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Phytoanticipins from banana (*Musa acuminata* cv. Grande Naine) plants, with antifungal activity against *Mycosphaerella fijiensis*, the causal agent of black Sigatoka

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Abstract The previously detected antifungal activity against *Mycosphaerella fijiensis* of aqueous infusions of healthy banana (*Musa acuminata* cv. Grande Naine) leaves, suggested the production of phytoprotectants by the plant. The bioassay-guided VLC-purification of the lyophilized infusion of the leaves of 4-month old healthy banana (*M. acuminata* cv. Grande Naine) plants, resulted in the purification of a phytoanticipin with strong antifungal activity against *M. fijiensis* Morelet, the causal agent of black Sigatoka, the most destructive and devastating disease of bananas and plantains in the world. The LC-MS analysis of the purified phytoanticipin suggests a steroidal saponin structure with four sugar units attached to the C-3 position of a diosgenin-like aglycone. This represents the first report of phytoanticipins occurring in *M. acuminata*.

Keywords Black Sigatoka · Diosgenin · *Musa acuminata* · *Mycosphaerella fijiensis* · Phytoanticipins · Saponins

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During their co-evolution with microorganisms, plants have developed physical, physiological and chemical defensive barriers to resist the attack of pathogens (Agrios 2005; Baker et al. 1997; Glazebrook 2005; Hammond-Kosack and Jones 2000; Van Etten et al. 1994). Chemical barriers are represented by low molecular weight secondary metabolites with antimicrobial activity, which are collectively known as phytoprotectants and are classified in two groups: constitutive and inducible. Inducible phytoprotectants are commonly known as phytoalexins, while constitutive phytoprotectants are called phytoanticipins. While phytoalexins are absent, or present at very low concentrations, in healthy plants, and their biosynthesis and accumulation can dramatically increase in response to both biotic and abiotic stress, phytoanticipins are preformed secondary metabolites with antimicrobial activity *in vitro* (Van Etten et al. 1994).

More than 350 phytoprotectants have been characterized from different plant species (Kuc' 1995). These metabolites have shown a wide diversity of chemical structures that include isoflavonoids such as the phytoalexins maackiain, produced by *Trifolium pratense*, and medicarpin, isolated from *Medicago sativa* (Van Etten et al. 1996); dithiocarbamates like brassinin, obtained from *Brassica napus*, and camalexin, produced by *Camelina sativa* (Pedras and Mukund 2004; Pedras and Ahiahonu 2005); and saponins such as the phytoanticipins α-tomatine, from *Lycopersicon esculentum*, and avenacin A1 and the avenacosides, from *Avena sativa* (Osbourn 1996). Each of these phytoprotectants have been isolated from important economic crops and they have all been demonstrated to play an important role in plant defense against pathogens.

Phytoalexins have received greater attention than phytoanticipins because of their potential as natural fungicides. However, their application has been limited because their production *in planta* depends on the activation of inducible defense mechanisms, and because the chemical synthesis of these products, in many cases, is not commercially viable (Kuc' 1995). Although phytoanticipins represent one of the first barriers encountered by the pathogen during its interaction with the host, they have received less attention. Yet, they represent an important source of low-cost antifungal agents with potential applications in agriculture (Molyneux 2002).

Banana (Musa spp.) is the fourth most important staple food crop in the world, after rice, wheat and maize (FAO 2003). Production of banana in the tropics, however, is affected by black Sigatoka, a destructive foliar disease caused by *Mycosphaerella fijiensis* Morelet (Carlier et al. 2000). It has been suggested that the natural resistance of some *Musa* spp. to *M. fijiensis* is related to the post-infection activation of different defense mechanisms in the plant, e.g. production of proteins related to pathogenesis (Lepoivre et al. 1993), hypersensitive response (Mourichon et al. 1997), changes in the structure of preformed metabolites (Hoss et al. 2000), and production of phytoalexins (Quiñones et al. 2000). Even though a number of phytoalexins have been reported from the crop (e.g. irenolone, emenolone and musanolones) (Harborne 1999; Luis et al. 1993, 1996), to date no phytoanticipins have been reported from *Musa* spp.

