SHORT COMMUNICATION

# Quantification of phenols in lesions caused by *Mycosphaerella fijiensis* Morelet in 'Cavendish naine'

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**ABSTRACT:** The presence of phenols was demonstrated in leaf lesions caused by *Mycosphaerella fijiensis* Morelet in inoculated plants of 'Cavendish naine'. Phenolic compounds were microscopically detected in plant tissue with symptoms in different development stages by using staining cathecol derivatives, and they were quantified by spectrophotometry. An accumulation of phenols was observed since the first lesions appeared and gradually increased as symptoms progressed. It varied from $29\mu g.cm^2$ in the early lesion stage to $79\mu g.cm^2$ in the last stage of the disease. Although phenols were not effective in preventing further disease development in this compatible interaction, these results constituted an evidence of the presence of these biochemical compounds as part of the plant defense response against pathogen infection.

**Key words:** Black Sigatoka, cathecol derivatives, histochemical detection.

## Cuantificación de fenoles en lesiones causadas por *Mycosphaerella fijiensis* Morelet en 'Cavendish naine'

**RESUMEN:** Se demostró la acumulación de fenoles en lesiones foliares causadas por *Mycosphaerella fijiensis* Morelet en plantas inoculadas de 'Cavendish naine'. Los compuestos fenólicos fueron detectados microscópicamente, en el tejido vegetal correspondiente a síntomas en diferentes estados de desarrollo, mediante la tinción de derivados del catecol y cuantificados por espectrofotometría. Se observó que la acumulación de fenoles ocurrió desde la aparición de las primeras lesiones e incrementó a medida que evolucionaron los síntomas, desde un valor de concentración de $29\mu g.cm^2$ en el estado más temprano de la enfermedad hasta $79\mu g.cm^2$ en el estado más avanzado. Aunque la inducción de fenoles no fue efectiva en el control del progreso de la enfermedad en esta interacción compatible, estos resultados constituyen una evidencia de la presencia de estos compuestos como parte de la respuesta de la planta ante la infección del patógeno.

**Palabras clave**: Sigatoka negra, derivados del catecol, detección histoquímica.

Histochemical techniques have been used in many kinds of studies of plant structures because of their simplicity and rapidness, which made them quite useful for a first approach to the phenomenon to be studied. They have been used in many plants to evaluate their morphological changes during growth (1), the pathogen spread in plant tissues, and also for observing the biochemical response of plant cells to pathogen infection in some pathosystems (2,3).

Accumulation of phenolic compounds in the host as a response to fungal attack to strengthen the cell wall is well known l (4), and it has been correlated with disease resistance in a number of plant pathogen interactions.

*Musa* sp.-*Mycosphaerella fijiensis* Morelet pathosystem has been studied from many perspectives with the aim of increasing the knowledge of this plant-pathogen interaction. In Cuba, some investigations have

been carried out in the field of the molecular biology related to this pathosystem (6) and the use of epidemiological variables and components of resistance to differentiate genotypes of *Musa* spp. (7). However, the use of biochemical techniques to detect and quantify molecular compounds involved in banana plant defence, such as phenols, has been little successful.

The symptoms caused by *M. fijiensis*, causal agent of Black Sigatoka, in susceptible *Musa* spp. plants are characterized by the presence of pronounced lesions with chlorotic halos since the stage 3 of the disease that suggests a photo-oxidative damage, the release of pathogen toxins and the triggering of the plant defense response (5). In this sense, obtaining of evidences of local accumulation of biochemical compounds, specifically phenols, in lesions of banana plants inoculated with *M. fijiensis*is is very important in studying this plant-pathogen interaction.

The objective of this work was the histochemical detection and quantification of phenolic compounds in the lesions caused by *M. fijiensis* in inoculated plants of 'Cavendish naine'.

'Cavendish naine' plants (*Musa* AAA, susceptible to Black Sigatoka) was used as the plant material. They were obtained from ¨La Cuba¨ enterprise (Ciego de Ávila, Cuba), propagated by tissue culture according to the protocol described by Orellana (8) and acclimatized during three months in a greenhouse until they reached 20cm of height and with more than three developed leaves. The pathogenic strain CCIBP-Pf83 of *M. fijiensis* from the microbial culture collection of Plant Biotechnology Institute (IBP, Cuba) was used.

