

Effect of H₂O₂ application during 'Grande naine' -*Mycosphaerella fijiensis* interaction

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ABSTRACT

In *Musa* spp. considerable economical lost are cause by *Mycosphaerella fijiensis* infection around the world, that is why the study of the pathosystem constitute a priority. However, the main mechanisms activated in banana after infection are still unknown and are a limitation for a better understanding of this complex relationship. The objective of this study was to determine the effect of hydrogen peroxide (H₂O₂) application, on leaves of 'Grande naine' plants, on black leaf streak disease development (BLS). For this purpose, the first three open leaves of banana plants were inoculated with the monoascosporic isolate of *M. fijiensis* CCIBP-Pf-83. At three days post-inoculation different H₂O₂ concentrations (10, 20, 30 and 40 mmol l⁻¹) were sprayed to these plants as well as to non-inoculated ones. During the time course of the experiment for inoculated, sprayed plants and for control plants (infected with *M. fijiensis*) epidemiological variables as well as the area of necrotic lesions at 49 dpi were measured. The findings of this analysis showed that the early application of H₂O₂ have influence on the BLS development.

Keywords: banana, black leaf streak disease, hemibiotrophic, hydrogen peroxide

Efecto de la aplicación del H₂O₂ durante la interacción 'Grande naine' -*Mycosphaerella fijiensis*

RESUMEN

En *Musa* spp. la infección con *Mycosphaerella fijiensis* Morelet causa considerables pérdidas económicas alrededor del mundo, por lo cual el estudio del patosistema constituye una prioridad. Sin embargo, los principales mecanismos activados en bananos después de la infección aún se desconocen y son una limitante para el mejor entendimiento de esta compleja relación. El objetivo de este estudio fue determinar el efecto de la aplicación de peróxido de hidrógeno (H₂O₂), en hojas de plantas de 'Grande naine', en el desarrollo de la enfermedad del rayado negro de la hoja (BLS). Para este propósito, las tres primeras hojas abiertas de las plantas de banano se inocularon con el aislado monoascospórico de *M. fijiensis* CCIBP-Pf-83. A los tres días posteriores a la inoculación diferentes concentraciones de H₂O₂ (10, 20, 30 y 40 mmol l⁻¹) se asperjaron a estas plantas así como a las no-inoculadas. Durante el curso de tiempo del experimento en plantas inoculadas, asperjadas y para las plantas control (infectadas con *M. fijiensis*) se determinaron variables epifitológicas así como el área de las lesiones necróticas a los 49 dpi. Los resultados mostraron que la aplicación temprana de H₂O₂ tiene influencia en el desarrollo de BLS.

Palabras clave: banano, enfermedad del rayado negro de la hoja, hemibiotrófico, peróxido de hidrógeno

INTRODUCTION

Black leaf streak disease (BLS) caused by the fungus *Mycosphaerella fijiensis* Morelet

[anamorph: *Pseudocercospora fijiensis* (Morelet) Deighton], affects bananas and plantains around the world (Churchill, 2011). In this sense, constitute a priority the search

of resistant cultivars as the best choice of disease control. However, to reach this goal the discovering and understanding of the main mechanisms involve in this complex relationship is essential.

In respond to pathogen attack, highly localized biochemical events are rapidly induced like the production of reactive oxygen species (ROS). At the same time, to keep the balance between their production and scavenging, plants possess a number of intracellular antioxidative machinery, to remove ROS and prevent some of the potential toxic effects of them (Mittler, 2017).

Some species like, superoxide (O_2^-) and hydrogen peroxide (H_2O_2), are produced during resistance response to biotrophic and the susceptible response to necrotrophic and hemibiotrophic pathogens respectively (Eloy *et al.*, 2015; Lehmann *et al.*, 2015; Camejo *et al.*, 2016; Lightfoot *et al.*, 2016). However, among the ROS compounds, H_2O_2 has been studied the most and has a special significance for a normal plant cell functioning (Petrov and Van Breusegem, 2012). Besides, it has been shown its direct antimicrobial effect and role in the cross-linking of cell walls, the induction of gene expression, signaling, hypersensitive cell death and in the induced systemic acquired resistance (Quan *et al.*, 2008; Barna *et al.*, 2012).

