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FUNGAL DISEASES

Production of hydrophilic phytotoxins by *Mycosphaerella fijiensis*

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Abstract We have established a reproducible strategy to purify hydrophilic phytotoxins present in the aqueous filtrate of *Mycosphaerella fijiensis* Morelet. The lyophilized culture filtrate is initially treated with activated charcoal, then successively purified using vacuum liquid chromatography and semipreparative high performance liquid chromatography. Phytotoxic activity was tested using a leaf-spot assay on healthy banana leaves.

Keywords Phytotoxins · *Mycosphaerella fijiensis* · Bananas · Leaf-spot assay · Black sigatoka

Phytotoxins are defined as low molecular weight, secondary metabolites produced by phytopathogens that may cause necrosis, chlorosis, wilting, or a combination of these symptoms in susceptible plant hosts (Huang 2001; Knoche and Duvick 1987; Prell and Day 2000). These metabolites have been classified according to their role in the infectious process as host selective toxins (HSTs) or non-host selective toxins (non-HSTs) (Ballio and Graniti 1991; Knogge 1996; Walton 1996; Wolpert et al. 2002).

HSTs are considered as primary determinants of pathogenicity because their production is required for the pathogen to colonize the plant. Furthermore, these metabolites can induce the symptoms of the disease even in absence of the pathogen and are toxic only to hosts of the pathogen that produces them (Markham and Hille 2001; Walton 1996; Wolpert et al. 2002). On the other hand, non-HSTs are not essential for the infection, but they contribute to the virulence of the pathogen and are thus recognized as secondary determinants of pathogenicity; these metabolites can affect a wider or different range of hosts, causing a variety of symptoms (Ballio and Graniti 1991; Knogge 1996; Mitchell 1984). Phytotoxins can exert their effects by inhibiting specific enzymes or by interfering with membrane functions or defense responses (Strange 2007). Potential applications of phytotoxins include their use as probes for studying the molecular basis of disease resistance and susceptibility in plants, as tools for the in vitro screening and breeding of disease resistant plants, and as natural alternatives to herbicides in weed control (Berestetskiy 2008; Harelimana et al. 1997; Okole and Schulz 1997; Strobel et al. 1991).

The hemibiotrophic fungal pathogen *Mycosphaerella fijiensis* is recognized as the causal agent of black sigatoka, the most destructive and devastating disease of bananas and plantains in nearly all banana-growing regions of the world (Carlier et al. 2000). The disease significantly decreases banana production by premature ripening of the fruits and lower photosynthetic activity from a reduction in leaf area (Sanchez and Cárdenas 2002). The symptoms of the disease include elongated, necrotic lesions surrounded by chlorotic zones, suggesting the involvement of phytotoxins (Upadhyay et al. 1990).

Even though a number of lipophilic phytotoxins have been reported from organic extracts of *M. fijiensis* (Hoss et al. 2000; Stierle et al. 1991; Upadhyay et al. 1990), to date no hydrophilic phytotoxins have been identified from this pathogen. Recently, phytotoxic activity was detected,

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using the leaf-spot assay on banana leaves, in both the organic extract and the aqueous residual phase obtained after the ethylacetate extraction of culture filtrates of *M. fijiensis* grown in V-8 juice medium. The differing solubilities indicated the production of two groups of phytotoxic metabolites by *M. fijiensis*, one lipophilic and the other of hydrophilic nature (Puch-Ceh 2001). These results did not agree with those reported by Stierle et al. (1991), who found no evidence of hydrophilic phytotoxins produced by *M. fijiensis* in M-1-D medium. Furthermore, while Stierle et al. (1991) reported that the phytopathogen produces a high yield of lipophilic fraction when cultured in M-1-D medium; culturing of *M. fijiensis* in V-8 juice medium results in a lipophilic fraction that is low in yield (21.3 mg/l) and moderately active (Puch-Ceh 2001). As part of our interest in the phytotoxic metabolites produced by *M. fijiensis* and the study of their role in the infectious process, we describe here the production of hydrophilic phytotoxins by *M. fijiensis*.

