. Spore morphology differed ($P \leq 0.05$) in conidial length, spore density, germ-tube development. Pathogenicity test showed significant differences ($P \leq 0.05$) in virulence amongst the isolates. All thirty isolates were pathogenic to two test cassava genotypes, causing necrotic lesions after artificial inoculation by stem puncture. Fungal isolates 05, 10 and 26 were more virulent, causing lesion size exceeding 20 mm in susceptible test cassava genotypes at 21 days after inoculation. The relationship among fungal growth parameters (mycelial growth, conidial length, spore germination and sporulation density) and virulence of the fungal isolates, showed a significant positive correlation between spore germination and sporulation ($r = 0.72$), spore germination and virulence ($r = 0.76$). Sporulation was significantly correlated with virulence ($r = 0.83$), but not correlated with conidial length ($r = 0.19$).

Key words: Morphology, pathogenicity, variability, *Collectrichum groeosporioides*

Introduction

There exist more than one thousand described form-species of *Colletotrichum*, but based on cultural studies only 20 species are recognized (Von Arx, 1974). The over 900 remaining form-species names appear to be synonyms. For instance, about 600 synonyms exist for *Colletotrichum goeosporioides*, the imperfect stage of the ascomycete *Glomerella cingulata*. *G. cingulata* is a ubiquitous ascomycetous plant pathogen causing anthracnoses of many different host plants (Alexopoulos and Mims, 1979).

Sutton (1980) reported that *C. gloeosporioides* shows excessive variation and indicated that forms could probably be differentiated by a combination of culture characteristics, morphology, host range and pathogenicity. *C. gloeosporioides* f. sp. *manihotis* is host specific to cassava, although the teleomorph, *Glomerella cingulata* (Stonem) Spaul. and Schenk has a wide range of host plants such as yam, banana, cocoa, citrus and many other cultivated and wild plants (Lozano and Booth, 1974). Cassava anthracnose disease (CAD) is one of the most important cassava diseases in the cassava growing regions of Africa (Hahn *et al*., 1989), South America (Lozano *et al*., 1981) and Asia (Nair *et al*., 1979).

The CAD fungus attacks tissues weakened by the insect *puncture (Pseudotheraptus devastans*) and lytic action of saliva. This induces dark-brown lesions which can either remain limited or become deep-brown cankers with lacerated epidermis (Theberge, 1985). CAD in some cases spreads and causes tip-dieback, leaf wilting, defoliation, petiole epinasty and poor sprouting (Lozano and Booth, 1974).

Accurate assessment of yield loss due to CAD has not been evaluated but far back in 1975, more than 90% of the cassava cultivars grown in Zaire for example were rated severely infected (PRONAM, 1977). Severe outbreaks of CAD can lead to significant losses in planting materials and total crop failure during an epidemic outbreak (Van der Bruggen *et al*., 1990; Ikotun and Hahn, 1991).

The taxonomic and nomenclatural history of Colletotrichum species has been discussed and the culture and morphology fully described and illustrated in other crops. However, information on the variability in culture and morphology and pathogenicity of *Colletotrichum* causing anthracnose in cassava is limiting. This pathogen is host specific and the fact that the disease is of major hindrance to cassava production in the Tropics needs an information base on the fungal features and its variability in culture and pathogenicity. There is the need to study and characterize this fungus in culture, isolated from the different agro-ecological cassava zones of Nigeria, a country that has become the World leading cassava producers (FAO, 1993).

This study was conducted to determine the culture, morphology and pathogenic variability of thirty isolates of *C. gloeosporioides* f.sp. *manihotis* processed from two hundred plant samples from the cassava growing regions of Nigeria, where the disease is prevalent. This study also attempts to provide useful information on the fungal features and also establish a relationship between the fungal morphology and pathogenicity.

