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ARTICULOS GENERALES

Effect of the *"in vitro"* subculture in conidial production and aggressiveness of *Mycosphaerella fijiensis* Morelet

Efecto de subcultivos *"in vitro"* en la produccion de conidios y la agresividad de *Mycosphaerella fijiensis* Morelet

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ABSTRACT. The effect of *in vitro* subculturing on the production of conidia and the aggressiveness of *Mycosphaerella fijiensis* Morelet isolates that were inoculated on the *Musa* cultivar ¨Grande Naine¨ was determined. The *in vitro* capacity of production of conidia and the behavior after artificial inoculation on ¨Grande Naine¨ were compared for isolates with a different number of *in vitro* subcultures. It was possible to obtain *M. fijiensis* conidia *in vitro* in all of the isolates. A great decrease in conidia production was observed when the number of subcultures increased, after eight subcultures this number of conidia was reduced in 80%. All the isolates occasioned symptoms in the plant inoculated leaves. Nevertheless, differences among *M. fijiensis* isolates were found regarding their aggressiveness. These results demonstrated that the aggressiveness and the capacity to produce spores of *Mycosphaerella fijiensis* isolate decreases after serial vegetative transfers on artificial substrates.

Keywords: Black Sigatoka, conidia, conservation method, *Musa* sp., *Pseudocercospora fijiensis.*

RESUMEN. Se determinó el efecto de los subcultivos *in vitro* de aislados de *Mycosphaerella fijiensis* en la producción de conidios y en su agresividad al ser inoculados artificialmente, en plantas de *Musa* del cultivar ¨Grande Naine¨. Fueron comparados aislados con diferentes números de subcultivos *in vitro* en cuanto a la capacidad de producir conidios *in vitro* y su efecto después de la inoculación artificial en plantas de ¨Grande Naine¨. Fue posible obtener conidios *in vitro* en todos los aislados de *M. fijiensis*. Se observó una disminución en la producción de conidios al aumentar el número de subcultivos, después de ocho subcultivos el número de conidios disminuyó en un 80%. Todos los aislados ocasionaron síntomas en las hojas inoculadas. Sin embargo, se observaron diferencias en la agresividad entre los aislados de *M. fijiensis*. Los resultados demostraron que la agresividad y la capacidad de producir esporas de *M. fijiensis* decrecieron después transferencias sucesivas en sustratos artificiales.

Palabras clave: Sigatoka negra, conidios, métodos de conservación, *Musa* sp., *Pseudocercospora fijiensis.*

INTRODUCCIÓN

Black Sigatoka disease, also known as "black leaf streak", is caused by *Mycosphaerella fijiensis* Morelet (anamorph *Pseudocercospora fijiensis* Morelet Deighton (Crous *et al.,* 2002)). It is considered the most damaging and costly foliar banana disease in the world (Pasberg-Gauhl *et al*., 2000; Marín *et al*., 2003). *Musa* genetic breeding programs have been developed for several years to achieve resistance. The combination of recent advances in plant biotechnology and traditional methods for crop improvement provides useful tools to increase efficiency in *Musa* breeding program. In this breeding programs using methods of early evaluation and they require *M. fijiensis* isolates characterized.

Mycosphaerella fijiensis collections have been developed for several general functions, for example, the characterization of whole fungal populations (Molina and Kahl, 2004), the monitoring of their adaptation to fungicides (Romero and Sutton, 1997a; Guzman *et al*., 2000; Amil *et al*., 2007),

the observation of the pathogenic variability (Fullerton and Olsen, 1995; Donzelli and Churchil (2007)) and the study of banana-*M.fijiensis* interactions (Romero and Sutton, 1997b; Abadie *et al*., 2003).

When grown in culture, filamentous fungi exhibit a high tendency to changing spontaneously, either morphologically (e.g. sectorization) or physiologically (e.g. secondary metabolite production). Variants will be selected according to specific culture conditions. The variability will also depend on the number of generations. Culture collections have dealt with strain instability by developing empirical techniques aiming to maximize both longevity and stability of stock cultures (Ryan *et al*., 2003).

Whereas the collection and the culture of *Mycosphaerella fijiensis* isolates is routine (Molina and Kahl, 2004), the physiological and pathogenic stability are poorly attendant. The effect of *in vitro* subculture on the physiology of *M. fijiensis* is virtually unknown. Long term conservation techniques in *M. fijiensis* are not very well documented. The most common way of preservation is by using short term techniques, in slant tubes with PDA at 4ºC. These techniques have some disadvantages such as the necessity of high number of subcultures, which may affect important characteristics of the microorganism itself (Van Den Ende, 1993; Krokene and Solheim, 2001).

The objective of this study was to determine the effect of the number of *in vitro* subcultures on the production of conidia and the aggressiveness of *Mycosphaerella fijiensis* isolates.

MATERIALES Y MÉTODOS

Plant material

Grande naine (*Musa* AAA) (susceptible cv.) plants obtained by tissue culture via organogenesis, were acclimatized in greenhouse during 45 days on a substrate composed of casting (50%), compost (30%) and zeolite (20%). Plastic pots with a diameter of 10cm were selected to grow the plant material for another 45 days.