Technical-grade solvents, distilled in the laboratory, together with analytical (J.T. Baker, Xalostoc, Edo. de México, México) and high performance liquid chromatography (HPLC) grade (J.T. Baker) solvents were used in the various extraction and purification procedures. Solvents were evaporated under reduced pressure using a rotary evaporator (Büchi, Postfach, Flawil, Switzerland, model 111) equipped with a water bath (Büchi, model 461), which was kept at 40°C. Aqueous fractions were lyophilized using a LABCONCO freeze dryer model 7753020 (Kansas City, USA). Vacuum liquid chromatography (VLC) and column chromatography purifications were carried out using TLC-grade E.M. Merck (Darmstadt, Germany) silica gel 60GF and Aldrich (Milwaukee, WI, USA) silica gel (70–230 mesh), respectively.

The different fractions were monitored by analytical HPLC using a Waters 600 series system (Milford, MA, USA), equipped with a Waters 2487 UV/Vis detector and a Waters 600E controller loaded with the program Millenium. Separations were carried out using a Waters C18 Spherisorb analytical column (5 µm ODS2; 4.6 mm inner diameter, 250 mm long) and a 10:90 mixture of CH_3CN:buffer (K_2HPO_4, 25 mM, pH 2.5 adjusted with perchloric acid) as the mobile phase; with a flow rate of 0.3 ml/min and the detector set at both 240 and 260 nm. Phytotoxic fractions were purified by semipreparative HPLC using a Hypersyl C18 (Alltech, Deerfield, IL, USA) preparative column (120 8U; 10 mm inner diameter, 250 mm long), and the same 10:90 mixture of CH_3CN:buffer (K_2HPO_4, 25 mM, pH 2.5 adjusted with perchloric acid) as the mobile phase, with a flow rate of 0.6 ml/min, monitoring at 240 and 260 nm.

The strain of *M. fijiensis* (C-1233) was kindly donated by Dr. Jean Carlier (CIRAD, France). The banana plant material (corms and buds *of Musa acuminata* cv. Grande Naine) used for establishing in vitro cultures was provided by Alberto Mayo (UJAT, Villermosa, Tabasco, México).

Cultures of the fungus were grown in 500 ml flasks containing 200 ml of V-8 juice (Herdez, México, DF, México) medium with 2 g $CaCO_3$/l. The pH of the medium was adjusted to 5.5 with 1 N HCl prior to sterilization. Each flask was inoculated with 1 ml of an aqueous suspension of fresh mycelium and conidia, then incubated in an orbital shaker at 150 rpm (New Brunswick Scientific, Orbital Shaker Model G25), at 26 ± 2°C, for 30 days, under photoperiod (12 h light/12 h dark) conditions. After the incubation period, the culture broth was separated from the mycelial mat by filtration through two layers of gauze and paper towels, and the volume of the filtrate was concentrated in vacuo to 1/10 of its original volume. The resulting concentrated filtrate was then lyophilized.

The phytotoxic activity of the filtrate and the different purified fractions was evaluated using a leaf-spot assay on healthy leaves of banana plants (*M. acuminata* cv Grande naine) growing in pots under greenhouse conditions. The first or second youngest leaf of a 4-month-old banana plant was excised, then disinfected for 60 s using a 5% solution of commercial sodium hypochlorite (Clorox, Tlalnepantla, Edo. de México, México). The leaves were rinsed with sterile distilled water, dried between two paper towels and placed in a plastic container, previously disinfected with 70% ethanol, lined with five wet paper towels.

A 20 µl drop of the filtrate or different fractions (2 or 3% solution) was placed on a wound made with a scalpel in the adaxial face of the leaf. Two leaves were used for each treatment, and the container was kept at room temperature under natural light conditions. The phytotoxic effects (i.e., total damaged area, including necrosis and chlorosis) were registered at 72 h after treatment; the lesion area was calculated using a leaf area measurer (Li-3100; Li-Cor, Lincoln; Nebraska, USA). Both sterile uninoculated medium and water were used as controls. All evaluations were done in duplicate.