Materials and Methods

Collection, Isolation and Identification of the pathogen: Two hundred cassava stems showing symptoms of anthracnose infection (dark brown lesions, deep cankers) were collected from different cassava growing regions of Nigeria (Table 1). Small pieces of infected tissues were cut from advancing edges of the canker, surfaced-sterilized for approximately 3-5 minutes in 10% sodium hypochlorite solution and rinsed in five successive changes of sterile distilled water. The stem pieces were dried on sterilized filter papers and placed on potato dextrose agar containing 100 mg/dm^3 sodium novobiocin antibiotics for bacterial suppression (PDA + N). The plates were incubated at 25°C under 12 hours/day intermittent mixed irradiation from near ultra-violet and daylight fluorescent tubes (Philips T1 40W/8R5, F40 BLB and TL 40W/33R5 respectively), suspended 50cm above the plates. The maintenance and subculturing of the isolates were also on PDA + N.

Mycelial morphological features were observed on slides of mycelia and fruiting structures mounted in lactophenol cotton blue. Morphological characters compared comprised type of mycelium, fruiting structures, shape of conidia, presence or absence of setae and conidial length. The latter was estimated by measuring 100 spores per isolate using a graduated ocular

Table 1: Isolate source and cultural characteristics of *C. gloeosporioides* f. sp. *manihotis* at 25 ± 2°C on Potato dextrose agar.

Isolate number	Location		Cultural Characteristics		
	Source	State	Mycelial Colour	Growth Pattern	Media Colour
01	IITA (ES21)	OYO	Pinkish brown	Circular	Orange
02	IITA (ES18)	OYO	Greyish brown	Radial	Normal
03	IITA (BS13)	OYO	Whitish green	Radial	Yellow
04	IITA (ES5)	OYO	Milky white	Circular	Normal
05	IITA (ES24)	OYO	Darkish green	Radial	Normal
06	Moniya	OYO	Greyish green	Radial	Dark
07	Ilorin	Kwara	Darkish Green	Radial	Green
08	Edu	Kwara	Whitish	Circular	Yellow
09	Tanke	Kwara	Whitish	Circular	Dark
10	Asa	Kwara	Pinkish	Radial	Pink
11	Ilorin	Kwara	Whitish grey	Radial	Brown
12	Odeda	Ogun	Milky yellow	Radial	Yellow
13	Ago-Iwoye	Ogun	Darkish green	Radial	Normal
14	Odeda	Ogun	Pinkish	Circular	Pink
15	Abeokuta	Ogun	Greenish	Circular	Dark
16	Ikene	Ogun	Darkish grey	Radial	Normal
17	Ado-Ekiti	Ondo	Whitish green	Radial	Dark
18	Akure	Ondo	Milky yellow	Circular	Normal
19	Ajue	Ondo	Darkish green	Radial	Brown
20	Lafia	Plateau	Whitish green	Circular	Normal
21	Bauchi	Plateau	Pinkish green	Circular	Pink
22	Jos	Plateau	Whitish green	Radial	Normal
23	Vom	Plateau	Greenish grey	Radial	Dark
24	Ilesha	Osun	Darkish green	Circular	Brown
25	Akampka	Cross River	Pinkish	Radial	Pink
26	Ikang	Cross River	Whitish green	Circular	Normal
27	Calabar	Cross River	Pinkish	Circular	Brown
28	Tedo	Cross River	Greenish	Radial	Dark
29	Ife	Osun	Pinkish grey	Radial	Pink
30	Osogbo	Osun	Darkish grey	Circular	Normal

calibrated with the microscope stage. Media colour changes and mycelial pigmentation was visually assessed. All isolates were identified according to the procedures of Barnett and Hunter (1972). Radial growth, spore germination and spore density were also used as parameters for characterization. Plates with PDA + N, the bottom of which was marked with two perpendicular lines passing through the centre, were inoculated with a 5 mm diameter mycelial disc of each isolate and incubated for 48 h under alternate 12 h-light/12 h dark condition and a temperature of 25 ± 2°C for growth initiation. Radial growth was measured along the perpendicular lines and the mean was calculated for each treatment, which consisted of five replicate plates arranged in a randomized complete block design. Records of mycelial colony growth were taken at 3,5,7 and 9 days after growth initiation. For spore density measurements, spore suspensions were prepared from 7-day old cultures by flooding the plates with 5 ml of sterile distilled water and dislodging the spores with a small brush. The suspension was centrifuged at 2000 rpm for 3 mins and the supernatant was filtered through 2 layers of sterile muslin cloth. A drop of the resulting spore suspension was placed on a haemocytometer chamber and the number of spores/ml of sterile distilled water was estimated as the average of spores counted in 10 standard haemocytometer fields. Spore density was calculated using a formula adapted from Tuite (1969). Spore germination was recorded using standard procedures described by Kiraly *et al*. (1974). One hundred spores for each isolate were observed at 8 h and 16 h intervals and germination was based on microscopically visible germ-tube formation.

