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ARTICULOS GENERALES

Enhanced sporulation, morphological and pathogenic characterization of *Mycosphaerella fijiensis,* causal agent of *Musa* Black leaf streak

Incremento de la esporulación, caracterización morfológica y patogénica de *Mycosphaerella fijiensis*, agente causal de la Sigatoka negra

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ABSTRACT. The present work focuses on enhanced sporulation, morphological and pathogenic characterization of *Mycosphaerella fijiensis* (CCIBP-1 and CCIBP-88) strains. They were incubated with continuous fluorescent light and temperature was maintained for 10 days at 20 ºC, 3 days at 5 ºC and 10 days at 20 ºC, respectively. Conidia number was counted per culture media. Random conidia and conidiophores were measured and cultural characteristic were also evaluated. Pathogenecity test was performed on eight-week-acclimatized plants of Grande naine (AAA. Cultural colonies characteristics of strains CCIBP-Pf 80 and CCIBP-Pf 88 were raised, velvety, dark stromatic mycelia and compact. Green, pink and grey colors were predominant on superficial mycelia of both strains. *Pseudocercospora* conidia types were produced on all assayed cultures media in both strains but Potato Carrot Agar and V-8 media were the most appropriated for conidia production. Morphological characteristics of conidia and conidiophores were similar for both strains. In the pathogenesis test both strains produced characteristic symptoms of Black Sigatoka on all inoculated cultivars. First symptoms caused by both strains were observed 14 to 20 days after infection in all banana inoculated cultivars. Disease development time was around 70-75 days when appeared the first matures necrotic lesions with dry centers.

Key words: Banana breeding, black leaf streak, morphological characteristics, phytopathogen fungus, *Pseudocercospora fijiensis*, sporulation.

RESUMEN. El presente trabajo utilizó diferentes medios de cultivos sólidos y condiciones de incubación para mejorar la producción de conidios de *Mycosphaerella fijiensis* (CCIBP-Pf 80 y CCIBP-Pf88) así como caracterizar morfológica y patogénicamente las cepas en estudio. Para la producción de conidios se utilizaron cámaras climatizadas con luz constante y temperaturas de incubación de 20 ºC durante los primeros 10 días, 5 ºC durante tres días y 10 días a 20 ºC. Se determinó la concentración de conidios en cada medio de cultivo, utilizando un microscopio clínico, la cámara de Neubauer y un contador manual. Para cada cepa se midieron las dimensiones de conidios y conidióforos así como su capacidad patogénica sobre plantas aclimatizadas de *Musa* spp. de ocho semana de edad. Los caracteres culturales se correspondieron con colonias elevadas, aterciopeladas, compactas, con reverso negro y micelio estromático, se observaron diferentes colores (verde olivo, grises y rosadas) en las colonias de ambas cepas. En todos los medios de cultivos utilizados se observaron conidios del tipo *Pseudocercospora,* aunque las mayores concentraciones se obtuvieron con los medios V-8 modificado, PDA y PCA. Los primeros síntomas en el Grande naine, se observaron a partir de los 14 hasta los 21 días posteriores a la inoculación artificial. Con los resultados de este trabajo, se logró mejorar la producción *in vitro* de conidios de *M. fijiensis* así como comprobar los caracteres morfológicos culturales y patogénicos del agente causal de la Sigatoka negra en casa de cultivo.

Palabras clave: Mejoramiento de bananos, sigatoka negra, caracteres morfológicos, hongo fitopatógeno, *Pseudocercospora fijiensis*, esporulación.

INTRODUCTION

Black leaf streak caused by *Mycosphaerella fijiensis* Morelet (anamorph: *Pseudocercospora fijiensis*) is the most important fungal disease of banana and plantain in many areas where *Musa* sp are grown (Mourichon, 2000). Ascospores and conidia produced by this pathogen are the main structures for dissemination in natural condition (Burt, 1994, Gauhl, 1994 Burt and Rutter, 1997, Burt *et*

al., 1998). Artificial inoculation techniques are among the priority research needs since standardized inoculation procedures must be available to assess qualitative and quantitative expression of resistance on *Musa* sp. and to study pathogenic variability of *M. fijiensis* strains from different geographical areas.

