Secondary metabolite profile and phytotoxic activity of genetically distinct forms of *Colletotrichum gloeosporioides* from yam (*Dioscorea* spp.)

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ABSTRACT

Highly virulent slow growing grey (SGG), moderately virulent fast growing salmon (FGS), and avirulent/weakly virulent fast growing grey (FGG) forms of *Colletotrichum gloeosporioides* have been described from yam (*Dioscorea* spp.) but little is known about their chemodiversity or the role of toxins in their pathogenesis. Secondary metabolite profiles in high performance thin layer chromatography showed that the pathogenic SGG

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and FGS forms have a chemotype (A or B) that is distinct from the non-pathogenic FGG form (chemotype C). Crude extracts of 35-day-old Czapek-Dox yeast broth cultures of FGS and SGG isolates caused tissue necrosis on treated yam leaves but not those of FGG isolates. Extract from uninoculated broth cultures showed no phytotoxic activity. Toxicity of the culture filtrate was not host specific and toxic substances were thermostable. *Dioscorea* genotypes with varying levels of resistance to anthracnose differed in their sensitivity to crude toxin extract of FGS (Cg33) and SGG (Cg25) isolates, indicating that these extracts may be useful in evaluating host resistance to anthracnose *in vitro*. Analysis of two toxin fractions unique to the pathogenic FGS and SGG forms using mass spectrometry and nuclear magnetic resonance suggested the presence of a low molecular weight amide peptide. However, possibly due to low yield and the presence of impurities, the chemical structure of the compound(s) could not be fully elucidated.

Keywords: Anthracnose, chemotype, chromatography, *Colletotrichum gloeosporioides*, *Dioscorea* spp., metabolite profiling, mass spectrometry, NMR, phytotoxin

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Introduction

Anthracnose is among the most widespread and economically important diseases of water yam (*Dioscorea alata* L.) worldwide. The disease is caused by *Colletotrichum gloeosporioides* Penz. and Sacc. (teleomorph, *Glomerella cingulata* [Stonem.] Spauld. and Shrenk) (Abang *et al*. 2002). It attacks all above-ground plant parts: die-back of shoots and eventual death of affected plants may result in over 80% yield loss when

susceptible varieties are grown under high disease pressure and conducive environmental conditions (Nwankiti *et al*. 1984; Green 1998; McDonald *et al*. 1998; Ano *et al*. 2002).

The presence of a distinct chlorotic zone surrounding dark brown anthracnose lesions on yam suggests the likely effect of a pathogen-induced toxin (Molina & Krausz 1989; Abang *et al*. 2003). The possibility that toxic metabolites play a role in the development of anthracnose on yam has been suggested (Amusa 1993, 2000; Alleyne 2001; Moura-Costa *et al*. 1993), and efforts have been made to extract and test the phytotoxic principle on yams with varying levels of resistance to anthracnose. The advantages of using *in vitro* plantlets rather than whole plants for assaying phytotoxins have been reviewed (Amusa 2006; Daub 1986; Soledade & Pedras 2004; Ŝvábová & Lebeda 2005). Experiments with pathogen culture filtrates have shown that tissue response *in vitro* may correlate with disease reaction of the host species/variety and, where this occurs, the use of culture fitrates may allow selection of important traits in disease resistance *in vitro* (Amusa 2006; Daub 1986; Moura-Costa *et al*. 1993).

Amusa *et al.* (1993) and Ahoussou (1989, cited in Moura-Costa *et al*. 1993) extracted phytotoxic substances from *C. gloeosporioides*-infected yam leaves, which induced necrotic lesions similar to that produced by the pathogen on yam leaves. The toxin extracted by Amusa *et al.* (1993) gave a fluorescent band similar to that produced by toxic metabolites of the pathogen in culture, indicating that it is a vivotoxin. In preliminary studies on the chemical characterization of culture filtrates, Ahoussou (1989, cited in Moura-Costa *et al*. 1993) showed that phytotoxicity of toxic fractions of a West African isolate of *C. gloeosporioides* could be attributed to an as yet unidentified glycoprotein and an associated polysaccharide. Similar results were obtained by Alleyne

(2001) who partially purified and characterized phytotoxic metabolites exuded by *C. gloeosporioides* from yam in Barbados. Water-soluble glycoprotein-type compounds were observed, and the protein fraction of the toxin complex showed host selectivity when tested on a wide range of yam and non-yam hosts. The chemical structure of the phytotoxin(s) remains elusive.