The lyophilized crude filtrate (A, 10 g) was resuspended in water (1:50 lyophilized filtrate to water) and treated with activated charcoal (1:5 lyophilized filtrate to activated charcoal). The resulting suspension was left to stir slowly overnight at 40°C, and then filtered. The clarified filtrate was concentrated to 1/10 of its original volume and then lyophilized to produce a pigment-free fraction B (7.1 g, 71%). Extraction (methanol) of the residual activated charcoal yielded 0.2 g (2%) of fraction C.

A portion of pigment-free fraction B (1 g) was purified by VLC using a stepwise gradient elution with dichloromethane/methanol/water (14:7:1 to 10:10:1) mixtures, which yielded 12 fractions (D1 to D12). Leaf-spot assay

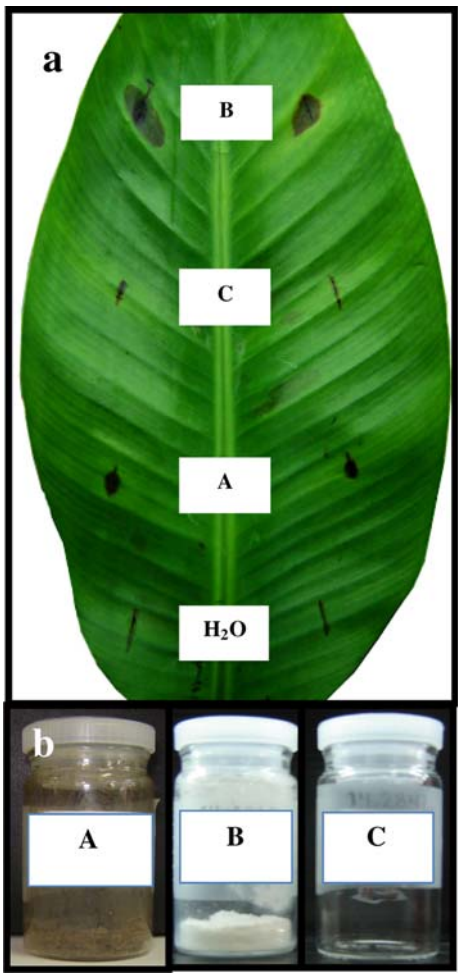

Fig. 1 Phytotoxic damage on banana leaf caused by the crude filtrate of *Mycosphaerella fijiensis* (*A*) and the pigment-free fraction (*B*) tested at 3% (w:v). **a** The pigment-free fraction (*B*) caused more damage than did the original crude filtrate (*A*). Fraction C, from the methanol extraction of the residual activated charcoal, did not cause significant damage when tested at 3% on banana leaves. Both sterile uninoculated medium and water (H_2O) were used as controls. Damaged tissue around the wound (necrosis and chlorosis) was quantified using a foliar area measurer (Li-COR, Lincoln; Nebraska, USA Li-3100). **b** Physical appearance (dark powder) of the lyophilized culture filtrate of *M. fijiensis* (*A*) before treatment with activated charcoal; lyophilized pigment-free fraction (*B*); and methanol extract of the residual activated charcoal (*C*)

