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ORIGINAL PAPER

Expression of *MfAvr4* in banana leaf sections with black leaf streak disease caused by *Mycosphaerella fijiensis*: a technical validation

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Abstract The objective of this study was to validate the use of banana leaf sections as a technique to study the molecular interaction between *Mycosphaerella fijiensis* and *Musa* spp. without the interference of biotic and abiotic factors that commonly occur under field conditions. The growth of *M. fijiensis* in banana leaf sections was evaluated and compared with the growth of the fungus in leaves under field conditions. Growth comparison was carried out through the absolute quantification by real-time PCR of a segment of the *β-tubulin* gene of *M. fijiensis*. Validation of the banana leaf sections technique consisted in monitoring *M. fijiensis MfAvr4* gene expression and its relative quantification by real-time PCR in banana leaf sections. With this technique, it was shown that the growth of *M. fijiensis* and *MfAvr4* gene expression were similar to those observed in infected leaves in the field. These quantitative real-time PCR results support the suitability of using banana leaf sections for molecular studies of gene expression in *M. fijiensis-Musa* spp. interactions.

Keywords Absolute quantification · *β-tubulin* gene · Lectin-like peptide · qRT-PCR · Relative quantification

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Introduction

Black leaf streak (BLS) disease caused by the fungus *Mycosphaerella fijiensis* affects bananas and plantains worldwide. This fungus causes leaf necrosis, reduces the photosynthetic area and indirectly induces premature maturation of fruits, leading to large economic losses (Marín et al. 2003). Nevertheless, little is known about the fungal genes involved in infection, adhesion of asexual or sexual spores to the foliar surface and their germination and penetration through the leaf stomata. Some molecular tools have been reported, such as the fungal transformation protocol developed by Balint-Kurti and Churchill (2001) using the expression of Green Fluorescent Protein, and the cDNA library of early infection in genotype Calcutta IV constructed by Mendoza-Rodríguez et al. (2006). The sequencing of both the *M. fijiensis* genome by the Department of Energy Joint Genome Institute (2010) and of the banana genome by D'Hont et al. (2012) has been essential to study the molecular *M. fijiensis-Musa* spp. interaction. Gene markers of *M. fijiensis*, such as *MfAvr4*, which encodes a lectin-like peptide that protects the fungal cell wall against the action of chitinases (Stergiopoulos et al. 2010), enzymes present in plants that degrade chitin (Hamid et al. 2013), and an *ABC* transporter highly expressed in the early stages of *M. fijiensis* pathogenic development (Couoh-Uicab et al. 2012) have been cloned. Furthermore, to study the pathogen-plant interaction, techniques have been essential to eliminate influences of biotic and abiotic factors in the field on the infection process; the use of banana leaf sections has permitted the rapid and efficient detection of *Musa* genotypes that are resistant to BLS (Twizeyimana et al. 2007). However, the use of this technique in pathogen-plant interaction studies has been controversial as reviewed in detail by Churchill (2011). To validate and standardize usefulness of in vitro techniques

to address specific questions and to understand the pathogen-plant interaction, it is necessary to correlate in vitro results with observations in field conditions.

Quantitative real-time PCR (qRT-PCR) is a suitable molecular tool to validate techniques, since this methodology is highly sensitive and can detect small quantities of nucleic acids and quantify specific nucleic acid sequences in a sample (Radonic et al. 2004). For example, the detection of *M. fijiensis* by qRT-PCR was first reported by Arzanlou et al. (2007), who quantified one pg of gDNA from *Mycosphaerella* spp. in pure cultures and 1.6 pg of gDNA from *M. fijiensis* per mg banana leaves infected with *M. fijiensis.* More recently, a qRT-PCR approach was used to quantify the biomass of *M. fijiensis* in banana leaves collected from plantations with different fungicide applications (intensive and semi-intensive) and to monitor the effect of fungicides on the pathogen in the field (Sánchez-Rodríguez 2012). In this study it is shown that banana leaf sections can reliably be used to study gene expression in the *M. fijiensis*-*Musa acuminata* (cv. 'Grand Nain') interaction by absolute quantification of the fungal *β-tubulin* gene and relative quantification of *MfAvr4* gene expression during BLS disease infection by qRT-PCR.