Three forms of *C. gloeosporioides* have been described from yam (Abang *et al*. 2002). Sequence analysis of the rDNA internal transcribed spacer (ITS) region confirmed that the moderately virulent fast-growing salmon (FGS) and the highly virulent slow-growing grey (SGG) strains, which cause typical anthracnose symptoms, belong to *C. gloeosporioides* (Abang *et al*. 2002). The avirulent/weakly virulent fast-growing grey (FGG) strain appeared unrelated to *C. gloeosporioides* and it remains unclear whether it should be retained within the *C. gloeosporioides* species complex (Abang *et al*. 2002; Johnston & Jones 1997). Secondary metabolites could be used as an additional criterion in chemotaxonomic studies of pathogenic and non-pathogenic forms of *C. gloeosporioides* from yam (Sutton 1992, Frisad *et al.* 2008). Although the 'blueprint' of each form is represented by the genome, its behaviour is expressed as its phenotype, i.e. growth characteristics, cell differentiation, response to the environment, the production of secondary metabolites and enzymes. Therefore, the profile of (secondary) metabolites - fungal chemodiversity – is important for understanding functional genomics in this pathosystem and the elucidation of host-pathogen interactions.

In this paper, genetically distinct forms of *C. gloeosporioides* from yam were further characterized based on their secondary metabolite profile. Also, bioassays were used to determine the phytotoxicity of toxin fractions both *in vitro* and *in vivo*, and the

response of diverse *Dioscorea* genotypes to anthracnose was evaluated using the toxins. Attempts were made to purify two toxin fractions from a virulent FGS strain, and elucidate their chemical structure using mass spectrometry and nuclear magnetic resonance.

Materials and methods

Fungal cultures, growth media, and metabolite extraction procedure

Twenty seven isolates, representing the FGS, SGG and FGG forms of *C. gloeosporioides* were used in this study (Table 1). Morphological, virulence and molecular-genetic characteristics of these morphotypes were described previously (Abang *et al*. 2002, 2003, 2004, 2005, 2006). These strains have been deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany.

To characterize all the 27 the isolates based on the banding pattern of their secondary metabolites on silica gel plates, three discs of mycelium (5 mm diam) were obtained from the advancing edge of colonies of each isolate and placed in a separate 250 ml conical flask containing 100 ml Czapek-Dox yeast (CDY) medium. The cultures were incubated at 28°C by shaking at 120 rpm under cool-white fluorescent light with an alternating 12-h photoperiod for 35 days. Uninoculated CDY served as control. After incubation, 0.2 ml of 85% phosphoric acid was added and the flasks were shaken vigorously. The contents were passed through two layers of cheesecloth to reduce fungal biomass and further filtered using a Schleicher and Schüll (S & S) folded membrane (125 mm) under suction. Ethyl acetate was used as solvent for extracting the phytotoxic

fraction from the culture filtrates. The organic solvent was used at a ratio of 1:1 (v/v) (culture filtrate to organic solvent). The extraction process was repeated twice. Concentrated Na_2SO_4 was added to the combined extract from two extractions to remove water and incubated overnight, after which the solvent was evaporated in a rotary evaporator (Büchi Rotavapour R-114, Büchi, Switzerland) under reduced pressure (240 mbar) at 40°C, then dissolved in 250 µl ethyl acetate and stored at -20 °C.

For detailed chemical and biological characterization of *C. gloeosporioides* phytotoxin isolates Cg19, Cg25, Cg29, Cg33 and Cg66 were selected as representatives of different morphotypes and virulence phenotypes (Table 1). In this experiment, 15 discs of mycelium (5 mm diam) were obtained from the advancing edge of colonies of each isolate and placed in a separate 1 l conical flask containing 500 ml CDY medium. Four conical flasks were inoculated for each isolate making a total of 2 l CDY medium per isolate. One ml of 85% phosphoric acid was added and the flasks were shaken vigorously. Finally, the combined extract was evaporated to dryness in a rotary evaporator at 40 °C and stored in 5 ml ethyl acetate at -20 °C. This was considered the crude toxin extract based on which dilutions were made. Both experiments were repeated once.

High performance thin-layer chromatography (HPTLC) analysis

The ethyl acetate extracts of all isolates were separated by applying the extracts to a Merck HPTLC pre-coated silica gel glass plate (Merck Kieselgel 60 F_{254} Model 1.05628). A mixture of *n*-hexane (Merck) and ethyl acetate (Baker) at a ratio of 3:1 (v/v) was used as solvent after tests with different solvent combinations and ratios showed that this

solvent gave best results. Compounds were detected under ultra-violet light at 254 and 366 nm. R_f values for observed bands were calculated using the formula:

R_f = distance travelled by the spot / distance travelled by the solvent.