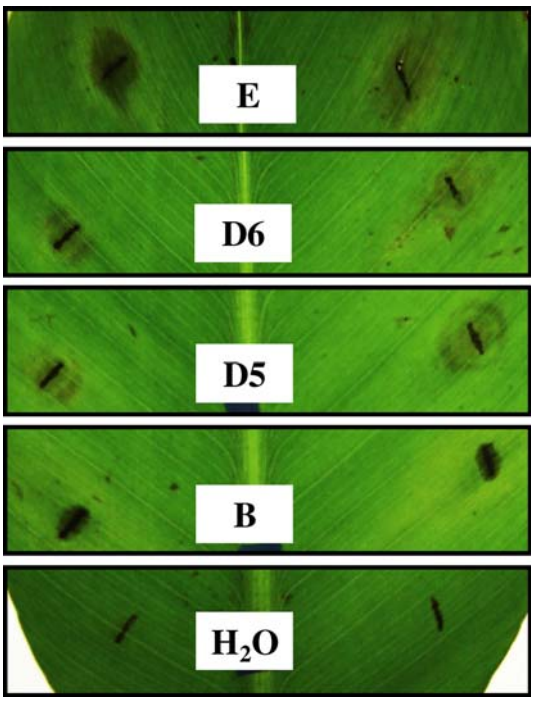

Fig. 2 Phytotoxic damage of banana leaf caused by fractions *D5* and *D6*, obtained by vacuum liquid chromatography purification of fraction *B* from *Mycosphaerella fijiensis*. Fractions *D5* and *D6* were phytotoxic when tested at 3% (w/v) in the leaf-spot assay. Fraction *E*, resulting from the combination of fractions *D5* and *D6*, also was phytotoxic at 3% (w/v). Sterile uninoculated medium and water (H_2O) were used as controls. Damaged tissue around the wound (necrosis and chlorosis) was quantified using a foliar area measurer (Li-COR, Lincoln; Nebraska, USA Li-3100)

evaluation of all fractions at 3% indicated that phytotoxic activity was located in fractions D5 (167.8 mg) and D6 (138.6 mg).

The phytotoxic fractions D5 and D6 were combined to produce fraction E. Semipreparative HPLC purification of fraction E (250 mg) produced fractions F3 (peak at t_R 18.8 min) and F4 (peak at t_R 28.9 min), which were lyophilized and then desalted by column chromatography (isocratic elution, dichloromethane/methanol/water 14:7:0.5), to yield the corresponding fractions G (2.2 mg) and H (1.5 mg), each a major component in HPLC. The phytotoxic activity of all fractions was evaluated at 2% using the leaf-spot assay.

It is important to mention that initial attempts to purify the hydrophilic phytotoxins from the lyophilized crude culture filtrate of *M. fijiensis* using a number of different chromatographic techniques and fractionation procedures, e.g., solid-phase extraction [Amberlite XAD-2 (Supelco, Bellefonte, PA, USA) and Diaion HP-20 (Nippon Rensui, Kanagawa, Japan)], reverse-phase (C18) (Sigma-Aldrich, St. Louis, MO, USA) chromatography, and gel filtration chromatography (Sephadex LH-20, Uppsala, Sweden), were hampered by the pigments in the lyophilized crude filtrate. However, treatment of the lyophilized crude filtrate (A) with activated charcoal produced a pigment-free fraction (B), which caused more phytotoxic damage than did the original lyophilized crude filtrate (A) (Fig. 1a). This finding suggests that the hydrophilic phytotoxins produced by *M. fijiensis* and present in the pigment-free fraction B (Fig. 1b), are different than those previously identified and reported as melanin shunt pathway metabolites (Stierle et al. 1991).