Recently, during the optimization of the culturing conditions for *M. fijiensis*, strong antifungal activity against *M. fijiensis* was detected in aqueous infusions prepared with healthy leaves of banana plants (*M. acuminata* cv. Grande Naine) (Puch-Ceh 2001). These results suggested the production of phytoprotectants by *M. acuminata*. As part of our interest in the role of natural products in plant-pathogen interactions, this report describes the isolation and preliminary identification of a phytoanticipin produced by *M. acuminata*, with antifungal activity against *M. fijiensis*.

The banana plant material (corms and buds of *Musa acuminata* cv. Grande Naine) used for establishing the *in vitro* cultures were provided by Alberto Mayo (UJAT, Villermosa, Tabasco, México). Healthy leaves (500 g) of 4, 6, 8, 10, or 12-month-old banana (*M. acuminata* cv. Grande naine) plants growing in pots under greenhouse conditions, were cut in small pieces and boiled in 1 l of distilled water for 30 min. The infusion was separated from the leaves by filtration through two layers of gauze and lyophilized using a LABCONCO freeze dryer to produce 4.3 g (0.86%) of freeze-dried (powdered) infusion.

A portion (0.5 g) of the lyophilized infusion from the leaves of 4-month old banana (*M. acuminata* cv. Grande naine) plants was purified by vacuum liquid chromatography (VLC) using E.M. Merck silica gel 60GF (column size: 3 cm diameter, 5 cm height) and a stepwise gradient elution with dichloromethane/methanol/water mixtures (four 100 ml fractions of 14:7:1 and four 100 ml fraction of 10:10:1), to produce eight main fractions (**A** to **D** and **E** to **H**, respectively). Testing of all fractions for antifungal activity against *M. fijiensis* (all evaluations were done in triplicate), demonstrated that only fraction **C**, which showed a major component by TLC, was active. Analytical TLC was carried out using aluminum-backed silica gel ($60F_{254}$) plates (E.M. Merck, 0.2 mm thickness). The various components in the chromatograms were visualized by dipping the plates in a solution of phosphomolibdic acid (20 g) and ceric sulfate (2.5 g) in 500 ml of sulfuric acid (5%), followed by drying and gentle heating. Technical-grade solvents, distilled in the laboratory, together with analytical (J.T. Baker) and 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, model 111) equipped with a water bath (Büchi, model 461) which was kept at 40°C.

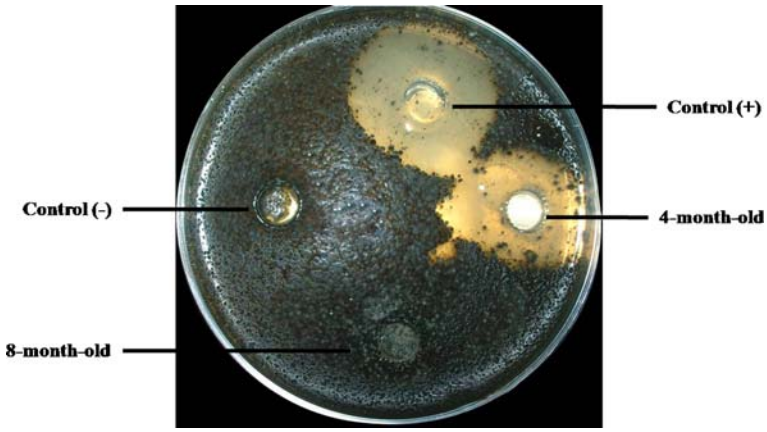

Fig. 1 Antifungal activity of aqueous infusions obtained from healthy leaves of 4 and 8-month-old banana plants (*M. acuminata* cv. Grande Naine) tested at 5% (w:v) against *Mycosphaerella fijiensis*. Neomicol at 1% and sterile water were used as positive and negative controls, respectively

