

Effect of artificial inoculation of *Mycosphaerella fijiensis* on the induction of defence-related enzymes in two *Musa* genotypes

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ABSTRACT

Black Sigatoka, caused by *Mycosphaerella fijiensis* Morelet (anamorph: *Pseudocercospora fijiensis* (Morelet) Deighton), is regarded as the most economically important foliar disease of bananas and plantains worldwide. Although exists numerous studies about plant-pathogen interaction in many pathosystems it is very limited the knowledge of the molecular mechanisms in *Musa*-*M. fijiensis* compatible or incompatible interaction. For this reason, the objective of the present study was to evaluate the effect of artificial inoculation of *Mycosphaerella fijiensis* on the induction of some defence-related enzymes in two *Musa* genotypes, under greenhouse conditions. Enzymatic activities of peroxidase (PO), phenylalanine ammonia-lyase (PAL) and β -1, 3-glucanase were determined at different times post infection. An increase in enzymatic activities were observed in 'Calcutta 4' inoculated plants and similar results were obtained in 'Grande naine' inoculated plants, except in β -1, 3-glucanase activity in which no differences between inoculated and non-inoculated plants were observed. The biochemical changes in 'Calcutta 4' plants in response to *M. fijiensis* infection were observed early than in 'Grande naine' and seem to offer new evidences of collective mechanisms of plant resistance to the invasion of the pathogen.

Keywords: Black leaf streak, Calcutta 4, β -1,3-glucanase, Grande naine, peroxidase (PO), phenylalanine ammonia-lyase (PAL).

Efecto de la inoculación artificial de *Mycosphaerella fijiensis* en la inducción de enzimas relacionadas con la defensa en dos genotipos de *Musa*

RESUMEN

La enfermedad denominada Sigatoka Negra es causada por el hongo *Mycosphaerella fijiensis* Morelet (anamorfo: *Pseudocercospora fijiensis* (Morelet) Deighton) y es clasificada como la enfermedad foliar de plátanos y bananos más importante económicamente, en todo el mundo. Aunque existen numerosos estudios sobre la interacción planta-patógeno en otros patosistemas, es muy limitado el conocimiento de los mecanismos moleculares en la interacción *Musa*-*M. fijiensis*, tanto en la respuesta compatible como en la incompatible. Es por ello que el objetivo de este trabajo fue evaluar la inducción de enzimas relacionadas con la respuesta de defensa en dos genotipos de *Musa* ante la inoculación artificial con *Mycosphaerella fijiensis*, en casa de cultivo. Se determinó la actividad enzimática peroxidasa (PO), fenilalanina amonio-liasa (PAL) y β -1,3-glucanasa, a diferentes tiempos después de la inoculación. Como resultado se observó un incremento en la actividad de las enzimas evaluadas en plantas de 'Calcutta 4' inoculadas. Resultados similares se observaron en el genotipo 'Grande naine', con excepción en la actividad β -1, 3-glucanasa donde no se encontraron diferencias entre las plantas inoculadas y la no inoculadas. Estos cambios bioquímicos en plantas del genotipo resistente 'Calcutta 4' en respuesta a la infección por *M. fijiensis* ocurrieron más rápido que en el cultivar susceptible 'Grande naine', lo que ofrece nuevas evidencias de los mecanismos de resistencia de la planta ante la infección por este patógeno.

Palabras clave: Calcutta 4, fenilalanina amonio-liasa (PAL), β -1,3-glucanasa, Grande naine, peroxidasa (PO), Sigatoka Negra.

INTRODUCTION

Bananas and plantains constitute the main source of food for about 400 millions people and they play an important role in the economy of several developing countries. Black leaf streak, caused by *Mycosphaerella fijiensis* Morelet (anamorph: *Pseudocercospora fijiensis*

(Morelet) Deighton), is regarded as the most damaging and economically important foliar disease of bananas and plantains worldwide (Marín *et al.*, 2003).

Economically and environmentally viable control options for Black Sigatoka on *Musa* sp. have not been established and the development

of resistant genotypes is still insufficient (Milgate *et al.*, 2005).