Following fractionation by HPTLC of the ethyl acetate extract of isolates Cg19, Cg25, Cg29, Cg33 and Cg66, individual bands were concentrated and scanned on a UV detector. Plates were sprayed with anisaldehyde stain solution and then heated to 110 °C on a thermoplate. For activity-directed purification, specific toxic activity was determined by placing a 20 µl droplet of a 1:1 (v/v) dilution of each fractionated sample in 20% methanol on detached leaves of a susceptible water yam cultivar (*D. alata* cv. TDa 92-2). The bioactive bands on silica gel plates were eluted with ethyl acetate. The bands were scraped off the glass plates and the silica gel was allowed to stand overnight in the solvent. The eluant was reduced to dryness by exposure to a stream of nitrogen gas and stored in the freezer at -20 °C. The eluant from each fraction (band) was redissolved in 500 µl $CDCl_3$ (deuterated chloroform). A Bruker DMX-600 NMR spectrometer (Bruker, Germany) operating at 600 MHz was used to take an NMR spectrum of the extract. It was not possible to identify any potentially useful compound(s) following fractionation with HPTLC. The ^1H spectra and the mass spectra were too heterogeneous to allow a meaningful interpretation.

High performance liquid chromatography (HPLC) analysis

Further fractionation/purification of the original culture filtrate from isolates Cg19, Cg29 and Cg25 was carried out by HPLC using the Waters Alliance Separations Module (Waters 2690) and a 996 photodiode array detector (Waters, Milford, MA). Separation

was achieved using a Nucleosil 100-5 C18 column (250 long x 4.0-mm internal diam; Machery-Nagel, Düren, Germany) using gradient elution of 20-80% methanol-water. The running time was 50 min at a flow rate of 1 ml/min. The column effluent was monitored by following the absorption using a detector with a wavelength range of 210-400 nm. The chromatograms were run at room temperature (23 °C). Fractions were collected every two min with an automatic fraction sampler and a total of 25 fractions were collected for isolates Cg19, Cg25, Cg29 and Cg33. Column effluents were collected and the methanol/water mixture was evaporated in a stream of nitrogen gas mounted on a metal block thermostat (Liebisch, Bielefeld, Germany) at room temperature.

NMR analysis

1D and 2D NMR (1D: ^{1}H; 2D: COSY) spectra of HPLC fractions 12 and 13 were recorded in deuterated chloroform ($CDCl_3$) at 300 K on Bruker DMX-600 (Bruker, Germany). ^{1}H chemical shifts are given in ppm relative to the internal standard, tetramethylsilane (TMS), and couplings are in units of frequency (Hz).

Mass spectrometry (MS) analysis

Electron ionisation (EI), chemical ionisation (CI) and desorption chemical ionisation (DCI) using NH_3 as reagent gas of HPLC fractions 12 and 13 in the negative and positive modes was performed on a Finnigan MAT 95 spectrometer (ThermoFinnigan, Bremen, Germany) at 3 kV accelerating voltage. Resolution was set to 1:1500. The extracts were also analysed with electrospray ionisation (ESI) in the negative and positive modes in a quadrupol-time-of-flight (QTOF) mass spectrometer.

183

184 ***rDNA ITS restriction fragment length polymorphism (ITS-RFLP) analysis***

185 DNA from the 27 fungal strains was extracted using a CTAB method as described
186 previously (Abang *et al*. 2002). Using the two universal primers, ITS1 and ITS4, PCR
187 products of the ITS1-5.8S-ITS2 region (about 500–600 bp) were obtained and digested
188 with five restriction enzymes (*Alu*I, *Hha*I, *Hae*III, *Msp*I and *Rsa*I). Enzyme treatments
189 were made by taking a 3 µL aliquot of the PCR reaction and incubating it with 1 U of the
190 respective enzyme for 1–2 h at 37°C using the digestion buffer specified by the
191 manufacturer. The restriction fragments generated were analysed by electrophoresis in
192 2% agarose gels in TAE buffer.

193

194 ***Biological assays***

195 Bioassay of the fractions was carried out on leaves of the anthracnose-susceptible *D.*
196 *alata* cv. TDa 92-2. Fractions were tested irrespective of whether they corresponded to
197 particular peaks on the chromatograms or to particular bands on HPTLC plates. When a
198 particular HLPC peak corresponded to a fraction with high phytotoxicity, efforts were
199 made to identify the fraction (band) using HPTLC. The HPTLC plates were examined
200 under UV light at 254 and 366 nm, and stained with anisaldehyde and ninhydrin spray
201 solutions.