Authors like Mourichon *et al*. (1987), Fullerton and Olsen (1995), and Romero and Sutton (1997) have inoculated *Musa* plants with *M. fijiensis* by artificial techniques. However, ascospore and conidia production under laboratory condition is a tedious task, because *M. fijiensis* is a very slow growing fungus culture.

One of the major constraints to study Black Sigatoka under controlled condition is the lack of reliable methods to produce quantifiable inoculum. Sporulation of *M. fijiensis* on common culture media is sometimes sparse. In order to mimic natural infection of pathogen in controlled conditions, large amounts of conidia are needed. Different authors have make great efforts to obtain conidia from *M. fijiensis* (Stover, 1976; Mourichon *et al*., 1987). Jacome and Schuh (1993) produced a conidial suspension on Mycophil agar plates but concentration was around $5x10^3$ conidia/mL. Fullerton and Olsen (1995) used conidia production in a concentration of 2.0×10^4 conidia/mL to evaluate pathogenic variability in 63 *M. fijiensis* strains. Romero and Sutton (1997) induced conidia on Mycophil agar plates at 25 ºC to evaluate sensitivity of *M. fijiensis* isolates to the fungicide Propiconazole, nevertheless they did not quantify the final conidia concentration. Ronald *et al*. (1997), obtained conidia from cultures of *M. fijiensis* on PDA and Mycophil agar media incubated at 20 ºC and 24 ºC, these structures were used to evaluate the reaction of four *Musa* genotypes at different temperatures from different geographical areas.

Regarding to the difficulty to obtain enough conidia of *M. fijiensis* by means of artificial inoculation, this study was focused on enhancing sporulation, morphological and pathogenic characterization of *Pseudocercospora fijiensis* strains by means of artificial inoculation on banana acclimatized plants, an important aspect to support *Musa* breeding program.

MATERIALS AND METHODS

Pseudocercospora fijiensis isolation. Leaves at stage 6 (Fouré, 1982) from the susceptible cultivar Grande naine (AAA) showing typical symptoms of black leaf streak, were collected from natural not sprayed fields. Pieces of 2 cm x 2 cm were cut and submerged in sterile water for 15-20 min and placed in the lid of an *in vitro* culture plant container. One slide containing 2 mL of 3 % water-agar was placed in a container. After 2-6 hours, discharge occurred and single ascospores could be easily and aseptically removed from each slide, using an Olympus microscope (100 x magnifications) and transferred to Petri dishes containing Potatoe Dextrose Agar (PDA, Fluka) medium. Plates were incubated during 20 days at 25 ºC in the dark. Mycelium fragments from the young colony surface were transferred to PDA slant tubes and incubated during 30 days at 25 ºC and dark. All the tubes were stored at 4 ºC.

Cultures media and incubation conditions. Nine culture media were used: carboxymethyl cellulose medium (CMC) at 1 % (p/v) pH 6.8; filter paper wetted with sterile distilled water; filter paper wetted with 2 mL of $CaCO_3$ Solution at 3 % (p/v); Potato Dextrose Agar pH 5.6; agar-mycophil 3.6 % (p/v), pH 5.6 (Jacome et al. (1993)); water agar 3 % (p/v) pH 5.6; agar poor synthetic nutrient medium (KH_2PO_4 1g; KNO_3 1g, Mg SO_4 $7H_2O$ 0.5 g, KCl 0.1 g, Sucrose 0.1 g, for 1000 mL of final volume) pH 5.6; Potato Carrot Agar [(PCA) (200 g of potato, 200 g of carrot, boiling during 30 min in 1000 ml of water, complete to 1000 ml)] pH 5.6 and V-8 (200 ml of V-8 juice, 0.2g of $CaCO_3$, Agar 30 g) pH 6.8.

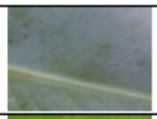
Ten Petri dishes per treatment were used and they were covered with parafilm. All plates were incubated in a cooler Gallenkamp illuminated incubator with continuous fluorescent light of 20 $mol/m^2/s$. The temperature was modified as follows: Ten days at 20 ºC, 3 days at 5 ºC and ten days at 20 ºC.