Material and methods

Fungal material

Mycosphaerella fijiensis C1233 isolate (accession number IMI 392976, International Mycological Institute, CABI Bioscience Centre, Egham, UK) was obtained from Yucatan, Mexico. This strain was cultured in vitro to obtain conidia as reported previously by Peraza-Echeverría et al. (2008). The conidial suspension was adjusted to 200 conidia/μL.

Plant material and growing conditions

Banana leaves of 'Grand Nain' (*Musa acuminata*, AAA, Cavendish Subgroup) naturally infected with *M. fijiensis* were harvested at the Uxmal Experimental Field Station of the National Institute for Forestry, Agriculture and Livestock Research (Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias -INIFAP) in Yucatan, Mexico (20°25′ 36″N; 89°45′20.3″W; 29 m a.s.l.). Naturally infected banana leaves were collected twice from 6 to 9-month-old plants, and samples showing each phase of BLS disease according to Fouré (1985) were selected and weighed; these were then frozen in liquid nitrogen and stored at −80 °C until use.

For artificial inoculation, banana leaf sections were obtained from the two first leaves of 4-month-old 'Grand Nain' plants grown in vitro and transferred to pots containing a mixture of soil and organic matter (2:1) in a greenhouse with a mean temperature of 28 °C, 12-h photoperiod, and 128 μmol/m^2·s light intensity. Whole banana leaves without midrib were treated with sodium hypochlorite (Cloralex® Mexico) (0.6 %; *v*/v) to remove contamination and cut into sections (25 cm^2) with a scalpel, using acrylic molds (25 cm^2) to facilitate cutting and decrease damage to the plant material. They were then placed in Petri dishes containing 0.5 % (*w*/*v*) water agar (Merck, Damstadt, Germany) and 200 mg/L benzimidazole (Fluka, Steinheim, Germany) to delay senescence, and finally were incubated at 26 to 30 °C, and a 12-h photoperiod at 72 μmol/m^2·s light intensity for three days to ensure asepsis before inoculation**.**

Artificial inoculation of banana leaf sections

The abaxial portion of the leaf sections (25 cm^2) was inoculated in vitro with 125 μL conidial suspension (1×10^3conidia/cm^2) using a camel-hair brush, and incubated as mentioned before, prior to nucleic acid extraction. Disease progress was monitored at 0, 5, 10, 15, and 30 days post-inoculation (dpi), sampling 1 cm^2 of leaf sections each time and staining them with 50 μL aniline blue (Sigma Aldrich, USA) (0.025 %; *w*/*v*) during 12 h to observe the conidia and its mycelial growth under an optical microscope (AxionPlan Zeiss®, Germany). Two independent experiments were performed with four replicates for each sample. The control was banana leaf sections with no inoculation and with benzimidazole.

DNA extraction

To quantify the biomass of *M. fijiensis* via the absolute quantification of its *β-tubulin* gene, the protocol of gDNA extraction described by Weising et al. (1991) was followed with modification. One 1 μL ribonuclease (Sigma 50 ng/μL) was added to 50 μL gDNA in 100 μL final volume, and incubated for 1 h at 37 °C. The gDNA was then precipitated with 6.6 μL 3 M sodium acetate, pH 5.0, and 94.4 μL ultrapure water, mixed with 400 μL cold ethanol, incubated on ice for 25 min, and centrifuged at 17,949 *g* for 10 min. The pellet was washed twice with 500 μL 70 % ethanol, and recentrifuged under the same conditions for 5 min. The dry pellet was resuspended in 50 μL of ultrapure water until use. The quantity and quality of gDNA was evaluated using a Nano Drop 1000 analyser (Thermo Scientific, USA).

RNA extraction and cDNA synthesis

To quantify *MfAvr4* gene expression, total RNA was extracted from leaf sections inoculated in vitro and from naturally infected banana leaves following the protocol by Rodríguez-García et al. (2010). Total RNA of *M. fijiensis* mycelia was extracted following the protocol by Islas-Flores et al. (2006) to

obtain standard curves of *β-tubulin* and *MfAvr4* gene for relative quantification. The quality and quantity of total RNA were evaluated using a Nano Drop 2000c analyser. RNA integrity was verified by visualising denaturated-RNA samples in a native agarose gel (1.2 %; *w*/*v*) as reported by Rodríguez-García et al. (2010).