Preparation of *M. fijiensis* mycelia suspension, artificial inoculation and evaluation of the symptom development and evolution were carried out following the protocol described by Alvarado-Capó *et al.* (9). The inoculum concentration was adjusted to approximately $10^5$ mycelia fragments.mL$^{-1}$. The first three leaves of 20 plants were inoculated with the fungal suspension and other 20 plants were inoculated only with 1% (w/v) gelatin and included as controls. Plants were kept under greenhouse conditions with 80% of humidity and with a sun light intensity of 3 841 µmol.m$^2$.s (measured with Extech Light Meter 401025, USA).

For the histochemical detection and quantification of phenolic compounds, 20 leaf segments (2cm$^2$) with one lesion per symptom stage (from 1 to 5), according to the scale proposed by Alvarado *et al.* (9) and without lesions were randomly sampled from inoculated 'Cavendish naine' plants and from control plants, respectively.

Leaf segments (samples) were placed on clean glass microscope slides before staining. The accumulation of phenolic compounds was visualized by the protocol described by Reeve (10) for cathecol derivatives. One drop of 10% (v/v) aqueous sodium nitrate, one drop of 10% (v/v) aqueous acetic acid and one drop of 20% (w/v) aqueous urea were added. After 3 min, two drops of 2N NaOH were finally added. Samples were examined with a compound light microscope (Olympus, 200X) and photographed immediately with a digital camera (Canon PC1201). A deep cherry red colouration was observed in the presence of phenolics.

The total phenol concentration was determined colorimetrically according to Bray and Thorpe (11). The samples were grounded in liquid nitrogen and extracted in 10 ml of 80% (v/v) ethanol, boiled at 50ºC for 30 min, and then centrifuged at 8 000 g for 10 min. Reaction volume included 1 ml supernatant, 3 ml distilled water, 1 ml Folin Ciocalteau reagent and 2 ml 20% (w/v) sodium carbonate. The ethanolic extract was heated for 1 min in a boiling water bath and cooled in tap water. The solution was diluted to 10 ml with distilled water and the intensity of the blue colour was measured at 750 nm in a spectrophotometer UV-visible (Genesys 6, Thermo Electron Corporation, USA) using a blank (the blank was obtained with 3 ml distilled water instead of the extract and the colour was developed as described above). A standard curve was prepared from known concentrations of gallic acid to calculate the concentration of phenolic compounds in the samples. The concentration of phenolic compounds was expressed as total phenol concentration (µg. cm$^{-2}$).

The data were analyzed by using the Statistic Package for Social Science (SPSS) version 19.0 for Windows. The values obtained were analyzed statistically by nonparametric tests of Mann-Whitney after verifying the assumptions of normality and heterogeneity of variance.

The accumulation of phenols was detected in the foliar tissue as deep red coloured zone corresponding to lesions in all the symptom stages, which increased gradually while lesions progressed (Fig. 1). Similarly, it was observed in the total phenol concentrations (Fig. 2), where the highest values were obtained in the stages 4 and 5.

It has been demonstrated that, after inoculation, many structural and physiological changes occurs in the cells surrounding the infection site for restricting pathogen spread and for reducing fluid loss (12).