A number of studies have attempted to elucidate the role of H_2O_2 in different pathogen-host interactions. It has been reported that H_2O_2 accumulation arrest biotrophic whereas necrotrophic pathogens are favored or even stimulated its production (Horbach *et al.*, 2011; Barna *et al.*, 2012). Conversely, in hemibiotrophic life style there is still not a clear definition about its action mode during the infectious process, which remains elusive (Shetty *et al.*, 2003; Shetty *et al.*, 2007; Shetty *et al.*, 2008). Specifically, in 'Calcutta' *M. fijiensis* interaction, the H_2O_2 is related with the hypersensitivity-like reaction (Cavalcante *et al.*, 2011).

In the *M. fijiensis*-*Musa* spp. pathosystem, some studies reveal the role of ROS during the interaction. Specifically, in *Musa acuminata* L.A. Colla subsp. *burmannicoides* E.A. cultivar (cv.) 'Calcutta' (*Musa* AA), the

induction of peroxidase (POX) activity and the cellular accumulation of H_2O_2 have been observed by several authors (Sánchez-García *et al.*, 2009; Sánchez-García *et al.*, 2010; Cavalcante *et al.*, 2011; Torres *et al.*, 2012). In addition, the up-regulation of *Pox* (Passos *et al.*, 2012; Rodríguez *et al.*, 2016). In this cultivar the early defense response against *M. fijiensis* seems to be related with the activity of this enzyme.

Based on a previous study made by Oloriz and Ocaña (2014) to determine the influence of H_2O_2 on *in vitro* growing of *M. fijiensis*, we aimed to investigate the causal relationship between the H_2O_2 application and BLS development *in vivo*. To address this question, we manipulate different H_2O_2 concentrations, which were sprayed to leaves of 'Grande naine' plants. Afterwards, some epidemiological variables and the area of necrotic lesions were determined. The new biochemical elements acquired with this study will contribute to a better comprehension of *M. acuminata* response to fungal infection.

MATERIALS AND METHODS

Establishment of host-pathogen system

Plants from the susceptible *M. acuminata* subgroup Cavendish cv. 'Grande naine' (*Musa* AAA) were obtained from the *in vitro* germplasm collection from Instituto de Biotecnología de las Plantas, Cuba. The *in vitro* propagation of them was realized according to the protocol described by Orellana (1994). Rooted plantlets after eight subcultures of multiplication were transferred to plastic pots (1 l capacity) containing humus, compost and zeolite mixture 5: 3: 2 (v/v) ratios. Plants were acclimatized in the greenhouse until they reached 20 cm tall and at least four active leaves. During the time course of the experiment, a photoperiod of 12 h light/ 12 h dark with an average temperature of 30 ± 2 °C during the day and a relative humidity of $80 \pm 5\%$ was maintained.

The *M. fijiensis* CCIBP-Pf-83 strain was used to prepare the fungal inoculum according to Portal *et al.* (2011). The artificial inoculation of banana plants with a mycelial suspension of this fungus was done in agreement with

the protocol described by Leiva-Mora *et al.* (2010). In this way, the first three open leaves of banana plants were inoculated.

Effect of H₂O₂ application on BLS D progression

At 3 days post-inoculation (dpi) with *M. fijiensis* inoculated and non-inoculated banana plants were sprayed with different H₂O₂ concentrations (10, 20, 30 and 40 mmol l⁻¹) for the abaxial leaf surface. The non-inoculated plants were used to observe any possible toxic effect of H₂O₂. Banana plants inoculated with the pathogen were considered as control. Each treatment included a group of six plants and it was evaluated every seven days.

During the time course of the experiment, for every treatment including inoculated and sprayed plants and for control plants, some epidemiological variables such as: incubation period (PI) (days), time of symptom evolution (TES) (days) and time of disease development (TDE) (days) as proposed by Leiva-Mora *et al.* (2010) were determined. Besides, the area of necrotic lesions at 49 dpi following the method described by Leiva-Mora *et al.* (2015) was measured. For this analysis, lesions in stage three and four of disease development according to the scale described by Alvarado-Capó *et al.* (2003) were included, considering the feasibility of their measurement.