Despite being the number one fruit crop in the world, very little is known about the phylogeny and molecular biology of banana (Thomas-Hall *et al.*, 2007) and yet there is little experimental evidence of what constitutes the plant defense response in the *Musa* spp.-*M. fijiensis* pathosystem. However, some genomic studies have been conducted in *Musa* spp., for example, in *Musa acuminata* subsp. burmannicoides 'Calcutta 4' (Vilarinhos *et al.*, 2003), *Musa balbisiana* 'Pisang klutuk Wulung' (Šafá *et al.*, 2004) and *Musa acuminata* 'Tuu' (Ortiz *et al.*, 2005). Similarly, Portal (2008) identified genes involved in the defensive response of *Musa* spp. to *M. fijiensis*, on plants of the cultivar 'Grande naine' (AAA).

In spite of the advances and results in genomic studies of *Musa*-*M. fijiensis* interaction, the knowledge of the molecular mechanisms involved in resistance is still limited. For this reason, biochemical studies are required to allow an integral understanding of this interaction.

It is known that the accumulation of certain enzymes for the biosynthesis of important secondary metabolites is a common characteristic of the induction of active defence mechanisms in plants (Collinge *et al.*, 1997, Benhamou, 2005). The primary defence response at the inoculated site of the plants is often accompanied by biochemical changes such as the over-production and accumulation of phenolic, deposition of lignin-related materials, plant enzymes such as peroxidase, and phenylalanine ammonia-lyase and the expression of pathogenesis related proteins (PR proteins) in the host by pathogens (van Loon *et al.*, 2006).

According to the genotypes resistance to Black leaf streak 'Calcutta 4' and 'Grande naine' were classified as resistant and susceptible respectively, by Fouré *et al.* (1990). The study of the mechanisms involved in plant defense response in both genotypes can offer new evidences of this plant-pathogen interaction.

For this reason the objective of the present study was to evaluate the effect of *Mycosphaerella fijiensis* artificial inoculation in some defence-

related enzymes such as peroxidase, phenylalanine ammonia-lyase and β -1, 3-glucanase, in 'Calcutta 4' and 'Grande naine' plants, under greenhouse conditions.

MATERIAL AND METHODS

Plants

'Calcutta 4' plants from INIBAP Germoplasm Bank (ITC 0249) and 'Grande naine' plants from INIVIT (Instituto Nacional de Viandas Tropicales) were propagated by tissue culture (organogenesis) according to protocol described by Orellana (1994) and acclimatized during three month in greenhouse. Plants with approximately 20 cm of height and with more than three leaves were used.

Fungal material

For inoculation, the CCIBP-Pf83 strain of *Mycosphaerella fijiensis* (Morelet) obtained from Microbial Culture Collection of Phytopathology Laboratory of Instituto de Biotecnología de las Plantas, Cuba, was used. The mycelia suspension for artificial inoculation was prepared following the protocol from Alvarado *et al.* (2003). Evaluation of symptoms evolution was carried out daily, according to the scale proposed by Alvarado-Capó *et al.* (2003).

Sample collection for enzymatic analysis

The first three leaves from three inoculated and control plant (non-inoculated) of 'Calcutta 4' and 'Grande naine' genotypes were collected at 3, 6, 8, 10, 14 and 16 days post infection (dpi). Samples were homogenized by using a pre-chilled pestle and mortar, 0.5 g were extracted in 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) with the addition of protease inhibitor cocktail for general use (Sigma). The homogenate was centrifuged at 10 000 g for 20 min at 4°C and the supernatant was used as enzyme extract for the enzymatic assays (peroxidase (PO), phenylalanine ammonia-lyase (PAL) and β -1, 3-glucanase). Three replicates from each sample were used in both genotypes.

Statistical analysis

The data were analyzed by using the *Statistic Package for Social Science* (SPSS) version

16.0 for Windows. The Monte Carlo Test was used for to increase the number of samples.

Estimation of PO activity

Peroxidase activity was colorimetric assayed (Cakmak *et al.*, 1993), with modifications. The reaction mixture consisted of 1.5 ml of 0.05% guaiacol, 0.5 ml of enzyme extract and 0.5 ml of 1% H₂O₂. The reaction mixture was incubated at room temperature. The increase of absorbance due to guaiacol oxidation was recorded at 470 nm ($\epsilon = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$) at 20 sec interval for 3 min and the boiled enzyme preparation served as blank (Hammerschmidt *et al.*, 1982). Mean differences were analyzed by using the Kruskal-Wallis Test. Enzyme activity was expressed in $\mu\text{moles}/\text{min}/\text{g}$ of fresh weight.