Phenol accumulation in foliar lesions is an evidence of the triggering of the defensive response in plants to pathogen attack, which has been described as one of the most important molecular events (4). In addition, it
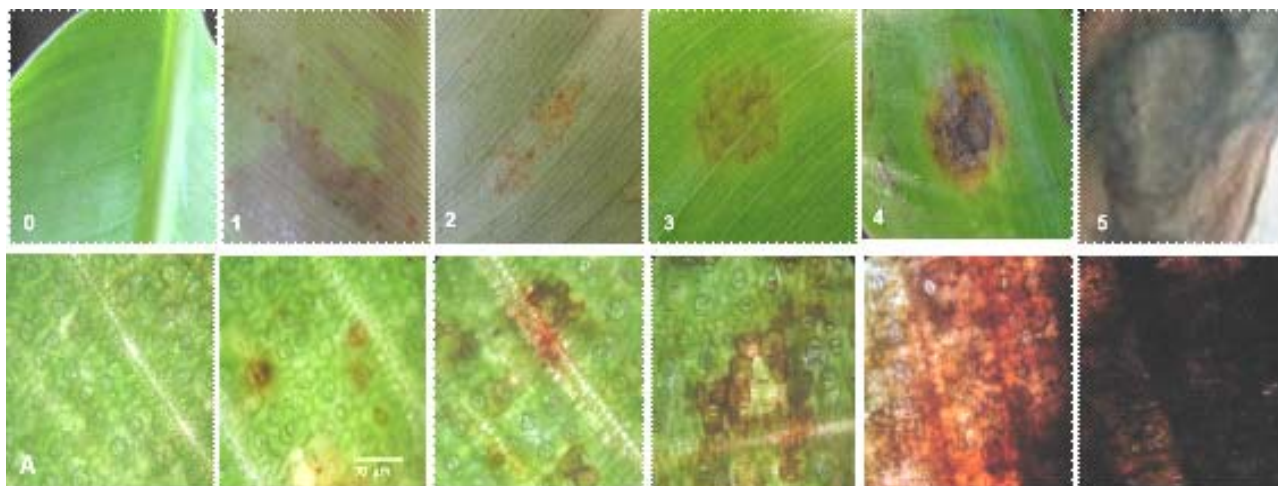
**FIGURE 1.** Histochemical detection of phenolic compounds (A, red stain) in foliar lesions caused by *M. fijiensis* in 'Cavendish naine' (Musa AAA) plants. Leaf tissue without lesions (0) and with lesions in different stages (1-5). / *Detección histoquímica de compuestos fenólicos (A- tinción de color rojo) en lesiones foliares de plantas de 'Cavendish naine' (Musa AAA) causadas por* **M. fijiensis**. *Tejido foliar sin lesiones (0) y con lesiones en diferentes estados (1-5).*
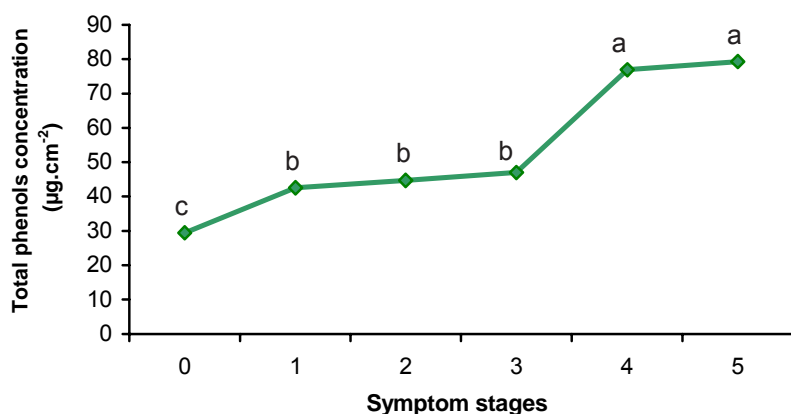


**FIGURE 2.** Concentration of phenolic compounds in foliar lesions caused by *M. fijiensis* inoculation in 'Cavendish naine' plants. Tissue without lesions (0), and in different symptom stages (1-5). Means with different letters are significantly different at p<0.05./ *Concentración de compuestos fenólicos en lesiones foliares de plantas de 'Cavendish naine' inoculadas con* **M. fijiensis**. *Tejido sin lesiones (0) y con lesiones en diferentes estados (1-5). Medias con letras diferentes son significativamente diferentes para p<0.05.*

has been demonstrated that phenol accumulation after infection are directly toxic to pathogens (12) and their polymerization makes the cell wall thicker and stronger (11). This phenomenon has been observed in many pathosystem such as *Sorghum vulgare* Pers.-*Sclerotium rolfsii* Sacc. (15), *Solanum lycopersicum* L.-*Botrytis cinerea* Pers. (2), *Eucalyptus* sp.-*Mycosphaerella* sp. (3) and *Lactuca sativa* L.- *Bremia lactucae* Regel (16).

In *Musa* sp.- *M. fijiensis* interaction, those evidences are very limited; however, Portal (6) mentioned the possible relationship of the phenol metabolic pathway in *Musa* defense response to *M. fijiensis* in the susceptible genotype 'Grande naine' by using a subtractive gen library. Accordingly, the results of this work provide a new direct evidence of a local accumulation of phenolic compounds in foliar lesions of 'Cavendish naine' plants after *M. fijiensis* inoculation as part of the defence response of banana plants in this compatible interaction. At the same time, they validate the use of this histochemical tool to detect biochemical compounds as a simple and rapid alternative for testing different resistance phenotypes under controlled conditions, which can be applied in *Musa* breeding programs. In general, they may also contribute to a better understanding of this important plant-pathogen interaction.

## ACKNOWLEDGMENT

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