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RELATIONSHIP BETWEEN THE FUNGUS *CERATOCYSTIS FIMBRIATA COFFEA* AND THE CANKER DISEASE OF THE COFFEE TREE

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Key Word Index—*Ceratocystis fimbriata coffea*; Ophiostomatales; fungus; canker disease; mycotoxins.

Abstract—The active fraction of an extract of the fungus *Ceratocystis fimbriata coffea*, which is responsible for the canker disease of the coffee tree, provided 13 metabolites which come mainly from the polyketide pathway. In addition to the five isocoumarins previously described, eight further metabolites are reported. Some of the isolated compounds showed necrotic symptoms on the coffee tree at concentrations of *ca* $1-4 \times 10^{-3}$ M. Previously unpublished spectroscopic data of some of the isolated compounds are reported.

INTRODUCTION

Ceratocystis fimbriata is a perithecial ascomycete, which was first described in 1890 as a pathogenic agent causing the sweet potato black rot [1]. This fungus, also named *Ceratostomella fimbriata*, *Ophiostoma fimbriatum* or *Endoconidiophora fimbriata*, also infects several highly valued commercial crops and trees (e.g. coffee, cocoa, hevea, plane, oak and mango). The strain currently investigated was isolated in Colombia on the coffee tree (*Coffea arabica* L.). The fungus causes on its host a disease named 'Ilaga macana' or canker disease of the coffee tree [2]. The aim of this study was to determine whether this disease could be related to the production, by the fungus, of some phytotoxic metabolites.

RESULTS AND DISCUSSION

After three weeks of incubation in liquid culture on a synthetic medium [3], the culture broth of *Ceratocystis fimbriata coffea* was filtered to remove the dark-coloured mycelium. The greenish filtrate was then extracted with ether and afforded a brown–yellow extract. This crude extract was first evaporated and the volatile fraction obtained was analysed by GC–mass spectrometry. All these volatiles were identified. There were four esters (ethyl to isoamyl acetates), four branched saturated C_6 hydrocarbons, two alcohols (ethanol and isobutanol), one ketone (propane-2-one) and one unsaturated hydrocarbon (pent-2-ene). The dry residue of the extract was partitioned into an acidic and a neutral fraction by washing it with 1 M $NaHCO_3$. The acidic fraction was acidified to pH 2 with HCl, extracted with ether and dried. It was then chromatographed on cellulose with the eluent butanol–ethanol–H_2O (5:2:1:1). The neutral fraction was dried and chromatographed on a silica gel column using hexane–ethyl acetate–acetic acid (50:50:1). Final purification of isolated compounds occurred either by TLC on silica gel or cellulose, or by reverse phase HPLC C-18 (methanol–H_2O). 8-Hydroxy-3-hydroxymethyl-6-methoxyisocoumarin (**1**), 6,8-dihydroxy-3-(2-hydroxypropyl)isocoumarin (**2**), 6,8-dihydroxy-3-hydroxymethylisocoumarin (**3**), 8-hydroxy-3-methyl-6-methoxyisocoumarin (**4**), 6,8-dihydroxy-3-methylisocoumarin (**5**), 3-methyl-2-indolinone (**7**), gliotoxin (**8**), 3,4-dihydro-3,4,8-trihydroxy1(2*H*)naphthalenone (**9**), vermelone (**10**), scytalone (**11**) and tyrosol (**13**) were isolated from the neutral fraction. From the acidic fraction, **3**, 3-indoleacetic acid (**6**), **9**, **11** and *cis*-4-hydroxyscytalone (**12**) were isolated. All compounds were identified by spectroscopic methods.

Isolation of **1**–**5** was recorded previously [1]. These compounds (except **5**) produce a necrotic symptom on coffee tree leaves at concentrations of *ca* $2-4 \times 10^{-3}$ M. This toxic activity could be due to the ability of isocoumarins to bind with some enzymes [4].

Compound **6** was isolated and identified by its ^{1}H NMR spectrum. An excess of auxin can inhibit tissue lignification and enhance the rate of both respiration and transpiration [5]. This particularity could partly explain the withering of plants infected by *Ceratocystis fimbriata*. In our case, **6** produced a necrotic symptom on the coffee tree leaves at 3×10^{-3} M. Compound **7** is a byproduct of **6**; it was identified by comparison with reported data [6]. These authors mention the absence of auxin-like activity in this compound.

Compound **8** was identified mainly by comparison of

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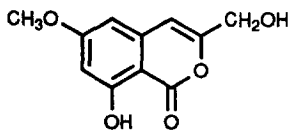

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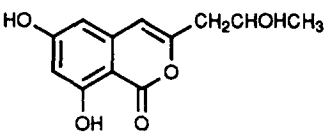

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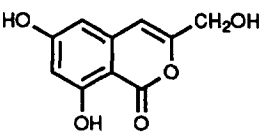

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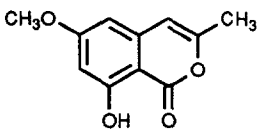

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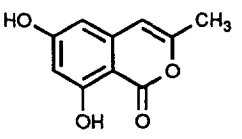

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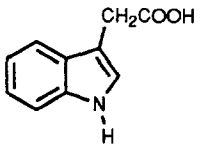

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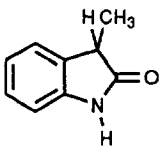

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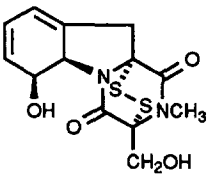

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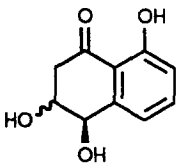