Estimation of PAL activity

The PAL assay was conducted for the method described by Ross and Sederoff (1992). The assay mixture was contained 100 μl of enzymatic extract 500 μl of 50 mM Tris HCl (pH 8.8) and 600 μl of 1mM L-phenylalanine, was incubated for 60 min. The reaction was stopped by adding 2 N HCl. Later 1.5 ml of toluene was added, vortexed for 30 sec, centrifuged (1 000 rpm, 5 min) and toluene fraction containing trans-cinnamic acid was separated. The toluene phase was measured at 290 nm against the blank of toluene. A standard curve was drawn with graded amounts of cinnamic acid in toluene as described earlier. Mean differences were analyzed by using the Kruskal-Wallis Test. Enzyme activity was expressed as nmol trans-cinnamic acid released/min/ g of fresh weight.

Estimation of β -1, 3-glucanase activity

The enzyme activity was colorimetric assayed (Pan *et al.*, 1991). The activity of β -1, 3-glucanase was determined by measuring the release of reducing sugars by using laminarin as substrate and glucose as standard. Crude enzyme extract of 62.5 μl was added to 62.5 μl of 4% laminarin and incubated at 40°C for 10 min. The reaction was stopped by adding 375 μl of dinitrosalicylic acid (DNS) and heated for 5 min on boiling water bath (DNS prepared by adding 300 ml of 4.5% NaOH to 880 ml containing 8.8 g of DNS and 22.5 g potassium sodium tartarate). The resulting coloured solutions were diluted with distilled water,

vortexed and the absorbance was read at 500 nm. The crude extract preparation mixed with laminarin at zero time incubation served as blank. Mean differences were analyzed by using the Kruskal-Wallis Test. The enzymatic activity of β -1, 3-glucanase was expressed as μg of glucose released/min/ g/ of fresh weight.

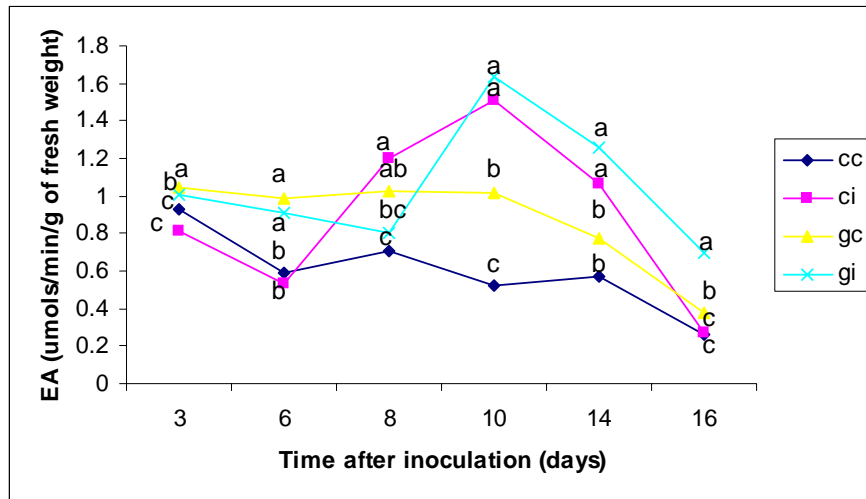
RESULTS AND DISCUSSION

Symptoms characteristics and their evolution during the days of the experiment in both genotypes were possible to observe by using the artificial inoculation method. In infected 'Cavendish naine' plants the appearance of the first symptoms (incubation period) occurred at the 14 days after inoculation, while in infected 'Calcutta 4' plants the incubation period it was approximately of 6 days. The time of symptoms evolution in the susceptible genotype 'Grande naine' it was of 52 days, from the stage 1 to 5 of the scale which corresponds with the results described previously by Alvarado-Capó *et al.* (2003) in this genotype. In the resistant genotype 'Calcutta 4' the time of symptoms evolution it was of 30 days approximately and symptoms only evolved to the stage 1 to 4, similar results were described by Leiva (2008) in the resistant genotype 'Yangambi km. 5'.