Before cDNA synthesis, 2 μg of each total RNA sample (40 ng/μL) was incubated at room temperature for 15 min with 1 μL desoxyribonuclease I (1 U/μL)(Sigma, Aldrich, USA), 1 μL of 10× buffer solution and ultrapure water (10 μL). After incubation, 1 μL stop solution (1 mM ethylenediaminetetraacetic acid) was added and the mixture was incubated at 70 °C in a water-bath for 10 min, and then rapidly cooled on ice. cDNA synthesis was performed using the TaqMan® Reverse Transcription Kit, according to the manufacturer's instructions (Applied Biosystems, Waltham, Massachusetts, USA). The PCR conditions were: one cycle of 25 °C for 10 min, 37 °C for 60 min, and 95 °C for 5 min. The quality of cDNA synthesis was verified by conventional PCR by detecting the actin cDNA (277 bp) of *M. fijiensis* using the primers reported by Rodríguez-García et al. (2010), MfactF (5′-CTTGACTC CGGTGACGGTGTCACTC-3′), and MfactR (5′-CGTCAGGAAGCTCGTAGGACTTCTC-3′) (accession number: Pr032816296). The PCR conditions were: 95 °C for 5 min, 33 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 7 min (Mastercycles).

Absolute quantification of the *β-tubulin* gene using real-time PCR

The specific primers and the Taqman probe reported by Arzanlou et al. (2007) to amplify a fragment (134 bp) of the *β-tubulin* gene from *M. fijiensis* were used. The reaction mix contained: 1× Taqman Universal PCR Master Mix (Applied Biosystems), 0.333 μM primers (MFBF/MFBR), 0.05 μM Taqman Tubulin-FAM (MFBP), and 25 ng gDNA in 15 μL total volume. A StepOne qRT-PCR detection system was utilized (Applied Biosystems). The PCR conditions were: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

To quantify the *β-tubulin* gene from mycelia of *M. fijiensis*, gDNA was extracted following the protocol described by Johanson (1995), and five serial dilutions of 1:10 (10,1,0.1,0.01,0.001 ng) of 100 ng/μL were prepared to construct a standard curve. The reproducibility of real-time assays was evaluated with an intra-assay experiment (six replicates of the same DNA sample, i.e., infected banana foliar fragments, and banana leaves naturally infected in the field, analysed in a single run); an inter-assay experiment (five replicates of the same DNA sample; banana leaves naturally infected in the field, on different days); and an inter-sample assay (three different gDNA samples of banana leaves naturally infected in the field, analysed in a single run).

Relative quantification of the *MfAvr4* gene using real-time PCR

The Taqman probe (CCCACAAGTCGAGTTCT) and the primers MfAvr4F (5′-GGAATGACAATCAGAAGTGG TGTGA-3′) and MfAvr4R (5′-GATCGTACCGCCCG TGTT-3′) (accession number: Pr032816295) of the *MfAvr4* gene of *M. fijiensis* were used to quantify its expression (68 bp) during infection. The probe and primers were synthesized by Applied Biosystems. The reaction mix contained: 1× TaqMan Gene Expression Assay (MfAvr4), 1× Taqman Universal PCR Master Mix (Applied Biosystems), and 50 ng cDNA in 15 μL total volume. The PCR conditions were: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min.

The *β-tubulin* gene of *M. fijiensis* was the housekeeping gene (endogenous control) used to validate the real-time assay, and was amplified using the primers (accession number: Pr032805662) MfBTubulinF (5′-TTCATCTCCAAACCGGCCAG-3′) and MfBTubulinR (5′-ATGCAGATGTCGTAGAGAGC-3′) designed from cDNA (600 bp). The reaction mix was: 2× Platinum Sybr Green qPCR SuperMix ®, MfBTubulinF/MfBTubulinR (0.2 μM), 6-carboxy-X-rhodamine (Rox) (50 nM), cDNA (from 2 μg total RNA) in 25 μL total volume. The PCR conditions were: 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min (StepOne Applied Biosystems).

The relative expression of the *MfAvr4* gene was calculated using the equations reported by Livak and Schmittgen (2001).