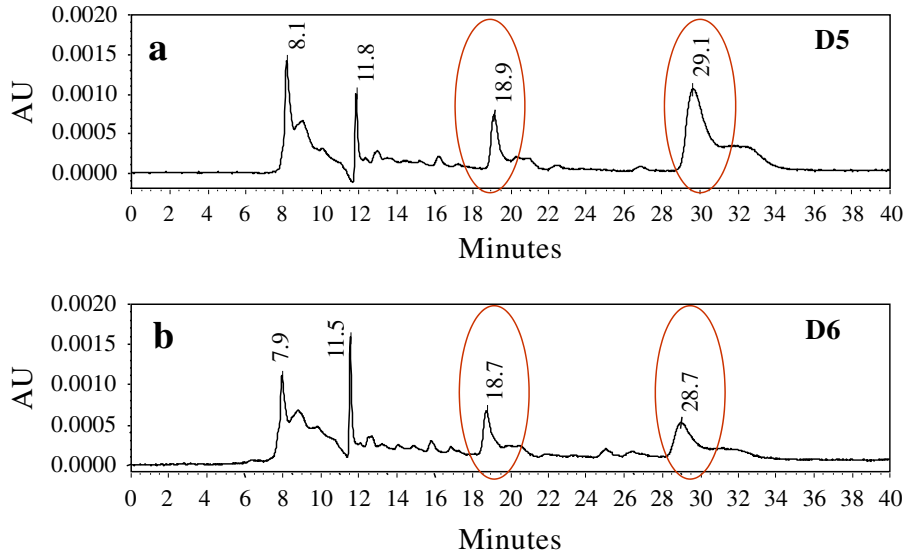

Fig. 3 Chromatographic profiles (HPLC) of phytotoxic fractions obtained by vacuum liquid chromatography of culture filtrate of *Mycosphaerella fijiensis*. **a** Fraction D5. **b** Fraction D6. In the HPLC chromatographic profile of both phytotoxic fractions there were two similar components at t_R 18.8 min and t_R 28.9 min. The fractions were analyzed by analytical HPLC on a Waters C18 Spherisorb analytical column (5 µm ODS2; 4.6 mm inner diameter, 250 mm long), with a 10:90 mixture of CH_3CN:buffer (K_2HPO_4, 25 mM, pH 2.5 adjusted with perchloric acid) as the mobile phase, with a flow rate of 0.3 ml/min, and the detector set at 240 and 260 nm

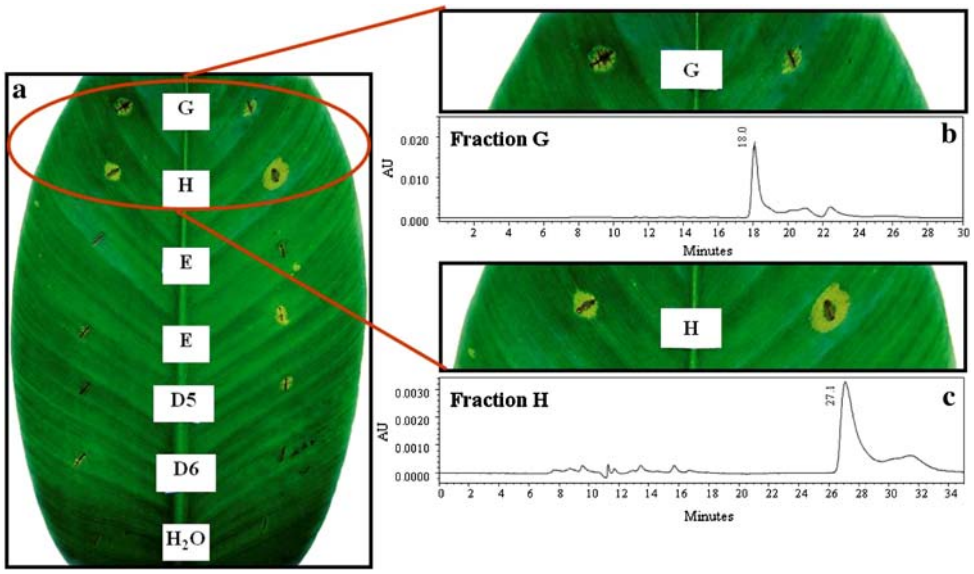

Fig. 4 Phytotoxic damage of banana leaf caused by purified fractions from culture filtrate of *Mycosphaerella fijiensis* tested at 2% (w/v). **a** Fractions *G* and *H* caused more damage than did the original sample (*E*). Sterile uninoculated medium and water (H_2O) were used as controls. **b** Chromatographic profile (HPLC) of fraction *G*. **c** Chromatographic profile (HPLC) of fraction *H*

Attempts to fractionate the pigment-free fraction B, using solid-phase extraction, reverse-phase, and gel filtration chromatography, were unsuccessful. However, VLC purification of fraction B resulted in the isolation of two semipurified fractions (D5 and D6) with phytotoxic activity when tested at 3% (w:v) (Fig. 2). The HPLC analysis of the two phytotoxic fractions showed the presence of two common components at t_R 18.8 min and t_R 28.9 min (Fig. 3a, b).

Purification of the combined phytotoxic fractions D5 and D6 (to produce phytotoxic fraction E, Fig. 2) by semipreparative HPLC yielded two fractions, F3 (t_R 18.8 min) and F4 (t_R 28.9 min) which, after a desalting procedure by column chromatography, yielded fractions G and H, each showing a major component on HPLC (Fig. 4b, c), and both causing more phytotoxic damage than did the original fraction E, when tested at 2% (w:v) (Fig. 4a).

This report is the first on hydrophilic phytotoxins being produced by *M. fijiensis* and to present a simple strategy to purify them. Presently, we are isolating more of fractions G and H. The identification of the chemical structure of the purified metabolites will soon be published.

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