Estimation of PO activity

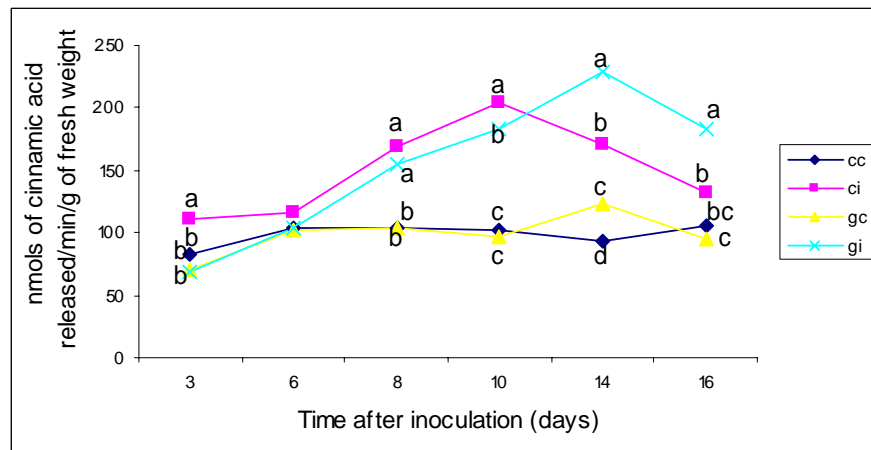
Peroxidase represents a component of an early response in plants against to pathogen attack and plays a key role in the biosynthesis of lignin witch limits the extent of pathogen spread (Bruce and West, 1989).

A significant increase in PO activity was observed in inoculated 'Calcutta 4' plants respect to the control and to 'Grande naine' inoculated plants starting from the sixth day, moment that coincides with the appearance of the first symptoms in the resistant genotype, (Figure 1). In the susceptible genotype, the increase in peroxidase activity was significantly different from the control but no from the inoculated 'Calcutta 4' plants, starting from the 10 to the 14 dpi (Figure 1), this time corresponds to period of the first symptoms appearance 'Grande naine' infected plants. In both genotypes occurred an increase in peroxidase activity about the third day, that which could be properly related with the effect of the inoculation process.



Mean with different letters at the same time differ by Kruskal-Wallis Test for $p < 0.05$.

Figure 1. Effect of *Mycosphaerella fijiensis* inoculation on peroxidase (PO) activity in 'Calcutta 4' inoculated (ci) and in non-inoculated (cc) plants (A) and in Grande naine inoculated (gi) and in non-inoculated (gc) plants (B), in different days post-infection. Values are the mean of three replications.

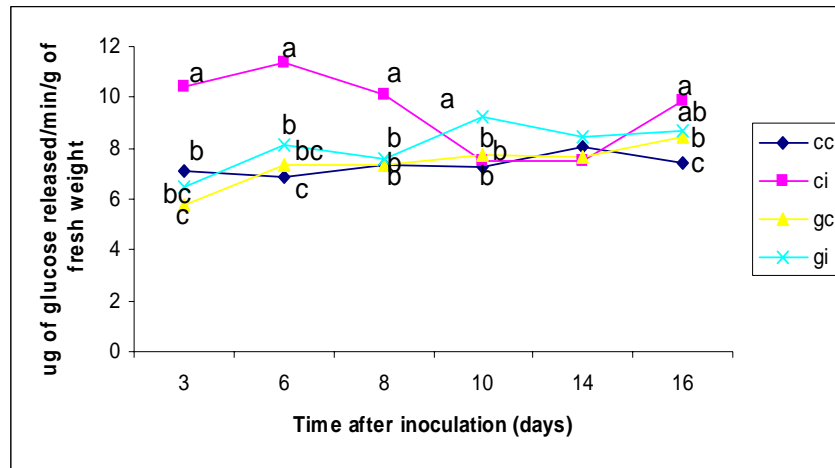


Mean with different letters in the same time differ by Kruskal-Wallis Test for $p < 0.05$.

Figure 2. Effect of *Mycosphaerella fijiensis* inoculation on phenylalanine ammonia-lyase (PAL) activity in 'Calcutta 4' inoculated (ci) and in non-inoculated (cc) plants (A) and in 'Grande naine' inoculated (gi) and in non-inoculated (gc) plants (B), in different days post-infection.

Induced peroxidase in plant-pathogen interaction has been informed by many authors. Chen *et al.* (2000) reported higher PO activity in cucumber roots treated with *P. corrugata*. Also Sendhil Vel (2003) reported induced synthesis and accumulation of PO, PPO, PAL, β -1, 3 glucanase, chitinase, catalase and total phenols in grapevine plants pre-treated with *P. fluorescens* and challenge inoculated with downy mildew and powdery

mildew pathogens. Anand *et al.* (2007) has also made similar observations in tomato plants pre-treated with *P. fluorescens*. It has been observed that in leaves of infected *Musa* plants with culture filtrate of *Fusarium oxysporium* f. sp. *cubense* race 1 the peroxidase activity increases in resistant genotypes regarding susceptible genotypes, as plant response to pathogen infection (Companiononi *et al.*, 2005).