Collection of conidia. Conidia production was evaluated after 23 days of incubation. From the different culture media five milliliters of distilled water with 0.1 % (v/v) of Tween 20, was added to each

Petri dish, and mycelium was scraped with a camel brush. One ml of each solution was mounted on Neubauer haemocytometer to determine the conidial concentration under an Olympus light microscope.

Plant material. Plants from the susceptible cultivar Grande naine (AAA) obtained by tissue culture via organogenesis, were acclimatized during 8 weeks on a substrate composed by 50% of casting, 30 % of compost and 20 % of zeolita. Plastic pots of 10 cm of diameter were chosen for growing the plant material. Plants with 20 cm of height and three to four fully open leaves were chosen for the artificial inoculation.

Table 1. Descriptions of the stages of symptoms development on *Musa* spp. *in vitro* plants inoculated with *Mycosphaerella fijiensis* in greenhouse

Stage	Symptoms description	
0	Predominance of no symptoms on leaf.	
1	Predominance on abaxial leaf surface of reddish flecks. No symptoms on the upper surface.	
2	Predominance on abaxial leaf surface of regular or irregular reddish circular spots. No symptoms on the adaxial leaf surface.	
3	Predominance on adaxial leaf surface of regular or diffused light brown circular spots.	
4	Predominance on adaxial leaf surface of black or brown circular spots may be accompanied by yellow halo or chlorosis at adjacent tissues. Some green tissues areas could be seen.	
5	Predominance on adaxial leaf surface of black spots with dry center of grey color. Leaf completely necrosed and sometimes holds down.	

Pathogenicity test. Ten plants of Grande naine per strain were used for artificial inoculation. Five not inoculated plants were used as control. Conidia were produced on PCA medium under the condition described above. Conidia suspensions were filtered through two layers of gauze after centrifugation at low speed, resuspended in sterile water and adjusted to 10^4 spores per ml with 1 % gelatin.

The first three open leaves of each plant were inoculated in the abaxial leaf surface using a camel brush. Inoculated leaves were marked on the adaxial side with a black marker. The plants were allowed to dry for two hours and then, humidity was maintained over 90% during the first three days by spraying water continuously. Afterwards, the humidity was saturated only during the night by spraying water.

Disease evaluation. The evaluations began 15 days after inoculation in each inoculated leaves and carried out every week until the 70 day after inoculation.

Table 1 shows the scale used to evaluate the symptoms development according to the stage of symptoms.

The incubation period (IP, time measured in day between inoculation and the appearance of first red speckles) and Disease development time (time, measured in days, between inoculation and the appearance of mature necrotic lesion) were determined according to Carlier *et al*., 2002. The evolution symptoms time (time, measured in days between one symptom stage and the next one) were determined for both strains.

Three random stage 1 symptoms in 10 inoculated leaves were circle with a black marker in each cultivar-strains combination and the symptom stage evolution (days) was recorded.

The statistical analysis was carried out using SPSS software, version 9.0 for Windows, was used for statistic analyses. Conidia concentration of *Pseudocercospora fijiensis* strains on different cultures media were compared by Dunnett´C non parametric test. Conidia and conidiophores lenght, width, septa number, were compared by Dunnett´C non parametric test. Symptoms stages evolution and severity was compared by Dunnett´C test. Incubation period was compared by Duncan test.

RESULTS

The two strains CCIBP-1 and CCIBP-88 used in this study showed typical cultural characteristics of *M.fijiensis*. Colonies were raised to almost hemispherical, velvety, with dark stromatic mycelia, compact and slow growing. Green, grey and pink colors were predominant on colonies of both strains. Both strains produced *Pseudocercospora* conidia types in all culture media (Table 2 and Table 3). The higher conidia production, however, was obtained on Potato carrot agar and V-8 media. Filter paper, Filter paper with $CaCO_3$ and Agar poor synthetic nutrient medium were the lower conidia producer media.