For the evaluation of antifungal activity, Petri dishes were first filled to ¼ of their volume with "agar-agar" (water agar) medium (Agarmex®, 20 gl^{-1} of water). Separately, a portion (ca 1 cm^2) of mycelium from a *M. fijiensis* parent culture (strain C-1233, kindly donated by Dr. Jean Carlier, CIRAD, France) was ground with 7 ml of water and mixed with 500 ml of PDA medium at 36ºC, and the resulting suspension was poured onto the Petri dishes containing the "agar-agar" medium and cylindrical molds (0.5 cm in diameter) placed on the surface to form wells. The samples (lyophilized infusion and purified fractions) were dissolved at 5% in water and sterilized by filtration with Millipore® filters (0.45 μm) and 150 μl of sample were utilized to fill the wells. Neomicol® (15 μl at 2%) and water were used as positive and negative controls, respectively. The Petri dishes were incubated for 15 days, at room temperature and under natural light conditions.

Fraction **C** (8.6 mg) was dissolved in 600 μl of a MeOH/H_2O (1:1) mixture and centrifuged. The supernatant was analyzed by LC-MS using a Surveyor HPLC system coupled to a Deca XP^{plus} ion trap mass spectrometer (both Thermo Electron). Injections of 2–10 μl were passed through a Luna C-18(2) reverse-phase column (Phenomenex; 100 mm long, 2 mm inner diameter, 3 μm C-18 particles) using the following gradient of acetonitrile (solvent B) and 0.1% formic acid in water (solvent A) at 30ºC and 300 μl min^{-1}: 0 min, 20% B; 3 min, 25% B; 20 min, 50% B; 30 min, 80% B; 32 min 80% B; 33 min, 20% B; 45 min, 20% B. Detection was by UV (190–600 nm) and positive electrospray MS (ESI-MS). Spray chamber conditions were 55 units sheath gas (arbitrary units of the manufacturer), 5 units aux gas, 350ºC capillary temperature, and a spray voltage of 5.2 kV. Data-dependent MS2 and MS3 spectra were collected using a collision energy of 35% and isolation width of 3.0 *m/z*.

A portion (250 mg) of the lyophilized infusion was dissolved in 30 ml of H_2O and 40 ml of HCl 1 M; the mixture was stirred slowly, refluxing at 85°C, for 2 h. The reaction mixture was extracted with CH_2Cl_2 (three times: 2:1, 1:1, 1:1 v/v) and the organic fraction was washed with a solution of $NaHCO_3$ (5%). Evaporation of the solvent yielded 10.7 mg (4.28%) of hydrolyzed crude product.

The acid hydrolysis product was analyzed by GC-MS using an Agilent Technologies model 6890 N gas chromatograph, equipped with a MS detector 5975B (Iner MSD), and a 5% phenyl methyl silicone capillary column (HP-5 MS; 30 m long, 0.25 mm inner diameter, 0.25 μm film). GC conditions were as follows: helium as the carrier gas, with a flow of

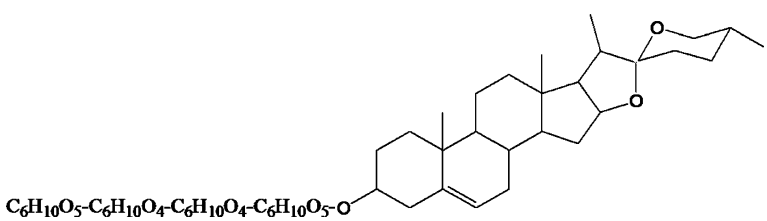

Fig. 2 Proposed structure of the steroidal saponin, having a diosgenin skeleton and four sugar units bonded to C-3, present in fraction **C**

0.25 ml min^{-1}; detector, 280°C; injector, 250°C; injected volume, 1 μl. The column was held for 3 min at 250°C and programmed at 8°C min^{-1} to a final temperature of 300°C for 20 min.