202 A simple leaf puncture bioassay was used for the rapid determination of
203 phytotoxic activity and for screening young, but fully expanded, leaves of *D. alata*. The
204 test fraction was dissolved in 20% methanol (1:1 v/v) and a 20 µl droplet of the diluted
205 extract was placed on the adaxial (lower) leaf lamina at the interveinal surface. The leaf
206 was carefully pierced with a sterile needle through the droplet. It was then placed in a

large (14 cm diam) petri dish containing moistened filter paper and sealed with parafilm. Petri dishes were incubated at 28 ^{o}C with alternate 12-h cool white fluorescent light (870 lux)/ 12-h dark for 48 h. All tests were repeated at least once. Filtrate from the control (uninoculated Czapek-Dox yeast) was also dissolved in 20% methanol and applied on the opposite side of the midrib. Phytotoxic activity indexing was on a scale of 0-5: 0, no lesion; +, lesions 1-3 mm diam; ++, lesions 4-7 mm diam; +++, lesions 7-10 mm diam; ++++, lesions >10 mm diam.

To determine the effect of temperature on the stability of the crude toxin extract, the partially purified toxin of isolate Cg29 was used. A 1:1 (v/v) dilution of the toxin extract was prepared in 20% methanol and incubated at 40, 50, 60, 70, 80, 90, 100 and 120 °C for 15 min (Jayasinghe and Fernando, 2000). Thereafter, phytotoxic activity of the heat-treated extracts was bioassayed using leaves of *D. alata* cv. TDa 92-2. There were three replications per treatment and the experiment was repeated once.

To determine whether the crude toxin extracts were host selective or not, detached leaves of cassava (*Manihot esculenta*), cucumber (*Cucumis sativus*) and tomato (*Lycopersicon esculentum*) were inoculated with extracts from isolates Cg19, Cg25, Cg29, Cg33 and Cg66. Inoculation, incubation and evaluation of phytotoxic activity were carried out as described above. Filtrate obtained from uninoculated Czapek-Dox yeast was dissolved in 20% methanol and served as a control.

Crude toxin extracted from Cg33 was used to screen leaves of five *D. alata* cultivars; TDa 289, TDa 87/01091, TDa 85/00250, TDa 95/00328 and TDa 92-2, for anthracnose resistance in the glasshouse. The crude toxin extract was dissolved in 20% methanol (1:1 v/v dilution) and a 20 µl droplet was used for inoculation as described

above. Filtrate obtained from uninoculated Czapek-Dox yeast was dissolved in 20% methanol and used as a control. The leaves were held flat following inoculation until the liquid had dried and were then covered with partly sealed, transparent polyethylene bags moistened with water (Jayasankar *et al*. 1999). Five leaves were inoculated and incubated for each cultivar and the leaves were observed for anthracnose symptoms after 2 d. All bioassays were repeated at least once.

Anthracnose resistance screening of yam tissue cultures using* Colletotrichum gloeosporioides *phytotoxin

In vitro tissue-culture plantlets (nodal cutting cultures) of seven yam genotypes used in these studies (Table 2) were obtained from the yam germplasm collection of the Tissue Culture Unit, International Institute of Tropical Agriculture (IITA), Ibadan-Nigeria (Ng & Ng 1994). Nodal cutting cultures of the seven genotypes were subcultured to initiate fresh cultures for use in the experiments. *In vitro* plantlets were grown essentially according to Ng & Ng (1994).

A partially purified culture filtrate of *C. gloeosporioides* Cg33, an isolate previously shown to be virulent on yam (Green *et al* 2000; Mignouna *et al*. 2001), was used in the development of the *in vitro* assay. To prepare a dilution series, the desired amount of crude toxin extract was evaporated to dryness in a stream of nitrogen gas and a 20% solution of methanol (prepared in sterile distilled water) was used to dilute the toxin extract and the uninoculated CDY extract. Single node cuttings (1-2 cm long) were obtained from tissue culture plantlets of TDa 95/00328 and TDa 92-2, and transferred to culture tubes containing 1.25 ml yam multiplication medium (YMM) (Ng & Ng 1994)

and Cg33 extract at concentrations (v/v) of 1:10, 1:25, 1:125, 1:250, 1:500, 1:1000 and 1:2000. As controls, cuttings were grown in culture tubes containing 1.25 ml YMM amended with the extract from uninoculated CDY, and also in tubes containing YMM only. For each dilution, six culture tubes (three per yam genotype) were used. Growth of the nodal cuttings was monitored for 5 weeks, after which the number of surviving cultures, presence/absence of shoots, and presence/absence of roots were recorded. The study was conducted under sterile conditions.