For pathogenicity/virulence studies, 30 isolates of *C. gloeosporioides* f. sp. *manihotis* collected from different cassava growing regions of Nigeria were used. Two cassava genotypes TMS 30211 (resistant) and TME1 (susceptible) (Ikotun and Hahn, 1991) were used as test plants. Stem cuttings 20 cm long, were planted in the greenhouse in sterilized soil in 30 cm diameter plastic pots. Two months after planting the plants were inoculated at the internodal region by stem puncturing (Muimba *et al*., 1983), using spore suspensions standardized at 10^5 spores/ml. Control plants received sterile distilled water and continuous watering was conducted to maintain a high relative humidity around the inoculated plants. Following inoculation, plants were observed and necrotic lesions diameter were measured at 7 day intervals for 21 days. A randomized complete block design with four replicates in split plot arrangement was used, with each cassava genotype representing the main plot and the fungal isolates representing the sub-plots.

Statistical analysis: The data for sporulation, spore germination, mycelial growth and necrotic lesions were subjected to analysis of variance (ANOVA) (SAS Institute,1989). Fischer protected LSD test of mean separation was performed only when the ANOVA showed significance. Correlation analysis was done to show the relationship between fungal growth paramters (mycecial growth, spore germination, sporulation, conidial length), with pathogenicity.

Results

Isolates varied in mycelial pigmentation, media colour, growth pattern and the presence or absence of setae (Table 1). Mycelial pigmentation varied from greenish-grey, dark, pinkish to milky white colour. The growth pattern was either circular with the mycelia showing a uniform growth pattern, or radial (mycelia

Table 2: Mycelial morphological characters of isolates *C. gloeosporioides* f. sp. *manihotis* at 25 ± 2°C on Potato dextrose agar on potato dextrose agar at 25 ± 2°C

Isolate number	Setae[a]	Acervuli[b]	Sporulation[c] x10^6 spores/ml	Conidial length[d] (µm)
1	Present	+	03.38n	10.45klm
02	Present	+ +	09.30u	07.15m
03	Present	+	07.60k	11.00kl
04	Present	+	11.94g	12.55hij
05	Present	+ + +	20.36a	13.75hij
06	Present	+	03.87m	11.55kl
07	Absent	+ + +	02.00r	15.40ghi
08	Present	+	13.50e	20.92cde
09	Present	+	06.23w	23.55bcd
10	Present	+ + +	14.58c	19.25efg
11	Present	+	10.56h	15.40ghi
12	Present	+ +	20.04b	20.35def
13	Present	+ + +	06.37i	15.95fgh
14	Present	+	02.45p	19.25efg
15	Present	+	01.10t	16.50fgh
16	Present	+ +	02.22q	13.75hij
17	Absent	+ + +	03.21o	08.25lm
18	Present	+ +	14.42c	13.75hij
19	Present	+ +	13.21f	16.50fgh
20	Present	+ +	04.10x	20.20def
21	Present	+ +	03.74n	24.20bi
22	Present	+ + +	06.53w	18.15efg
23	Present	+ + +	08.73j	11.55ijk
24	Absent	+	01.58s	30.25a
25	Present	+	03.23x	24.75b
26	Present	+ + +	13.93d	13.55hij
27	Absent	+ +	10.30i	29.70a
28	Present	+ + +	13.09f	16.55fgh
29	Present	+ +	07.98v	08.25lm
30	Present	+ +	01.23s	12.65ijk
CV(%)	-	-	17.33	03.35

+ = Few acervuli not united
+ + = Moderate acervuli and united
+ + + = Many acervuli all united
a,b = Each value is a mean of 5 replicates
c = Each value is a mean of 4 replicates
d = Value calculated from 10 standard microscope fields of 100 spores each.
* = Values in the same column followed by the same letters are not significantly different at P= 0.05 by Duncan multiple range Test.