Statistic processing

Data was processed with the Statistical Package for the Social Sciences (SPSS)

version 18.0 (SPSS Inc., Chicago, IL, USA) for Windows, with previous confirmation of the suppositions of normality and variance heterogeneity, with a $p \leq 0.05$. The non-parametric H of Kruskal Wallis and U of Mann-Whitney test was used after the generation of 10 000 samples, with a similar distribution to the real by means of Monte Carlo, with a level of significance $\alpha = 0.05$.

RESULTS

The artificial inoculation of 'Grande naine' plants using the mycelial suspension of *M. fijiensis* (CCIBP-Pf-83 strain), under greenhouse conditions, allowed an adequate development of BLS D symptomatology (Figure 1).

Effect of H₂O₂ application on BLS D progression

The hemibiotrophic pathogen *M. fijiensis* was able to complete its life cycle in 'Grande naine' leaves sprayed with different H₂O₂ concentrations. Specially, 30 and 40 mmol l⁻¹ had the main contribution to BLS D progression. It was evidenced by the higher area of necrotic lesions, observed at the time point of evaluation (49 dpi) (Table 1). Besides, during the time course of the experiment in inoculated banana plants with *M. fijiensis* as well as in non-inoculated ones, there was not any symptom of toxicity in leaves related with the application of different H₂O₂ concentrations (data not shown).

The incubation period for all treatments was 13 dpi except for 30 mmol l⁻¹ where the first



Figure 1. Symptoms of black leaf streak disease developed in leaves of 'Grande naine' plants according to the scale proposed by Alvarado-Capó *et al.* (2003). 0, Leaf symptoms mostly absent. 1, Reddish flecks on lower leaf surface. No symptoms on the upper surface. 2, Regular or irregular reddish circular spots on the lower leaf surface. No symptoms on the upper surface. 3, Regular or diffuse light brown circular spots on the upper leaf surface. 4, Black or brown circular spots, possibly with a yellow halo or chlorosis of adjacent tissues, on the upper leaf surface. Areas of green tissue sometimes present. 5, Black spots with dry centre of grey colour.

Table 1. Area of necrotic lesions in 'Grande naine' plants artificially inoculated with mycelial suspension of *M. fijiensis* CCIBP-Pf-83 strain, under greenhouse conditions and sprayed with different H₂O₂ concentrations at 49 dpi.

Treatment	Area of the lesions (mm ²)	
	Mean	Mean rank
GE + <i>Mf</i> + H ₂ O ₂ 10 mmol l ⁻¹	253.00	68.43 b
GE + <i>Mf</i> + H ₂ O ₂ 20 mmol l ⁻¹	216.66	65.65 b
GE + <i>Mf</i> + H ₂ O ₂ 30 mmol l ⁻¹	576.19	122.00 a
GE + <i>Mf</i> + H ₂ O ₂ 40 mmol l ⁻¹	406.85	102.33 a
GE + <i>Mf</i> (Control)	219.62	61.48 b

Mf: *M. fijiensis*, *GE*: 'Grande naine'. Means followed by different letter indicate differences among the ranges according to H of Kruskal Wallis and U of Mann Witney test ($p \leq 0.05$) $n=30$

Table 2. Time of symptom evolution and disease development in 'Grande naine' plants artificially inoculated with mycelial suspension of *M. fijiensis* CCIBP-Pf-83 strain under greenhouse conditions and sprayed with different H₂O₂ concentrations.

Treatment	Time of symptom evolution (days)	Time of disease development (days)
GE + <i>Mf</i> + H ₂ O ₂ 10 mmol l ⁻¹	36	49
GE + <i>Mf</i> + H ₂ O ₂ 20 mmol l ⁻¹	36	49
GE + <i>Mf</i> + H ₂ O ₂ 30 mmol l ⁻¹	24	35
GE + <i>Mf</i> + H ₂ O ₂ 40 mmol l ⁻¹	36	49
GE + <i>Mf</i>	36	49

Mf: *M. fijiensis*, *GE*: 'Grande naine'

symptoms appeared at 11 dpi. In spite of, 30 and 40 mmol l⁻¹ of H₂O₂ have the same incidence in disease development. The spraying of the lower concentration to banana leaves produced a reduction of 12 days on the TES and 14 on TDE with respect to the others treatments including control (Table 2). For all assays, the complete development of the disease under greenhouse conditions was reached earlier than informed by Alvarado-Capó *et al.* (2003).

