Preliminary Studies of *in vitro* Stimulation of Sexual Mating among Isolates of *Mycosphaerella fijiensis*, Causal Agent of Black Sigatoka Disease in Bananas and Plantains

Ebimieowei Etebu,[1,*] Cornelia Pasberg-Gauhl,[2] Friedhelm Gauhl[3] and Lloyd Ayibo Daniel-Kalio[1]

Single-ascospore-derived isolates of *Mycosphaerella fijiensis* Morelet from false horn 'Agbagba' plantain leaves obtained from five different villages in southern Nigeria were stimulated to mate under artificial conditions. Pairs of isolates were incubated under blacklight on potato dextrose agar (PDA) with surface-sterilized plantain leaves or on PDA with autoclaved plantain leaves. Some isolates were observed to be sexually compatible by their ability to produce spherical to bulb-shaped fruiting body structures (FBS) and ascospores on pairing. FBS were observed to measure between 39–65 μm (smallest diameter) and 39–104 μm (largest diameter; mean 55.3×71.1 μm) in diameter, whereas ascospore lengths measured between 13.0 and 14.9 μm. Length of incubation time required for FBS production was dependent on the pair of isolates involved, the average being 40.1 days. With some pairs, ascospores were observed after 35 days of incubation.

KEY WORDS: *Mycosphaerella fijiensis*; mating; sexual reproduction; black Sigatoka; *Musa* sp.

INTRODUCTION

Black Sigatoka disease caused by the ascomycete fungus *Mycosphaerella fijiensis* Morelet is the most destructive leaf spot disease of plantains and bananas. *M. fijiensis* produces pseudothecia, ascospores and conidiospores prolifically on plantain and banana leaves. The major source of inoculum for infection is ascospores discharged from older infected leaves (6,9). Yield losses due to this disease on plantains and bananas have been reported to be between 20% and 90% (1,4,10,14,20).

Although resistance to black Sigatoka disease has been a breeding objective for many years (1,16,21), knowledge of the pathogenic potential of the pathogen and its capacity for sexual reproduction and genetic recombination has not been specifically addressed (5). Studies of the genetic structure of *M. fijiensis* on a global scale showed that populations can maintain a high level of genetic diversity and that recombination plays an important role in this pathogen (2,3). Furthermore, reports on the appearance of strains of *M. fijiensis* resistant to different fungicides abound (1,15,17,19).

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[1]Plant Pathology Unit, Dept. of Biological Sciences, Rivers State University of Science and Technology, Nkpolu, Port Harcourt, Rivers State, Nigeria. *Corresponding author [e-mail: eetebu@hotmail.com].

[2]P.M.B. 633-6150, Santa Ana, Costa Rica [cpasberg@racsa.co.cr].

[3]Chiquita Brands International, San José, Costa Rica [fgauhl@chiquita.com].

Knowledge of genetic recombination of *M. fijiensis* is therefore imperative for black Sigatoka breeding and resistance management, since recombination determines the appearance of new genotypes breaking host plant resistance or developing tolerance against fungicides. However, this can be achieved by first stimulating the pathogen to undergo sexual reproduction *in vitro*. *M. fijiensis* grows slowly on common culture media. Although pseudothecia are produced abundantly in the field, very little is known about laboratory studies of sexual reproduction of *M. fijiensis*. In 1990, for the first time, Mourichon and Zapater reported pseudothecia from sexually compatible *M. fijiensis* isolates under controlled conditions *in vitro* (11). However, these authors did not report the harvest of viable ascospores obtained *in vitro*. Since then, to the best of our knowledge, no other work has been published on *in vitro* sexual studies of *M. fijiensis*. Mengistu *et al.* (7) describe different methods to achieve sexual mating and the production of ascospores *in vitro* with single ascospore isolates of *Leptosphaeria maculans* (Desmaz.) Ces. & De Not.

There is a need for more reliable and repeatable methods of ascospore production for controlled mating and genetic analysis. This work aimed at gaining more insight on *M. fijiensis* using a simple methodology and compatible pairs to stimulate sexual mating among *M. fijiensis* isolates from southern Nigeria under artificial conditions.