Table 3: Isolate radial growth and spore germination of *C. gloeosporioides* f. sp. *manihotis* at 25 ± 2°C on Potato dextrose agar

Isolate number	Radial growth (mm) (days[a])				% Spore germination (hours[b])	
	3	5	7	9	8	16
01	17.00	38.67	58.67	71.33	23.33	38.00
02	24.33	50.00	60.33	77.00	42.33	60.00
03	23.33	40.33	54.67	64.33	36.67	72.33
04	23.67	39.00	52.00	56.67	24.33	36.00
05	40.00	67.33	83.00	90.00	65.67	85.00
06	32.67	63.00	75.67	82.33	46.33	57.33
07	31.33	51.33	68.00	74.67	31.00	38.67
08	35.67	48.33	66.33	72.33	51.67	85.67
09	29.33	45.67	58.00	65.33	49.33	70.67
10	33.67	57.33	78.67	85.67	65.67	85.00
11	21.67	39.67	52.00	61.33	34.33	49.67
12	32.67	63.67	72.33	86.00	71.00	93.33
13	33.33	44.67	63.67	72.00	55.33	82.00
14	23.00	42.67	60.33	73.67	15.52	24.00
15	30.67	50.67	77.33	85.33	63.67	86.33
16	27.00	48.67	65.67	74.33	43.67	64.67
17	15.33	37.00	59.67	72.33	27.33	37.67
18	29.67	55.33	70.33	86.67	76.00	84.33
19	18.67	40.67	53.00	60.00	47.00	62.67
20	18.33	34.00	53.00	66.33	37.00	46.33
21	30.67	50.67	77.33	85.33	63.67	86.33
22	37.67	53.33	70.33	79.00	79.67	84.67
23	19.00	36.67	65.00	82.00	57.00	70.67
24	24.00	40.00	79.33	88.67	14.33	24.33
25	32.67	56.00	77.33	87.00	41.00	70.67
26	30.67	55.33	74.00	82.33	79.33	94.00
27	40.67	53.33	69.67	83.33	54.67	71.00
28	39.00	58.33	76.66	89.66	56.00	80.33
29	27.67	42.00	69.67	77.33	28.00	40.70
30	0.00	0.00	0.00	0.00	0.00	0.00
Control	19.00	0.00	35.67	0.00	52.33	0.00
	62.33	0.00	43.33	0.00	58.00	0.00
Mean	28.10	47.97	66.10	76.00	46.93	64.07
CV(%)	18.73	08.32	08.26	06.23	08.67	08.24
LSD) (0.05)	04.04	06.76	09.07	07.73	05.26	07.33

a = Each value is a mean of 5 replicates
b = Each value is a mean of 4 replicates

growing in a ringlike pattern). Setae were produced by 26 of the 30 isolates. (Table 2). Acervuli were catigorized into three classes. There were a few acervuli not united, moderate and united and many acervuli all united into large masses. Conidial length and spore density differed ($p \leq 0.05$) amongst the isolates. Spore density was high in isolates 05, 12, 10, 18 and 08, with maximum values of 20.36 x 10^6 pores/ml of sterile distilled water recorded in isolate 05. The isolates with very low spore densities were 15, 30, 24 and 07, with the lowest spore load of 1.10 x 10^6 spores/ml. Maximum mean conidial length of 30.25 µm was recorded in isolate 24 and the least mean value of 7.15 µm was recorded in isolate 02 (Table 2).

Radial growth and spore germination differed ($P \leq 0.05$) amongst the isolates at the indicated periods of incubation (Table 3). After 9 days of incubation isolates 05, 28, 24 and 25 showed the highest radial growth rates, with diameters above 80mm. Greatest spore germination after 16 hours was recorded in isolates 26 (94%),12 (93.3%) and 21 (86.3%).