Serial dilutions (1:10) of cDNA (200, 20, 2, and 0.2 ng) from mycelia of *M. fijiensis* were prepared and these were plotted against the threshold cycle (Ct) values to obtain the standard curves, and to calculate the *MfAvr4* Taqman probe/primer PCR efficiencies.

Statistical analysis

The absolute and relative quantifications of *M. fijiensis* in banana foliar fragments were measured in three different samples with their respective biological replicates (in quadruplicate) at 0, 5, 15 and 30 days post-inoculation (dpi), and in naturally infected banana leaves at stages 1, 2, 3, and 4. The Ct values for each PCR reaction were manually set to intersect the exponential phase of the amplification curves, but the baseline was automatically set using the software of StepOne, Applied Biosystems. The PCR efficiency was calculated using the formula: $10^{(-1/\text{ slope})} - 1$ and the data (ΔΔCt values) were analysed by ANOVA and Fisher's multiple comparison tests. When the distribution of variables did not assume normality we applied a Kruskal-Wallis test ($P < 0.05$) (STATGRAPHICS Centurion).

Results

Conidia were observed on the leaf sections at time 0 after dpi (Fig. 1a); apical growth and branching of their hyphae on leaf sections with penetration by stomata at 5 dpi (Fig. 1b); hyphae emerging from stomata, suggesting a previous penetration, and a possible colonisation of substomatal chambers at 15 dpi (Fig. 1c) and a large degree of growth of the mycelia over almost all the area of the foliar fragment and anastomosis at 30 dpi (Fig. 1d).

Absolute quantification of the *β-tubulin* gene

Reaction volume, efficiency of the PCR reaction, and dynamic range of the standard curves were standardized prior to use real-time PCR to quantify the biomass (absolute quantification) of *M. fijiensis* in banana leaf sections, and in naturally infected banana leaves. An efficiency of 88.81 % was observed with a coefficient of determination (R^2) of 0.998 and a slope of −3.623 (Fig. SM1), using dilutions 1:10 (10, 1, 0.1, 0.01 and 1×10^{-3} ng) of 100 ng/μL (initial concentration) gDNA from *M. fijiensis* mycelia in 15 μL total volume. Efficiencies of PCR reactions of 99.05 % and 89.87 % were observed in banana leaf sections and in naturally infected banana leaves, respectively. The mean regression value was 0.99 and 0.983, and the mean slope was −3.345 and −3.591, respectively (Fig. 2a, and 2b).

The mean Ct values (detection threshold cycle) of the *β-tubulin* gene of *M. fijiensis* detected in banana leaf sections, and in naturally infected banana leaves for the intra-assays experiments were 26.91 ± 0.22 SD and 26.10 ± 0.57, respectively (Table SM1). The Ct value for the inter-assay experiment performed using a single sample (naturally infected banana leaves) for five measurements over a period of 15 d (Fig. SM2) was 26.35 ± 0.34 SD. Finally, the Ct value for the inter-sample assay was 29.07 ± 0.19 SD (Fig. SM3). At 0 dpi, 6.5×10^{-3} ng of *β-tubulin* gene from *M. fijiensis* was detected in banana leaf sections, and an increase of 19- and 51-fold in the level at 15 and 30 dpi, respectively (Fig. 3a); in naturally infected banana leaves, we detected 7.5×10^{-3} ng of *β-tubulin* gene of *M. fijiensis* at stage 1, a gradual increase (two-fold) at stage 2, a 20-fold increase at stage 3, and, finally, a slight decrease at stage 4 (Fig. 3b).

Relative quantification of the *MfAvr4* gene

Because the standard curves of the *β-tubulin*, and *MfAvR4* genes showed efficiencies of 96.72 % and 92.81 % respectively (Fig. SM4), the relative expression of *MfAvR4* was quantified using the ΔΔCt ($2^{-\Delta\Delta Ct}$) method; the calibrator to quantify the levels of *MfAvr4* gene expression in banana leaf sections was the*β-tubulin* transcript from time 0 dpi, and the same transcript was used in the first stage of naturally infected banana leaves.