Evaluation of the antifungal activity against *M. fijiensis* of infusions obtained from healthy leaves of 4, 6, 8, 10 and 12-month-old banana plants (*M. acuminata* cv. Grande Naine) showed that only the infusion from 4-month-old plants had significant activity (Fig. 1). These results confirm those previously reported (Puch-Ceh 2001) and are in agreement with reports that the concentration of phytoanticipins decreases with the age of the plant (Osbourn 1999).

Initial attempts to isolate the antifungal metabolites using a number of different chromatographic techniques and fractionation procedures, e.g., solid-phase extraction (Amberlite XAD-2 and Diaion HP-20), reverse-phase (C18) chromatography, and gel permeation (Sephadex LH-20) chromatography, were unsuccessful. However, VLC purification of the lyophilized infusion produced a fraction **C** which showed antifungal activity against *M. fijiensis* and a major component by TLC.

The LC-MS analysis of fraction **C** showed a main component at t_R 8.1 min of the ion chromatogram (Supplementary material Fig. S1). The mass spectrum of this main component showed a protonated molecular ion peak $[M+H]^+$ at *m/z* 1031 (Supplementary material Fig. S2), corresponding to a molecular weight of 1030 and suggesting a possible molecular formula of $C_{51}H_{82}O_{21}$. The analysis of the fragmentation pattern of the protonated molecular ion peak at *m/z* 1031 allowed the detection of major fragments at *m/z* 869 $[(M+H)\text{-}162]^+$, *m/z* 723 $[(M+H)\text{-}162\text{-}146]^+$, *m/z* 577 $[(M+H)\text{-}162\text{-}146\text{-}146]^+$, and *m/z* 415 $[(M+H)\text{-}162\text{-}146\text{-}146\text{-}162]^+$ (Supplementary material Fig. S3), corresponding to the sequential loss of a hexose ($C_6H_{10}O_5$), two deoxyhexoses ($C_6H_{10}O_4$), and a hexose ($C_6H_{10}O_5$), respectively. Additionally, the analysis of the fragmentation pattern of the ion fragment at *m/z* 415 showed a base peak at *m/z* 271, corresponding to the fragment resulting from the opening of ring E in steroidal saponins (Budzikiewicz et al. 1964), and a fragment at *m/z* 253, corresponding to the loss of a water molecule, in a fragmentation characteristic of a C-3 secondary alcohol in a triterpenoid structure. These results suggested that the main component in fraction **C** has the structure of a steroidal saponin, with the chain of sugars (hexose, two deoxyhexoses, and hexose) attached to the C-3 position of a diosgenin-type aglycone (Fig. 2).

To confirm the presence of steroidal saponins with a diosgenin-type aglycone in the infusion of healthy banana leaves, the lyophilizate was subjected to an acid hydrolysis. The TLC (Supplementary material Fig. S4) and GC (Supplementary material Fig. S5) chromatographic profiles of the crude hydrolysis product showed the presence of components having R_f values similar to those of diosgenin and sarsasapogenin. The identity of sapogenins in the hydrolyzate was confirmed by a co-chromatography analysis of the reaction product and two authentic samples.

This is the first report of phytoanticipins occurring in *M. acuminata*, with a saponin structure that is similar to those reported for other phytoanticipins, e.g. α-tomatine and the avenacosides (Osbourn 1996). This finding also represents an important contribution to the knowledge of the role of natural products in the banana-*M. fijiensis* interaction and opens the possibility to learn more about the importance of this type of saponins in the resistance or susceptibility of different banana varieties to black Sigatoka. Currently, the identification of the full chemical structure of the major saponin in fraction **C** is in progress. The results will be published in due course.

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