Table 2. Conidia concentration (conidia/mL) of *Pseudocercospora fijiensis* strain (CCIBP-88) on different cultures media after 23 days of growth

Fungal Culture Media	Conidia concentration x10^3
Filter paper + $CaCO_3$	1.16 **e**
Wetted filter paper	2.66 **e**
Agar poor synthetic nutrient medium	4.00 **d**
Carboxy methyl cellulose medium	7.16 **cd**
Water agar 3 %	12.00 **c**
Agar-Mycophil	13.33 **c**
Potato Dextrose Agar	49.50 **b**
V-8	218.33 **a**
Potato Carrot Agar	232.00 **a**

The mean difference is significant at the 0.05 level. Dunnet C test was used for media comparison. The SPSS version 12.0 package was used for data processing. The data represented the mean of conidial numbers collected on ten Petri dishes

Table 3. Conidia concentration (conidia/mL) of *Pseudocercospora fijiensis* strain CCIBP-1 on different cultures media after 23 days of growth

Fungal Culture Media	Real mean x10^3
Filter paper + $CaCO_3$	0.66 **d**
Wetted filter paper	1.16 **d**
Carboxymethyl cellulose medium	1.66 **d**
Agar poor synthetic nutrient medium	1.66 **d**
Water agar 3%	3.00 **d**
Agar-Mycophil	4.83 **d**
Potato Dextrose Agar	33.30 **c**
V-8	127.00 **b**
Potato Carrot Agar	188.50 **a**

The mean difference is significant at the 0.05 level. Dunnet C test was used for media comparison. The SPSS version 12.0 package was used for data processing. The data represented the mean of conidial numbers collected on ten Petri dishes

Morphological characteristics of conidia and conidiophores were similar for both strains (Table 4). *Pseudocercospora fijiensis* conidia were pale green or olivaceous, obclavate to cylindro obclavate in shape, 1-10 septas and straight or curved, with a visible and slightly thickened scar, obtuse tip and with different lenght (41.60 to 141.44 µm long). Conidiophores were pale to olivaceous brown, slightly paler toward the tip. They were straight to bent, often with several geniculations, rarely branched near the base, 0-5 septate, 8.32-91.52 µm long. They had, sometimes, basal swelling and one or more scars near the tip.

In the pathogenicity test, both strains were pathogenic to all Grande naine inoculated plants, producing characteristic symptoms similar to Black leaf streak disease on field young plants. In the figure1 it is shown the incubation period and the evolution symptoms time. Reddish circular spots in

abaxial leaf surface were observed on Grande naine inoculated leaves 35-40 days after infection. Diffused light brown spots in adaxial leaf surface were observed 50-55 days after inoculation. Black to brown elliptic or circular spots, accompanied sometimes by yellow chlorotic haloes were observed in the adaxial leaf surface around 60-65 days after inoculation. Disease development time of both strains on Grande naine inoculated plants was around 70-75 days after inoculation when appeared the first matures necrotic lesions with dry centres.

Table 4. Morphological characterization of the *Pseudocercospora fijiensis* strains CCIBP-Pf 80 and CCIBP-Pf 88

Structure	Strains	Size (µm)		
		Length	Width	Septa
Conidia	CCIBP-1	87.13 a	4.16 a	6.61 a
	CCIBP-88	98.59 b	4.16 a	6.08 a
Conidiophores	CCIBP-1	57.73 a	4.16 a	1.66 a
	CCIBP-88	61.93 a	4.16 a	1.51 a

The mean difference is significant at the 0.05 level. Conidia and conidiophores (length, width, septa number) were compared by Dunnett´C non-parametric test.

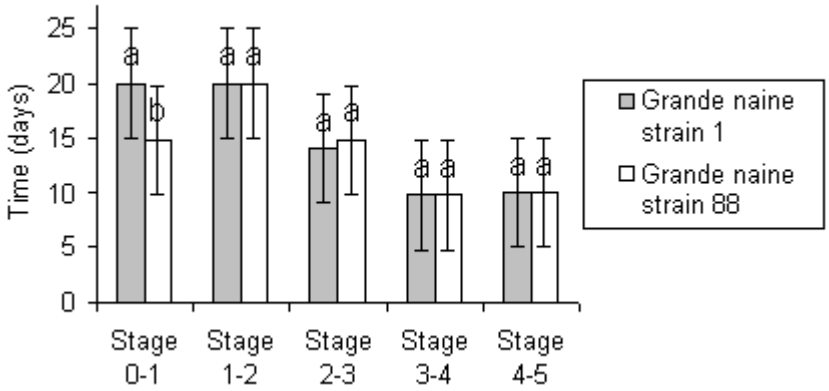


Figure 1. The evolution symptoms time (days between one symptom stage and the next one according to the symptoms evolution scale) determined for two *M.fijiensis* strains. Strain 1 (CCIBP 80) and strain 88 (CCIBP 88)