Pathogenicity/virulence showed that all the 30 fungal isolates were pathogenic to the test cassava genotypes with different levels of invasion capacity. There was no evident of necrotic lesion recorded for the control plants (Table 4). All isolates caused necrotic lesions on the young stem tissues after fungal stem puncture inoculation. There was a gradual increase in the size of necrotic lesion at indicated period of inoculation. Isolates 05, 10 and 12 were more virulent causing necrotic lesions above 20 cm in all the test cassava genotypes, at 21 days after inoculation for all isolates tested. Non-inoculated cassava stems showed small raised lesions, which failed to develop further.

The relationship among fungal growth parameters and virulence (Table 5), showed a significant positive correlation between spore germination and sporulation (r = 0.72), spore germination with virulence (r = 0.76). Sporulation was significantly correlated with

Table 4: Virulence of isolates of *Colletotrichum gloeosporioides* f.sp. *manihotis* on two cassava genotypes following stem puncture inoculation

Isolate number	Necrotic lesion diameter (mm) at indicated time after inoculation (days)[a]					
	TMS 30211 (Resistant)			TME1 (Susceptible)		
	7	14	21	7	14	21
01	4.20	6.70	8.33	5.67	8.00	15.70
02	6.33	7.75	9.55	6.67	11.00	14.33
03	3.86	5.92	6.67	8.00	11.33	15.67
04	6.67	7.33	9.00	10.33	14.67	19.67
05	7.33	9.45	10.60	12.00	16.67	24.67
06	4.33	6.33	7.33	5.33	9.67	13.67
07	4.67	8.35	8.98	7.30	10.74	14.25
08	3.00	4.67	7.55	7.67	11.33	16.00
09	5.10	5.70	7.25	10.33	13.67	19.33
10	6.60	9.47	11.35	15.00	18.67	25.33
11	5.67	7.15	8.55	5.67	9.33	18.00
12	8.66	10.10	12.40	14.67	19.33	25.67
13	4.28	6.18	7.90	8.33	11.33	15.67
14	5.30	5.89	6.33	8.70	11.00	17.35
15	4.67	5.70	7.55	6.60	8.63	15.57
16	5.65	7.35	8.66	6.00	8.33	16.67
17	3.65	5.55	6.95	7.00	10.33	17.00
18	5.00	6.22	7.85	11.67	15.00	19.33
19	6.67	7.79	9.17	6.33	9.33	16.53
20	6.33	9.53	10.60	7.00	10.67	19.55
21	4.10	5.45	6.39	5.33	8.00	17.13
22	6.67	7.35	8.15	9.33	11.67	15.00
23	3.00	5.67	6.50	6.00	10.00	16.33
24	4.22	6.45	7.30	8.00	10.33	14.65
25	5.00	5.85	6.74	10.33	12.00	15.33
26	6.80	8.19	10.55	14.00	17.00	21.33
27	3.77	4.80	5.52	8.00	11.67	19.55
28	5.33	5.97	6.68	5.67	10.00	14.67
29	5.42	6.33	8.75	4.67	8.63	16.00
30						
Control	5.82	0.00	6.10	0.00	8.43	0.00
	8.00	0.00	10.67	0.00	15.33	0.00
Mean	6.27	6.84	8.25	8.32	11.63	17.51
CV	18.13	15.32	11.83	18.63	14.78	11.53
LSD (0.05)	02.25	02.98	03.02	02.52	02.81	03.96

a = Each value is an average of five canker measurements/plant in three replicates.
TMS = Tropical Manihot Specie; TME = Tropical Manihot Exotic

Table 5: Relationship between fungal growth parameters and virulence in *Colletotrichum gloeosporiodes* f. sp. *manihotis*.

	Mycelial growth (mm)	Spore grem -ination	Conidial length	Sporul -ation	Virulence
Mycelial growth (mm)		0.32	0.15ns	0.58*	0.30ns
Spore germination			0.23	0.72**	0.76**
Conidial length				0.19ns	0.22ns
Sporulation					0.83**
Virulence					

*,** = Significant at 1% and 5% probability levels.
Ns = Not significant at 1% and 5% probability levels.

virulence (r = 0.83). The relationship between mycelial growth and virulence was non significant (r = 0.30).

Discussion

The significant variation in culture characteristics, mycelial morphology and pathogenicity amongst the test isolates indicated that *C. gloeosporioides* f. sp. *manihotis* can best be characterized by a combination of culture characteristics, morphology and virulence on host plants.