The relative expression of the *MfAvr4* gene in banana leaf sections increased 351-fold at 15 dpi in comparison with that at time 0 dpi, and decreased 65-fold at 30 dpi (Fig. 4a). Furthermore, a basal level of expression of this gene was observed in naturally infected banana leaves at stage 1, and the expression subsequently increased 75-fold and 4150-fold at stages 2 and 4 of infection, respectively (Fig. 4b).

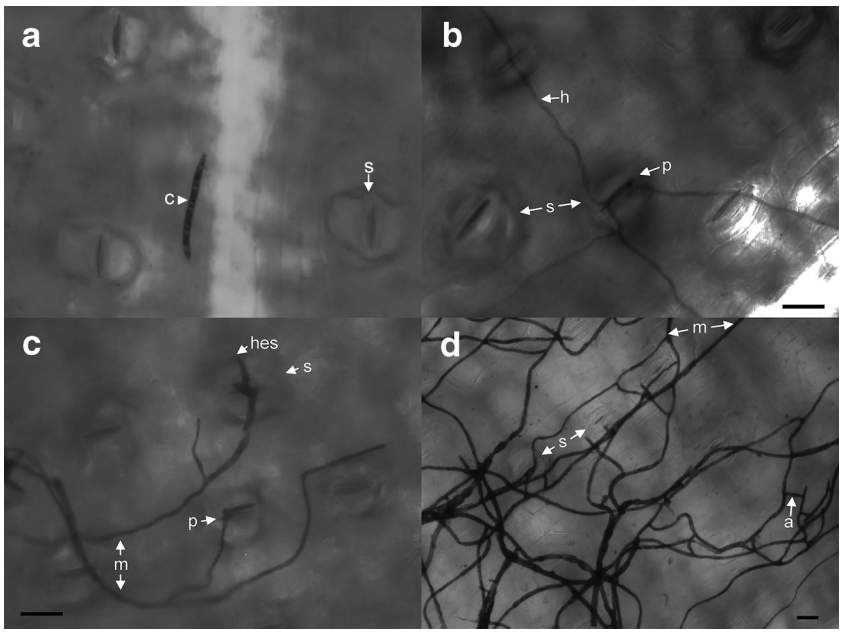

Fig. 1 Microscopic images of *M. fijiensis* growing in artificially infected banana leaf sections. **a** At 0 day post-inoculation (dpi), conidium (c); **b** 5 days dpi, penetration (p) of a stomate by a hypha (s); **c** 15 dpi, a hypha emerging from a stomate (hes); **d** 30 dpi, heavy mycelial growth (m) and an anastomosis (a). The samples were stained with aniline *blue* to observe the fungal infection. Scale bars = 20 μm