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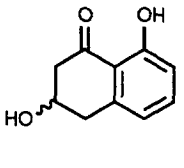

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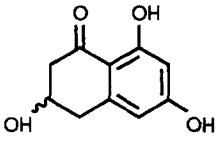

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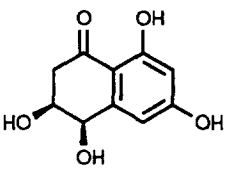

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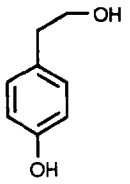

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its EI and CI mass spectra with reported data. This toxin is known for its antibiotic and toxic effects. We observed a necrotic symptom on coffee tree leaves at $3–4 \times 10^{-3}$ M. Compounds **9** and **12** were first isolated from *Pyricularia oryzae* [7]. Compounds **10** and **11** were mentioned from *Verticillium dahliae* [8] and several other fungi [9, 10]. These compounds are known to be intermediates in the synthesis of fungal melanin [8, 11, 12]. None of these compounds showed significant toxic activity on coffee trees at concentrations of *ca* 1×10^{-3} M.

Compound **13** has been already isolated from the fungus *Ceratocystis clavigera*, which is responsible for the blue stain of the American mountain pine [13]. This metabolite was reported for its toxic activity against rice and lettuce seedlings [14], but showed no activity against coffee trees at $3–4 \times 10^{-3}$ M.

We conclude that the *in vitro* culture of the fungus *C. fimbriata coffea* produces numerous toxic metabolites. As these compounds are active at relatively high concentrations and are described from other fungal sources, they can be defined as non-specified toxins. The symptoms observed on diseased coffee trees could be, at least partly, explained by a cumulative or a synergistic effect of these compounds. The presence of a host-specific toxin, active at very low concentrations, seems to be excluded in the relationship between *C. fimbriata coffea* and the symptoms of the canker disease of the coffee tree.

EXPERIMENTAL

Fungal strain and cultivars of *Coffea arabica* were kindly provided by Dr J. Leguizamon (Cenicafe, Chin-

china, Colombia). The methods used for *in vitro* cultures of the fungus and for the bioassays were described previously [1]. ^{1}H NMR spectra were recorded at 400 or 300 MHz. Solvents were MeCO-d_6, CD_3OD or $CDCl_3$. Int. standards were not used. Mass spectra were recorded at 70 eV. GC-MS were run with an HP5890 II GC coupled with a HP 5971A mass selective detector. Prep. TLC was carried out on precoated TLC glass plates CEL 300-10 UV_{254} 0, 1 mm, and on silica gel glass plates, 1 mm LS_{254}. Compounds were detected at 254 and 366 nm. Phenolic compounds were revealed with diazotized *p*-nitroaniline. CC was performed with a 0.015–0.040 mm size silica gel.

Spectroscopic data for isolated compounds (only unpublished data are reported). Dihydro-3,4-trihydroxy-3,4,8-1(2*H*)naphthalenone (**9**): COSY ^{1}H–^{1}H (MeCO-d_6, 400 MHz) δ 7.67/7.29, 7.67/6.96, 4.79/4.23, 3.18/2.85; HETCOR (short range) ^{13}C–^{1}H NMR (Me_2CO-d_6, 600 MHz): δ 137.2/7.67, 117.8/7.29, 116.4/6.96, 72.9/4.79, 71.4/4.23, 43.8/3.18, 43.8/2.85. Other spectral data have been described [7].

cis-Hydroxyscytalone (**12**). COSY ^{1}H–^{1}H (Me_2CO-d_6, 400 MHz) δ 6.57/6.19, 6.57/4.74 (weak), 4.74/4.23, 4.23/2.83; HETCOR (short range) ^{13}C–^{1}H NMR (Me_2CO-d_6, 200 MHz) δ 43.40/2.83, 70.12/4.23, 70.67/4.74, 101.9/6.19, 108.1/6.57. HETCOR (long range) ^{13}C–^{1}H NMR (Me_2CO-d_6, 400 MHz) δ 148.4/4.74, 148.4/4.23, 110.2/2.83, 110.2/2.83, 110.2/4.74, 110.2/6.57, 110.2/6.19, 201.5/2.83, 201.5/2.83, 201.5/4.23. NOEDIFF experiment (Me_2CO-d_6, 400 MHz) at δ 4.23 [H-C(3)] showed a stronger NOE at 4.74 [H-C(4)] (6.56%) than at 2.83 [Ha, b-C(2)] (4.38%). It allowed us to deduce that the relative configuration was *cis*. This was supported by the fact that homonuclear decoupling on H-C(4) produced a triplet for H-C(3), showing that H-C(3) was equidistant from Ha-C(2) and Hb-C(2). Other spectral data have been published elsewhere [6].

Methylation product of **12**. Me_2SO_4 was used for derivatization. The product was extracted in $CHCl_3$ and dried on $CaSO_4$. R_f (EtOAc, silica gel) = 0.6. ^{1}H NMR spectrum (200 MHz, $CDCl_3$) showed 16 protons, δ (ppm) = 7.18 [1H, *d*, *J* = 2.5 Hz, H-C(5)], AB system centred at 6.69 [2H, AB, *J* = 8.5 Hz, H-C(2), H-C(3)], 6.57 (1H, *d*, *J* = 2.5 Hz, H-C(7)], 3.96 (*s*, 3H, OMe), 3.95 (*s*, 3H, OMe), 3.93 (*s*, 3H, OME), 3.90 (*s*, 3H, OMe).

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