Sources of isolates and cultural condition

Three monospore isolates of *Mycosphaerella fijiensis* Morelet were provided by the Culture Collection of the Microbiología Aplicada Laboratory in the Instituto de Biotecnología de las Plantas (IBP, Cuba). These were isolated from two different Cuban natural, unsprayed fields (Villa Clara and Ciego de Ávila) by discharging the ascospores from leaves of the susceptible cultivar ¨Grande naine¨ (*Musa* AAA), following the method proposed by Stover (1976). The characteristics of the conidia and conidiophores in all of the isolates corresponded with those described by Meredith and Lawrence (1969) and Carlier *et al.* (2002). Strains were cultured in Potato Dextrose Agar (PDA) slant tubes and incubated at 25ºC in the dark. All the tubes were stored at 4ºC and subcultured every six moths.

Effect of *in vitro* subculture in conidial production

For the conidial production of each isolate, 300 µl of mycelial suspension ($5x10^5$ mycelial fragment.ml^{-1}) were added to 15 ml of V-8 solid medium (Mourichon *et al*., 1987) in slant tubes (six tubes per isolate). The tubes were incubated in an illuminated *Gallenkamp* incubator with continuous fluorescent light of 60 mol.$m^{-2}s^{-1}$ at 20ºC during 25 days. Five milliliters of distilled water with 0.05 % (v/v) Tween 80 were added to each tube and the conidial suspension was recovered using a vortex (Heidolph Top Mix 94 323) (Acosta *et al.,* 2004). The spore concentration was determined using a Neubauer haemocytometer under an *Olympus* light microscope (magnification 400x).

Isolates with different number of subculture (one, four and eight) were analyzed using two isolates for every subculture. Six replicates per isolates were used.

Pathogenicity tests

The selected isolates were: CCIBP-*Pf*-57, CCIBP-*Pf*-39 (four times subcultured *in vitro*), and CCIBP-*Pf*-1 from Villa Clara (sixteen times subcultured *in vitro*). *M. fijiensis* was grown in potato destroxa broth (PDB) medium for 15 day at 28 ºC on rotatory skaker at 120 rpm. The fungal inoculums was

prepared by mixing the 15 day-old mycelium in Ultra-Turrax T25 homogenize for one minute. The concentration of the mycelium fragments was determined using a Neubauer haemocytometer under an *Olympus* light microscope (magnification 400x).

Plants with 20cm of height and four active open leaves were inoculated by brushing mycelial suspension (10^5mycelial fragment.ml^{-1}) of each isolate on the adaxial leaf surface of the first three open leaves. Ten plants were inoculated per isolate and ten non-inoculated plants were used as control.

From the first until the 63th day post inoculation (dpi), the plants were visually examined and the stage of symptoms per leaf was recorded in correspondence with the criteria of Alvarado-Capó *et al.* (2003).

The incubation period (time measured between the day of inoculation and the appearance of the first symptoms red speckles), the symptom evolution time (time measured between the day of symptom stage and the next) and the disease index for each day post inoculation using McKinney's formule (McKinney, 1923) were also determined.

Statistical analysis

Data were subjected to parametric and non-parametric tests using the Statistical Package for the Social Sciences (SPSS) version 16.0 for Windows. For conidia concentration analysis a One way ANOVA testing was adopted as mean comparison for Dunnett´s C test. A Kruskal-Wallis test was used to analyze the pathogenicity assay, in these cases, the Monte Carlo technique, with a significance of 99% confidence, was used.

RESULTS

Effect of *in vitro* subculture in conidial production

Significant differences in conidia producing capacity were observed among isolates with different number of subcultures. When the number of subcultures increased, a decrease in the production of conidia was observed (Table 1).

Table 1. Conidia concentration of *Mycosphaerella fijiensis* isolates in different *in vitro* subcultures

Number of *in vitro* subculture	Isolates	Means of conidia concentration (x 10^5 conidia.ml^{-1})
One subculture	CCIBP- *Pf*-34	10.2[a]
	CCIBP-*Pf*-66	15.4 a
Four subcultures	CCIBP-*Pf*-39	6.0 b
	CCIBP-*Pf*-57	7.8 b
Eight subcultures	CCIBP-*Pf*-54	1.0 c
	CCIBP-*Pf*-64	2.2 c

The results of these studies indicate that *in vitro* subculturing of *M. fijiensis* on culture media affects the conidia producing capacity of the fungus. The number of conidia produced after one subculture was 12.8 x10^5 conidia.ml^{-1}. After eight subcultures this number was reduced in 80%.

Pathogenecity test

The mycelial suspension from *M. fijiensis* isolates produced symptoms in all inoculated leaves. In addition, they were capable to induce the typical symptoms of the different stages on ¨Grande naine¨ inoculated leaves. Symptoms corresponded, approximately, with the description made by Mourichon *et al.* (1987) for *in vitro* inoculated banana plants using conidia suspension. The developments of symptoms using mycelial suspension were similar to results obtained by Leiva *et al.* (2002) and Alvarado- Capó *et al.* (2003). The incubation period was the same for all inoculated isolates (14 days post inoculation).