MATERIALS AND METHODS

Media preparation Potato dextrose agar (PDA; Difco, Detroit, MI, USA) from dehydrated powder was prepared and sterilized according to the manufacturer's specification and plantain leaf pieces were added. Unexposed parts of 'Agbagba' false horn plantain leaves which had not unrolled (forming a 'cigar') were taken from plants already established in the field. Leaves were cut into small pieces of ~ 2 cm^2 and processed as follows:

(i) PDA with surface-sterilized plantain leaves (PSL): Four surface-sterilized (0.88% sodium hypochlorite, 7 min) plantain leaf pieces were dipped in molten sterile PDA and placed on top of already solidified, sterile PDA in each glass petri dish.

(ii) PDA with autoclaved plantain leaves (PAL): Instead of surface sterilizing, leaf pieces were put into glass petri dishes containing ~ 40 ml PDA each and autoclaved. Before the PDA solidified, leaf pieces were extracted from the medium with forceps and four pieces were placed on top of already solidified PDA in each glass petri dish.

Fungal Cultures Single-ascospore-derived isolates of *M. fijiensis* from five villages in southern Nigeria (Table 1) were subcultured on PDA at 25–29°C for 10–20 days in order to obtain actively growing cultures for mating experiments. Mycelia of the most vigorously growing isolates were scraped into small quantities of sterile distilled water to form separate suspensions.

Aliquots (~ 2 ml) of the fungal suspension of each selected isolate were mixed with aliquots of each of the others in separate petri dishes. Suspension mixtures were then placed directly on top of the four leaf pieces (PAL or PSL) in petri dishes. Then the petri dishes were sealed with Parafilm and incubated for 56 days under continuous blacklight, produced by two Sylvania fluorescent lamp tubes (F40/350 BL) fixed 40.5 cm above the culture plates. Each pair of isolates was thus cultured four times (replications) in the same petri dish per medium. Temperature was maintained between 25 and 29°C.

After 21 days of incubation, the top of each pair of cultures was scraped and viewed under a Leitz Laborlux S microscope x20 fitted with a Leica Wild MPS 52 camera, for

TABLE 1. Villages selected for the sampling of single ascospore isolates of *Mycosphaerella fijiensis* on false horn plantain Agbagba in southern Nigeria, 1995

Village	State	Latitude, N	Longitude, E	Isolate No.
Onne	Rivers	4°43'	7°11'	542
Kpite	Rivers	4°44'	7°18'	573
Igbofia	Cross River	5°30'	8°05'	639, 645, 650, 653
Umoghun	Edo	5°59'	5°57'	848, 853, 854, 862
Omotosho	Ondo	6°47'	4°38'	803

the presence of asci, ascospores, pseudothecia, and/or other fruiting body structures (FBS). Then the plates were resealed and incubated for re-examination after 28, 35, 42, 49 and 56 days.

Exp. 1: Stimulating sexual reproduction among isolates of *M. fijiensis* on different culture media Mycelia of isolates 639, 645, 650 and 653 from Igbofia and isolates 848, 853 and 862 from Umoghun were paired with mycelia of isolate 854 also from Umoghun (Table 2). Isolate 854 was used as a partner of all the other isolates, because in earlier experiments it had always produced the most vigorous cultures. In other words, cultures from isolate 854 grew faster than cultures from other isolates, and always produced conidiospores earlier than did other isolates (unpublished data). Suspension mixtures were placed directly on top of the four leaf pieces of both media, PAL or PSL, in petri dishes. Growing cultures were evaluated as described above.

Exp. 2: Compatibility among isolates from the five villages Single ascospore isolates of *M. fijiensis* from five villages were used to pair every isolate with each other (Table 3) and plated onto PAL, incubated, and evaluated as described above. Only PAL was used, since it was easier to prepare and handle. Additionally, previous results demonstrated that there was no difference in the mating of *M. fijiensis* when using PSL or PAL.

RESULTS AND DISCUSSION

Isolates from Umoghun and Igbofia were selected for Exp. 1 because these villages were most distant from each other (Table 1). Carlier *et al.* (3) and Müller *et al.* (12,13) reported that isolates from distant areas were genetically more different from each other, than isolates collected from more closely located areas. In Exp. 1, four of the seven combinations of artificially paired isolates produced FBS after 21 to 35 days of incubation (Table 2), indicating that some reproductive processes followed pairing under the conditions mentioned. To the best of our knowledge this is the first time that (partially) compatible pairs from isolates of *M. fijiensis* produced FBS and ascospores under the described conditions in the laboratory. In the material scraped from fungal colonies of pair 650*854, FBS were always present, irrespective of medium. Pairs 639*854 and 650*854 produced FBS also on both media, whereas pairs 853*854 and 653*854 produced FBS only on PAL or PSL, respectively. Of the four pairs that produced FBS, three also produced ascospores (Table 2). Pair 639*854 produced ascospores on both media, whereas pairs 853*854 and 653*854 produced ascospores only on PAL or PSL, respectively. However, it is also possible that artificially paired cultures without FBS or ascospores were not given enough time to produce (or not) fertile perithecia.