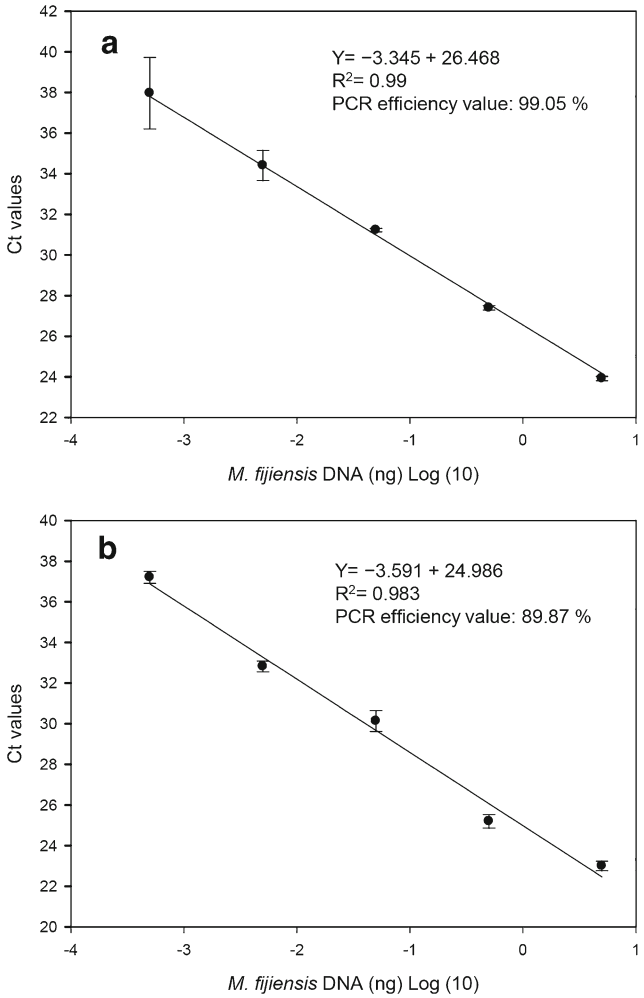

Fig. 2 Standard curve regression between the threshold cycle (Ct) values for the *β-tubulin* gene and the log10 of *M. fijiensis* DNA for the absolute quantification of artificially infected banana leaf sections (**a),** and in naturally infected banana leaves (**b).** Serial dilutions (1:10) of gDNA (100 ng/μL) from mycelia of *M. fijiensis* were prepared to construct the standard curve. *Black circles* represent the mean of data used to calculate the PCR efficiency value using the formula: $10^{(-1/\text{ slope})} - 1$

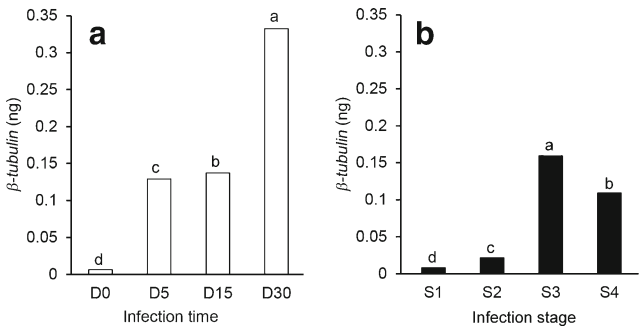

Fig. 3 Absolute quantification of the *β-tubulin* gene from *M. fijiensis* in artificially infected banana leaf sections (D0, D5, D15, and D30 days post-inoculation), and in samples of naturally infected banana leaves at stage1 (S1), stage 2 (S2), stage 3 (S3), and stage 4 (S4). Three different samples were measured with their respective biological replicates (in quadruplicate); different letters indicate statistically significant differences ($P < 0.05$) determined by ANOVA and Fisher tests

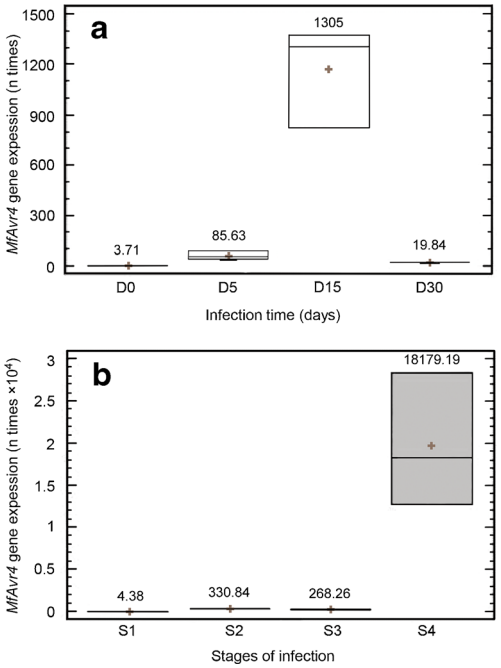

Fig. 4 Relative quantification of the *MfAvr4* gene of *M. fijiensis* in banana leaf sections inoculated in laboratory (**a**), and in naturally infected banana leaves (**b**). Three different samples were measured with their respective biological replicates (in quadruplicate). Statistical differences ($P = 0.05$) were determined by the Kruskal-Wallis test