Predominance of reddish flecks on the abaxial leaf surface (stage 1) was observed after 21 days post

inoculation for all of the isolates used. Nevertheless, the evolution of the symptoms was different among them.

Predominance of regular or diffused light brown circular spots on the adaxial leaf surface (stage 3) of the isolates CCIBP-*Pf*-57 and CCIBP-*Pf*-39 were observed at 35 dpi. Black or brown circular spots with a yellow haloes and chlorosis of adjacent tissues (stage 4) were observed after 56 dpi. Completely necrotic leaves with black spots and a grey, dry center (stage 5) were witnessed after 63 dpi. On the other hand, the symptom evolution of CCIBP-*Pf*-1 was very slow.

Differences among *M. fijiensis* isolates were found regarding their aggressiveness on ¨Grande naine¨ plants. This was expressed in the appearance of different disease index (DI) at the same time after inoculation (Figure 1). Until 21 dpi, no significant statistical differences in disease index were observed among isolates. During further development of the disease statistical differences in DI were observed. For instance, the isolate CCIBP-*Pf*-1 just reached 62% of DI at 63 dpi. The same isolate (with two subcultures) was reported by Leiva *et al.* (2002) in similar experimental conditions and they predominance of stage 5 after 63 dpi. However, isolates with fewer subcultures, CCIBP-*Pf*-57 and CCIBP-*Pf*-39, at 63 dpi reached more than 80% of DI. It was proven that the loss of aggressiveness of the isolate CCIBP-*Pf*-1 is correlated with the number of subcultures.

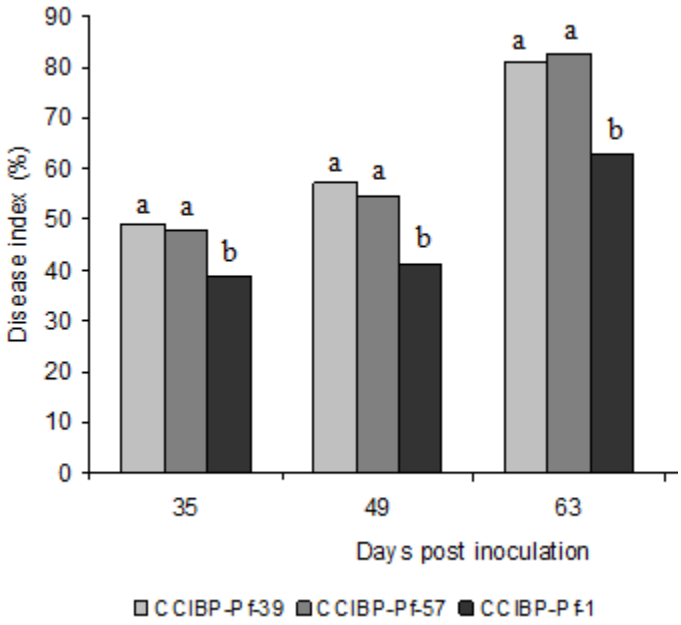

Figure 1. Disease index reached on ¨Grande naine¨ plants after artificial inoculation with mycelial suspension of different *Mycosphaerella fijiensis* isolates. Isolates with four *in vitro* subculture (CCIBP-*Pf*-39, CCIBP-*Pf*-57) and sixteen *in vitro* subculture (CCIBP-*Pf*-1).
Mean values with dissimilar letters at the same time differ by a non-parametric Kruskall-Wallis Test for $p < 0.01$

The results of the present study suggest that there is a relationship between the number of subcultures *in vitro* and the differences in aggressiveness and the conidia producing capacity.

DISCUSION

Some authors have reported that *in vitro* prolonged subculturing of *M. fijiensis* isolates causes the complete loss of conidia production capacity (Meredith and Lawrence, 1969). Jeger *et al.* (1995) showed that it is difficult, and in some cases impossible, to induce sporulation after a long period of storage, or repeated transferring. Stover (1976) determined that virulence and sporulation capacity can be easily lost using *in vitro* culture. Similar result were obtained by Van Den Ende (1993), who reported reduced virulence in *Mycosphaerella brassicicola* when fungal cultures were aged over six moths.

In other species of plant pathogenic fungi, has been reported that subculturing of isolates coved induce various mutations including those affecting pathogenicity and the capacity to produce spores (Newcombe *et al*., 1990, Van Den Ende, 1993; Tharreau, 1994; Krokene and Solheim, 2001).

According to this, reduced aggressiveness and other signs of senescence in laboratory cultures have been found to depend on how long a fungus is kept in culture (Gramss, 1991) and the number of serial transfers to which it has been subjected (Newcombe *et al*., 1990). The nature of the transfers is probably also important (Morrow *et al*., 1989).

This study has demonstrated that isolates of the *Mycosphaerella fijiensis* may lose their aggressiveness after repeated *in vitro* subculturing. It highlights the need to keep the following in mind when using isolates from culture collections for pathogenicity tests and other interactive studies with host

plants. Culture conditions, preservation procedures and the period of preservation may affect strain performance.

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