DISCUSSION

Colony surface colors of *M. fijiensis* observed on culture media corresponded with criteria reported by other authors (Meredith and Lawrence, 1969, Stover, 1976, Mourichon *et al*. 2000). CCibp1 and CCibp88 strains colonies had similar cultural characteristics although the surface color of colony of the same strains was variable (grey, pink and white).

In this study we showed that production of conidia can be enhanced by growing *M. fijiensis* strains on PCA and V-8 medium. Potatoes Carrot agar is an alternative culture medium with a higher conidia production respect to standard Mycophil and PDA culture media used by other researcher. PCA and V-8 are composed by natural components of root, tuber, or fruits. Nutritional supplement components such as inorganic and organic charcoal, nitrogen source, microelements like Ca, Mn, Zn, Cu, Mo, Fe and quantities of vitamins of these two culture media, could stimulate sporulation of several phytopathogen fungus (Rivas, 2001).

Conidia and conidiophores of *Pseudocercospora fijiensis* have been found frequently on lesions of infected leaves of *Musa* sp and they developed under favourable condition of temperature and high humidity. These structures sometimes differ when they are developed *in vitro* conditions, which could induce changes in conidia morphology. *In vitro*

conidia of *Pseudocercospora fijiensis* have been almost identical to those on naturally infected leaves but occasionally have been longer and more septate (Meredith and Lawrence, 1969).

Studies respect morphology of fungus have been commonly used for identifying causal agents of some plants pathological disorders and also to characterize phytopathogen fungal culture collection. Nishikama *et al*. (2002) described some morphological characteristics (stromata, conidia and conidiophore shape, septum number and size) of *Cercospora guatemalensis*, the causal agent of circular leaf spot of sweet basil (*Ocimum basilicum* L). Similarly, Furukawa and Kishi (2003), illustrated morphology of *Septoria chrysanthemella* Saccardo, the causal agent of leaf spot on *Aster savatieri* (Makino). In the present work there was a correspondence of conidia and condiophores morphology of both strains (CCIBP-1, CCIBP-88) and the results described by Meredith and Lawrence (1969) and Carlier et al. 2002, when they characterized these structures of *Pseudocercospora fijiensis.*

Symptoms induced in the pathogenicity test by *P. fijiensis* strains corresponded approximately with the description by Mourichon *et al.*,(1987), Fullerton & Olsen (1995), for *in vitro* plants inoculated with conidia and they were comparable to symptoms observed in young plant infected with natural inoculums at field condition. Other groups of authors have obtained equivalent symptoms pattern expression but using mycelium fragments of *M.fijiensis.* (Alvarado *et al*. 2003; Balint-Kurti *et al*., 2001)

The possibility to produce great amount of conidia under controlled condition, give more chances of early screening procedure in Musa breeding programs. Artificial inoculation methods are needed for rapidly screening Musa genotypes to assess plant response to *M. fijiensis*. However, *M. fijiensis* is a slow growing fungus and sporulation sometime is poor and time-consuming in many laboratories. *M. fijiensis* inoculation techniques are among the priority research needs since standardized inoculation procedures must be available to assess qualitative or quantitative expression of resistance on *Musa* sp. and for studying pathogenic variability of *M. fijiensis* strains from different geographical areas (Frison et al., 1997).

With the results discussed above it was possible to characterize morphological and pathogenically and to enhance sporulation of CCIBP-1 and CCIBP-88 strains of *Mycosphaerella fijiensis*. It made possible to develop a method for early evaluation of Black leaf streak on Musa acclimatized vitroplants.

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