TABLE 2. Formation of fruiting body structures (FBS) and/or ascospores among single ascospore isolates of *Mycosphaerella fijiensis* from southern Nigeria on different culture media, under continuous blacklight at 26–29°C, 1995

Pairs	Incubation time (days)					
	21	28	35	42	49	56
PAL[z]						
639*854	-	+	+x	-	+	-
645*854	-	-	-	-	-	-
650*854	+	+	+	+	+	+
653*854	-	-	-	-	-	-
848*854	-	-	-	-	-	-
853*854	-	-	x	-	-	+
862*854	-	-	-	-	-	-
PSL[z]						
639*854	-	+	x	-	-	-
645*854	-	-	-	-	-	-
650*854	+	+	+	+	+	+
653*854	-	+	x	-	-	-
848*854	-	-	-	-	-	-
853*854	-	-	-	-	-	-
862*854	-	-	-	-	-	-

[z]PAL: PDA with autoclaved plantain leaf pieces; PSL: PDA with surface sterilized plantain leaf pieces.
- Neither FBS nor ascospore(s) observed.
+ FBS only observed.
x Ascospore(s) only observed.
+x Both FBS and ascospore(s) observed.

TABLE 3. Formation of fruiting body structures (FBS) and/or ascospores among five single ascospore isolates of *Mycosphaerella fijiensis* from southern Nigeria after incubation on PAL[z], under continuous blacklight at 26–29°C, 1995

	Incubation time (days)					
Pairs	21	28	35	42	49	56
650*542	+	+	+	+	+	+
650*573	-	-	-	+	+	+
650*803	-	-	-	-	-	+
650*854	-	+	+x	+	+	+
542*573	-	-	-	-	-	-
542*803	-	-	-	-	-	-
542*854	-	-	-	-	-	-
573*803	-	-	-	-	-	-
573*854	-	-	-	-	-	-
803*854	-	-	-	-	-	-

[z]PAL: PDA with autoclaved plantain leaf pieces.
- Neither FBS nor ascospore(s) observed.
+ FBS only observed.
+x Both FBS and ascospore(s) observed.

All the pairs involving isolate 650 produced FBS in Exp. 2 (Table 3). In fungal material scraped from colonies 650*854, ascospores were also found after 35 days of continuous incubation (Table 3). The other pairs of isolates did not produce FBS and/or ascospores. In addition, during Exp. 1, pair 650*854 was the only one which on all observation dates and on both media, FBS were always found. This suggests that isolate 650 is sexually different from and (partially) compatible with the other isolates.

TABLE 4. Incubation time (IT) for production of fruiting body structures (FBS) from different pairs of *Mycosphaerella fijiensis* after incubation under continuous blacklight for 21–56 days at 26–29°C, southern Nigeria, 1995

Pair	Mean IT for FBS production (days)
650*542	28.0 a[z]
650*854	33.3 ab
650*573	47.3 bc
650*803	56.0 c

[z]Means followed by a common letter do not differ significantly at P=0.05 according to Duncan's Multiple Range Test.