Discussion

Black leaf streak is considered to be the most devastating foliar disease of bananas and plantains worldwide. Currently, its control depends of the application of synthetic fungicides, whose abuse has caused the appearance of resistant strains of *M. fijiensis*, and has had a negative impact on nature by soil and aquifers contamination (De Lapeyre et al. 2010). Many studies on *M. fijiensis-Musa* spp. interaction exist (Beveraggi et al. 1995; Hoss et al. 2000; Portal et al. 2011). Nevertheless, there are some that have used detached leaves and/or leaf sections to evaluate banana genotype resistance over a short time period, in limited space, similar conditions and most importantly, without other pathogens being present (Twizeyimana et al. 2007), and also to study the potential roles in pathogenesis of two β-1, 3-glucanosyltransferases (Gas) quantifying the *MfGAS1*, and *MfGAS2* expression during BLS disease (Kantún-Moreno et al. 2013). Leaf sections or detached leaves have also been used mainly to evaluate the resistance of cultivars to pathogenic fungi, such as *Phakopsora pachyrhizi* in *Glycine max* (Soybean) (Twizeyimana et al. 2007), and *Phytophtora colocasiae* in *Colocasia esculenta* (Taro) (Brooks 2008). Furthermore, leaf sections have also been used successfully to evaluate the pathogenicity and virulence of other phytopathogens, such as *Pseudomonas syringae* (Yessad et al. 1992) and *Phytophthora infestans* (Vleeshouwers et al. 1999).

Considering the advantages of the banana leaf section technique, and that at present, no validation exists to confirm the reliability of using this technique to study the molecular interaction between *M. fijiensis* and 'Grand Nain' (*Musa acuminata*), *MfAvr4* gene expression was analysed in *M. fijiensis* in banana leaf sections artificially infected in vitro and compared them with the expression of the *MfAvr4* gene from naturally infected banana leaves. First, it was found that the infected banana leaf sections also showed symptoms characteristic of BLS disease observed under field conditions, agreeing with the results reported by Abadie et al. (2008) and Peraza-Echeverría et al. (2008). These authors reported that the first symptoms of BLSD appeared after 18 to 20 dpi and that necrotic lesions appeared at 30 dpi on banana leaf sections from susceptible cultivars. It also was reported that the physiological condition of the plant for banana leaf sections is very important, to observe the late stages of the BLS disease; in this study, conidia germination, mycelium development, anastomosis, and penetration by stomata was observed, similar to what has been described for the infection of leaves grown under field conditions. The asexual cycle could be completed, since at late stages of in vitro infection, conidia were observed, these were cylindrical in shape with 4–5 septae and different lengths and widths (40–60 μm × 2–3μm), as well as new mycelia emerging from stomata. Ascospores were not observed, since a single strain was used and *M. fijiensis* is heterothallic. Secondly, the indirect quantification of *M. fijiensis* biomass in artificially infected leaf sections was equivalent to that found in naturally infected plants.

To obtain an absolute quantification of the *β-tubulin* gene of *M. fijiensis* in banana leaf sections using qRT-PCR, it was necessary to evaluate the Taqman probe and the *β-tubulin* primers via intra-assay, inter-assay and intra-sample assays as performed in other studies (Raymaekers et al. 2009; Winton et al. 2002). In this study, it was observed a reproducibility of the real-time assay of ±0.57, ± 0.34, and ±0.19 SE for the intra-assay, inter-assay and intra-sample assay, respectively. Altogether, these validation assays concerning amplification efficiency, and no variability due to equipment and reagents and reproducibility of the gDNA extractions, support that this molecular tool is amenable for the analysis of *Musa-M. fijiensis* interactions. In addition, the statistical analysis showed that the variability between replicates was negligible.

DNA of *M. fijiensis* at time 0 from banana leaf sections and at stage 1 from naturally infected leaves was detected using the qRT-PCR technique. Furthermore, the standard curves showed 98 % efficiency, a 0.98 mean correlation (R^2) and a mean slope of −3.34. All these values were greater than those reported by Arzanlou et al. (2007), which might be the result of the different extraction protocols and equipment used. These authors also reported that the quantification of *β-tubulin* from *M. fijiensis* using leaf sections was not reliable or consistent, however in our study a positive correlation was observed between the amount of mycelium and the progress of the disease. *Mycosphaerella graminicola* is another fungal pathogen that has been detected in artificially infected wheat leaves since early stages using qRT-PCT. In this case, the amount of fungi also showed a positive correlation with disease progression (Guo et al. 2006). *M. fijiensis* biomass increases throughout the progression of BLS disease, but a decrease was observed in its DNA at stage 4 in leaves naturally infected in the field. Similarly, Qi and Yang (2002) reported that the quantity of biomass of the fungus *Magnaporthe grisea* is not always proportional to the necrotic symptoms that developed in rice. Stage 4 of BLS disease was defined according to the scale of Fouré (1985); however, staging is a subjective parameter that probably does not correlate with the quantity of *M. fijiensis* DNA.