Earlier work by Chevaugeon (1956) distinguished three groups of *C. gloeosporioides* on the basis of pigmentation and spore production. Pigmentation could be used as a criterion for differentiating the fungal isolate at the preliminary stage of isolate selection for physiologic race studies. Hindorf (1973) showed that although culture characteristics vary greatly in most respects, groups based on similarities in colour and texture may be discerned. Sutton (1980) emphasized the *C. gloeosporioides* shows excessive variation and indicated that forms could probably be differentiated by a combination of culture characteristics, morphology, host range and pathogenicity, but conceded that *C. gloeosporioides* and its various forms were neither morphologically nor biologically sharply divided.

The differences in sporulation capacity among isolates could be a useful parameter for characterizing fungal isolates, due to the fact that variation in spore density among the fungal isolates was significant. Fungal isolates with high spore density has been shown to increase the the rate of invasion of cell walls of tropical fruit crops by *Colletotrichum* spp., (Jeffries *et al.,* 1990). Fokunang *et al*. (1995) reported that under favourable conditions, the isolates of *C. gloeosporioides* f. sp*. manihotis* consistently sporulated producing abundant secondary and tertiary inoculum for subsequent cycles of infection. Makambila and Bakala-Koumouno (1982), reported variability in radial growth and germ-tube development amongst *C. gloesporioides* f. sp. *manihotis*, which was assumed to result from heterokaryosis, parasexuality or mutation within the fungal isolates. Muimba *et al*. (1983), observed considerable variation in linear growth of fungal isolates in their generic diagnosis of *C. manihotis* which was based on a traditional approach using parameters such as appresorial morphology, conidial size and shape.

The differences in infectivity of the pathogen isolates to the test cassava genotypes showed the relationship between resistance and susceptibility of host to the fungus and also the rate of invasion of the fungus. Van der Bruggen *et al*. (1990) reported that *C. gloeosporioides* f.sp*. manihotis* colonized damaged tissues at an early stage to cause stem necrotic lesions. Van der Bruggen and Maraite (1987) showed that resistant cassava varieties react to the CAD pathogen invasion through the formation of regenerative zone, made of cork layers which are formed at the level of the initial necrotic lesions than in the primarily healthy stems. It is possible that the resistance of cassava genotypes to fungal isolates was due to pathogenic variability of the isolates or the differential reaction of host sensitivity of each cassava genotype. Boher *et al*. (1983) reported that the differences in isolate variability were evident with respect to their relative pathogenicity to different cassava genotypes as demonstrated in this paper.

Considerable variability was observed in isolates of *C. gloeosporioides* f.sp*. manihotis* in terms of colony, morphology, mycelial morphology and virulence. Tjamos *et al.* (1993), reported that genotypes of a fungal pathogen can differ from each other in many inherited characteristics, for example in morphology, physiology and pathogenicity. The variability which is of particular importance to plant breeders is that which

concerns the development and dissemination of new physiologic races, with the ability to attack varieties that were previously resistant.

The sporulating capacity of fungal isolates may have an important implication in the study of the epidemiology of CAD. The variability in fungal pathogenicity and the close relationship between sporulation and virulence could provide a useful information base for screening cassava germplasm collections for resistance to the pathogen and subsequent breeding programs for durable resistance. This is possible through the selection of highly sporulating and virulent fungal isolates for example to screen cassava genotypes and then tests their performance under different field conditions at different agro-ecological zones for resistance to the anthracnose pathogen.

Culture, morphology and pathogenic variability could be preliminary parameters for characterizing the fungal isolates. These parameters are limiting due to the fact that they can not give details about variation in races or pathotypes. Variation in pathotypes is essential for the understanding of the genetic structure of the population and would provide useful information programmes, epidemiological studies and disease management. Further progress in molecular studies is ongoing for a diagnostic approach and characterization *C. gloeosporioides* f.sp *manihotis* through the application of Random amplified polymorphic DNA (RAPD) assay. It is hoped that the result would provide a good molecular database for race/strain studies *on C. gloeosporioides* f.sp. *manihotis* causing cassava anthracnose disease, that has become an epidemic problem in Nigeria, one of the World's cassava producing nation.

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