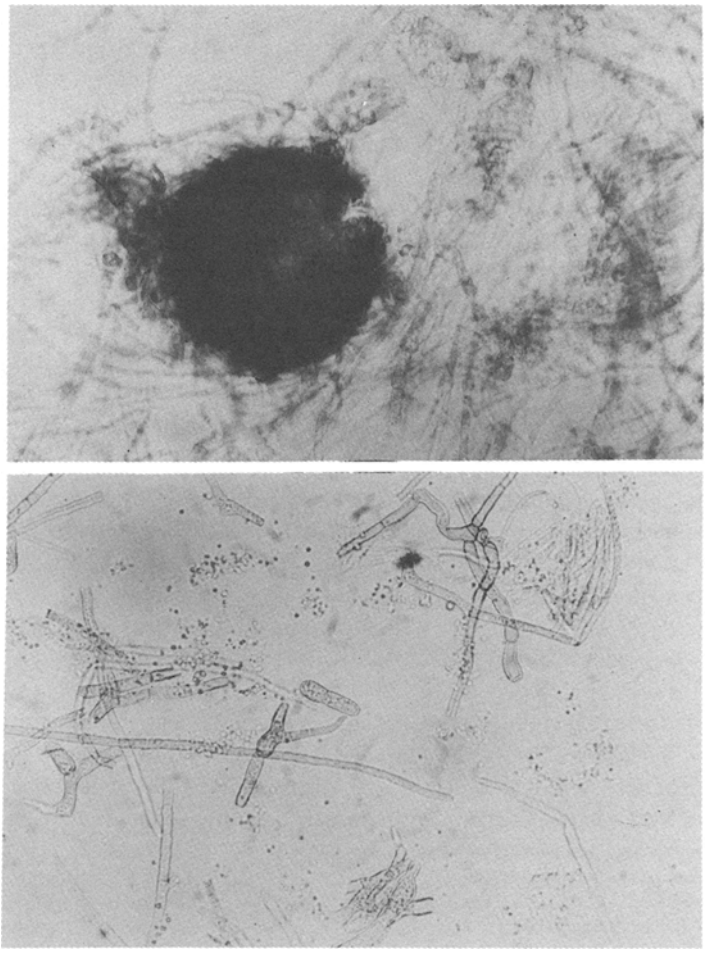

Fig. 1. Mating between single ascospore isolates of *Mycosphaerella fijiensis in vitro*. Top, fruiting body structure; bottom, released ascospore.

It was interesting to note that if ascospores were produced, they were observed after 35 days of incubation, irrespective of the medium and isolates involved (Tables 2 and 3). As incubation time increased, ascospores were no longer observed from these pairs or any

other pair. Under natural conditions it takes about 42 days for ascospores to be produced *in vivo* in false horn Agbagba plantain leaves (6). Mourichon and Zapater (11) inoculated young banana plants in the greenhouse with different pairs of *M. fijiensis* and observed formation of pseudothecia approximately 5 weeks after inoculation.

Of the seven pairs that produced FBS (Tables 2 and 3), four pairs representing 57% also produced ascospores, indicating that the FBS were fertile. Except for these combinations which produced ascospores, no complete compatibility was proven for the other combinations. It seems that sexually compatible isolates require a certain development period before pairing and conditions suitable for mating. Mourichon and Zapater (11) reported also cases of sexual incompatibility between isolates of *M. fijiensis* upon pairing *in vitro*. Thus, sexual mating among *M. fijiensis* isolates depends on their sexual compatibility, *i.e.*, two different mating types must unite to complete a sexual cycle. *M. musicola*, a closely related fungus, has been reported to be heterothallic also (18).

Although four pairs produced FBS in Exp. 2 (Table 3), the incubation time required for their production varied significantly (P=0.05) between 28 and 56 days (Table 4). Stover (18), working with *M. musicola*, observed pseudothecia in banana leaves after 4 to 6 weeks of inoculation with paired ascospores or conidial cultures. The significant differences observed could be due to their innate genetic composition, which probably affects their rate of genetic recombination which occurs during sexual reproduction (1,2,3,5,12,13).

Spherical to bulb-shaped FBS (Fig. 1, top) and ascospores (Fig. 1, bottom) were observed, resembling respectively pseudothecia and ascospores of naturally occurring *M. fijiensis*. While diameters of FBS from combinations of artificially paired isolates measured between 39–65 μm (smallest diameter) and 39–104 μm (largest diameter; mean 55.3–71.1 μm; n=18), ascospores lengths measured between 13.0 and 14.9 μm (Fig. 1), respectively. Measurements of diameters for naturally produced pseudothecia range between 50 and 85 μm (mean 63 μm) and lengths of ascospores range between 11.5 and 15.6 μm (mean 13.7 μm), respectively (8). The resemblance of observed FBS and ascospores to those described by Meredith and Lawrence (8) as occurring naturally within diseased leaf tissues suggests that sexually compatible pairs, mated *in vitro*, may have gone through a sexual reproductive process equivalent or similar to the natural one.

In summary, it was shown that sexually compatible isolates of *M. fijiensis* can be stimulated to reproduce sexually under artificial conditions, as proved by their ability to produce FBS and/or ascospores upon pairing. Incubation time required to produce FBS among pairs varied significantly (P=0.05) between 28 and 56 days from pair to pair depending on the isolates involved, indicating that there are differences in fertility among isolates. Single ascospores observed after 35 days of incubation could not be harvested because there were too few of them. More research is still needed to increase the capacity for pseudothecia and ascospore production.

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