To compare gene expression in both artificially infected banana foliar fragments and naturally infected banana leaves, *MfAvr4* was quantified, a gene of *M. fijiensis* that protects the fungus against the hydrolytic activity of the chitinases produced by the plant (Stergiopoulos et al. 2010). A basal expression was detected at the beginning of infection in both leaf sections and in field samples, and a huge increase in levels at the intermediate stage (15 dpi) for leaf sections and at stage 4 for the field samples. The basal expression of *MfAvr4* might be explained by the presence of chitinases in banana leaves, as reported by Sánchez-García et al. (2012). On the other hand, Cho et al. (2008) found one EST similar to the gene *Avr4* of *Cladosporium fulvum* in three cDNA libraries constructed from *M. fijiensis* cultured in different conditions, meaning that the expression of this gene could be constitutive. In addition, the expression of *MfAvr4* in leaf sections decreased at 30 dpi, but this trend was not observed with the field material, because the late infection stages were not included in the study. Nevertheless, a decrease in *MfAvr4* expression at late stages can be predicted, due to the decrease in chitinases resulting from the high degree of foliar damage. Similar pattern of expression has been reported for other genes of *M. fijiensis* (ABC transporter, *MfGAS1*, and *MfGAS2*) during interaction with banana (Couoh-Uicab et al. 2012; Kantún-Moreno et al. 2013). Marshall et al. (2011) reported an initial increase in *Mg3LysM* gene expression in *M. graminicola* during infection in wheat, followed by a decrease at later stages of the disease. They observed that this gene protected the fungus from chitinases, and that its expression was higher during the asymptomatic stage but decreased during the necrotic stage, suggesting that the expression of *Mg3LysM* may be not essential to eliminate chitinases, because of the presence of other wheat defense gene as *PR-1* (Pathogenesis-related), and that the Mg3LysM protein may be induced by the presence of living host cells and rapidly down-regulated during the activation of the host cell death response in the course of necrosis. Motteram et al. (2009) also reported a high expression of the *MgNLP* gene of *M. graminicola* during the asymptomatic

stage of the colonisation of a susceptible cultivar, followed by a drastic decrease in *MgNLP* gene expression during the formation of lesions. There is no clear role for this gene; although it shares homology with necrosis-like protein family, *MgNLP* causes no necrosis on wheat and it is dispensable for infection of susceptible wheat cultivars. High expression of the *SIX1* gene of *Fusarium oxysporum* was also reported during early stages of infection in cells of *Solanum lycopersicum*, and low or no expression was observed during the total colonisation of the roots. The *SIX1* gene encodes an effector secreted in xylem sap during colonisation of xylem vessels. Interestingly, it seems to play a role in distinguishing between living and dead plants since dead tomato seedlings failed to induce its expression but *SIX1* was overexpressed immediately upon penetration of living cells in the root cortex (Van der Does et al. 2008).

Similar efficiencies of amplification for *MfAvr4* (96.72 %) and *β-tubulin* (92.81 %) were obtained, suggesting that the Ct ($2^{\Delta\Delta Ct}$)method is an appropriate method to quantify the expression levels of these genes (Livak and Schmittgen 2001). The kinetic amplification of both genes (endogenous and target) must be similar to avoid errors in the method. The *MfAvr4* gene expression measured in banana leaf sections was similar to that in the samples from the field, suggesting that the in vitro fungal infection of banana leaf sections is similar to the fungal infection of naturally infected banana leaves at least in the BLS stages analysed. In summary, the use of the banana leaf sections technique was validated to study the molecular interaction of *M. fijiensis* with its plant host 'Grand Nain'(*Musa acuminata*), monitoring its biomass and quantifying the *MfAvr4* gene expression. According to our experience and to guarantee reproducibility and to ensure homogeneous growth conditions, it is recommended to use plants (the first two young open leaves) from greenhouse with similar physiological age (three or four months). The results presented here show that the in vitro banana leaf sections system is suitable for gene expression analysis in the *M. fijiensis-Musa acuminata* interaction.

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