Mean with different letters in the same time differ by Kruskal-Wallis Test for $p < 0.05$.

Figure 3. Effect of *Mycosphaerella fijiensis* inoculation on the activity of β -1, 3-glucanase in 'Calcutta 4' inoculated (ci), non-inoculated (cc) plants (A) and in 'Grande naine' inoculated (gi) and non-inoculated (gc) plants (B), in different days post-infection.

Estimation of PAL activity

Phenylalanine ammonia-lyase plays an important role in the biosynthesis of various defence chemicals in phenylpropanoids metabolism. A significant increase in PAL activity was detected in *Musa* leaves after inoculation with *M. fijiensis*, in both genotypes. The increase in PAL activity showed significantly differences between the inoculated and non-inoculated 'Calcutta 4' plants and it was higher that 'Grande naine' inoculated plants at the 10 dpi, while in 'Grande naine' inoculated plants reached the highest value latter at the 14 day after inoculation (Figure 2).

Several studies have shown that PAL activity is induced in plants upon treatment with pathogens Chen *et al.* (2000) reported that high levels of PAL were induced in cucumber roots inoculated with *P. aphanidermatum*. Sendhil Vel (2003) has also made similar observation in grapevine plants inoculated with *U. necator* and *P. viticola*. Also, authors like Karthikeyan *et al.* (2006) report an increase in the activity of PAL in coconut roots treated with biocontrol agents. In *Musa-M. fijiensis* interaction the pathogen infection is able to induce several defensive mechanisms in the host plant, which respond with an increment in the activity of the PAL enzyme (Hoss *et al.*, 2000). In this sense, the resistance in *Musa* to *M. fijiensis* seems to be closely related with post-transcriptional mechanisms activation, as well as for the

phytoalexins induction (Luis *et al.*, 1994), as products of the phenylpropanoids route.

Estimation of β -1, 3-glucanase activity

Pathogenesis-related proteins (PR proteins) are host coded proteins that are induced by pathogens and by abiotic stress. (Van Loon *et al.*, 1998). Some of the PR proteins such as β -1, 3-glucanase (PR-2) (Kauffmann *et al.*, 1987) have the potential to hydrolyze β -1, 3-glucan, which are one of the major components of fungal cell walls. This enzyme has been considered one of the main factors involved in the induction of resistance mechanisms of plants, as reported by Manandhar *et al.* (1999) and Liljeroth *et al.* (2001).

In this experiment the increase in β -1, 3-glucanase activity showed significantly differences between the inoculated and non-inoculated 'Calcutta 4' and also respect to 'Grande naine' infected plants from the first day to the eighth day post inoculation, while in 'Grande naine' plants, there was no differences between the inoculated and non-inoculated plants (Figure 3).

Authors like Castro and Bach (2004) reported the increased production of β -1,3 glucanase and proteins in *Bipolaris sorokiniana* pathosystem treated using commercial xanthan gum. In *Musa-M. fijiensis* interaction has been pointed out that the induction of PR proteins has a

narrow relationship with the resistance of the plants to pathogen infection, but the knowledge of the roll of these proteins is even limited in this pathosystem (Lepoivre *et al.*, 2003).

Nevertheless, authors like Dagert *et al.* (2002) carried out a subtractive library in 'Yangambi km5' (*Musa* AAA, highly resistant to the disease) inoculated with *M. fijiensis* and they found the differential expression of a glucanase, that demonstrates their narrow relationship with the defence process of the plant against the pathogen.

CONCLUSIONS

Mycosphaerella fijiensis inoculation induced defence-related enzymes in inoculated plants respect to the control in both genotypes as evidence of the defence mechanisms of plants to pathogen attack. In the resistant genotype 'Calcutta 4' the accumulation of these biochemical compounds in the early stage of the infection occurred quicker than in the susceptible genotype 'Grande naine'. These biochemical changes in the resistant genotype in response to *M. fijiensis* infection seem to offer new evidences of collective mechanisms of plants resistance to the invasion of the pathogen and wide the knowledge in this important pathosystem.

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