DISCUSSION

The progression of BLS on plants of the susceptible cv. 'Grande naine' inoculated with *M. fijiensis*, was in agreement with the protocol described by Leiva-Mora *et al.*

(2010). The feasibility of this procedure allow obtaining a successful development of the disease, in inoculated leaves of plants from the *in vitro* culture.

The knowledge about the main changes that takes place during *M. fijiensis* infection as well as, the time and type of response triggered in *Musa* spp. after its attack, are poorly understood at a biochemical level. In this hemibiotrophic pathosystem, the induction of oxidative stress could be an important defense strategy to protect plants from pathogen infection (Sánchez-García *et al.*, 2010; Cavalcante *et al.*, 2011; Torres *et al.*, 2012). In this sense, to continue working on deciphering the subtle action mechanism involve in ROS metabolism and regulation

throughout the infectious process, offer new opportunities for a better understanding of the disease.

In our experimental conditions, the successful progression of BLSD in banana plants, under an aggressive and highly oxidizing environment, showed the survival capacity of *M. fijiensis*. Previously, Beltrán-García *et al.* (2009) and Oloriz and Ocaña (2014) observed the adaptation and tolerance of this pathogen to higher concentrations of H₂O₂. In this sense, some authors refer that plant pathogenic fungi have developed an enormous array of strategies to defend themselves and to infect their host plants (Horbach *et al.*, 2011).

According with the results founded in the present work, with the exogenous application of 30 and 40 mmol l⁻¹ of H₂O₂ to banana leaves, it seems reasonable their influence in accelerating the BLSD progression. Formerly, Oloriz and Ocaña (2014) under *in vitro* conditions also obtained the higher growing of *M. fijiensis* at 30 mmol l⁻¹ of H₂O₂. Besides, in our time of analysis, the reduction of TES and TDE at this concentration respect to another treatments even control, suggest it as a possible trigger of BLSD.

In the compatible interaction, there is not a clear definition about oxidative stress role throughout the time course of BLSD. Through the long symptomless biotrophic stage of *M. fijiensis*, low H₂O₂ level in 'Grande naine' leaves is expected. Previously, no early detectable amounts of H₂O₂ in 'Grande naine' (Cavalcante *et al.*, 2011) and 'Williams' cv. (Torres *et al.*, 2012) during the same compatible reaction was observed. In this sense, the lack of recognition of the pathogen (Torres *et al.*, 2012) and the activation of the antioxidant systems in the fungus (Beltrán-García *et al.*, 2009) could contribute to this.

In this interaction H₂O₂ application could be favoring the necrotrophic life style of the pathogen, which have been informed before by several authors in another pathosystems (Shetty *et al.*, 2007; Horbach *et al.*, 2011; Barna *et al.*, 2012). Later on, during *M. fijiensis* infection it is possible that the pathogen suppresses host response to

maintain ROS level. Consequently, H₂O₂ production by the plant as a defense response plus the exogenous application of 30 and 40 mmol l⁻¹ of H₂O₂ maybe cannot be neutralize by the cellular antioxidative systems which carry out to accelerate BLSD. In the interaction this molecule could be also acting as a signal for pathogen recognition which was recently reported by Camejo *et al.* (2016).

Considering the relevance of *M. fijiensis*-*Musa* spp. pathosystem as an important threat affecting banana and plantain production around the world, the understanding of biochemical pathways that control intercellular ROS levels constitute a scientific challenge.

Even when some evidences highlight the possible role of H₂O₂ during the compatible response of *Musa* spp. to *M. fijiensis* infection, there is still not a clear understanding about it. ROS production can be harmful to plants as well as pathogens (Barna *et al.*, 2012; Lehmann *et al.*, 2015). Therefore, to maintain a balance between their generation and scavenging is an important aspect to consider for H₂O₂ role as protective, damaging or signaling factors at the proper site and time (Quan *et al.*, 2008; Oliveira *et al.*, 2014; Camejo *et al.*, 2016; Mittler, 2017).

CONCLUSION

This study showed that H₂O₂ application contributed to BLSD progression having 30 mmol l⁻¹ the higher incidence in disease development. Taking into account the complexity of ROS metabolism and its relationship with other pathways during plant response to pathogen infection further studies are therefore necessary in *M. fijiensis*-*Musa* spp. pathosystem. This is in accordance with, in the interaction many elements related with the fine-tune regulation of ROS are still limited.

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