The above experiment showed that a dilution of 1:25 was optimal for differentiating the yam genotypes based on their response to the crude toxin extracts *in vitro*. To evaluate the tissue-culture based technique developed, the seven yam genotypes were tested for their reaction to crude extract of the highly virulent isolate Cg25. A 1:25 dilution of Cg25 was prepared (using 20% methanol as solvent) in culture tubes containing 1.25 ml YMM. Single node cuttings (1-2 cm long) were obtained from the tissue culture plantlets of the seven genotypes and used to inoculate the culture medium. There were ten replicate single node cuttings per genotype, making a total of 10 tubes for each genotype.

Data analysis

Consistently produced metabolites (as visualized under UV 360 nm and/or stained with anis-aldehyde) and unambiguously scorable ITS-RFLP bands were scored for presence and absence and used as markers. The combined data was used to produce a binary matrix consisting of the 27 *Colletotrichum* strains and 21 markers (19 ITS-RFLP bands and 2 metabolites). A phenetic tree was constructed using MEGA 2.1 software (Kumar *et*

al. 2004). Similarity was calculated as the proportion of markers at which the two isolates compared had the same score (SMxy= (n11 + n00)/n; where n is the number of markers scored). The distance is 1_SM. Cluster analysis was performed using the unweighted pair group method with arithmetic averages (UPGMA). Bootstrap values were calculated in 1,000 permutations and presented in percentages.

Results

High performance thin-layer chromatography (HPTLC) analysis

Attempts were made to differentiate *C. gloeosporioides* isolates based on their secondary metabolite profile. Ethyl acetate extracts prepared for each of twenty-seven fungal isolates with known morphological and virulence characteristics were chromatographed on HPTLC plates. A single band (R_f = 0.66) was identified for most FGG and FGS isolates when visualized under UV light at a wavelength of 366 nm (Fig. 1). The band was conspicuously absent in samples of the SGG isolates and the control. Although the absence of the band appeared characteristic of SGG isolates, some FGS isolates (Cg1, Cg13 and CgS_6) also did not produce a band at Rf 0.66. To visualize non-UV absorbing compounds, HPTLC plates were developed with para-anisaldehyde stain. A more complex pattern of bands was observed following this treatment with a pink/red-stained band (R_f = 0.08) that was consistently observed for FGS and SGG isolates. The band appeared either pink or red depending on the intensity of the secondary metabolite production. This band was absent in all samples of the FGG isolates (Fig. 2). Combined analysis of these secondary metabolite profiles showed that the pathogenic SGG and FGS

300 forms have a chemotype (A or B) that is distinct from the non-pathogenic FGG form
301 (chemotype C) (Table 1).
302
303 ***Biochemical assays***
304 Bioassay of the crude ethyl acetate extracts revealed phytotoxic metabolites of SGG and
305 FGS isolates while extracts from FGG isolates did not induce symptoms on yam. Extracts
306 prepared from SGG and FGS isolates caused anthracnose-like symptoms on TDa 92-2
307 similar to those induced by *C. gloeosporioides* on yam.
308 Crude toxin extracts prepared from *C. gloeosporioides* isolates Cg19, Cg25 and
309 Cg29 were further subjected to HPLC. Extracts from isolates Cg25 and Cg29 produced
310 similar chromatograms with slight differences in intensity of peaks. UV absorption
311 spectra of compounds detected from toxin extracts of isolates Cg19 (FGG) and Cg29
312 (FGS) are shown in Fig. 3. Each of the 25 fractions resolved in HPLC was assayed for
313 biological (phytotoxic) activity on TDa 92-2. Fractions 12 and 13 from Cg29 were
314 selected for further analysis because of their high bioactivity (necrotic lesions > 7 mm
315 diam). These corresponded to peak/compound A (retention time 20.69 - 21.74 min with
316 peak at 21.00 min) and peak/compound B (retention time 21.74 - 22.53 min with peak at
317 22.02 min) respectively, on the chromatograms obtained for isolates Cg25 and Cg29 (Fig.
318 3 a). Fraction 13 produced a distinct and uniquely pink-coloured band when stained with
319 para-anisaldehyde. Comparison of the HPTLC chromatogram of fraction 13 (compound
320 B) with that of the crude toxin extract from Cg29 revealed that the R_f value of compound
321 B (0.12) was identical to that of a similarly pink-stained compound in the crude extract
322 (Fig. 4).

323 A further analysis to resolve ^{1}H NMR spectra of fractions 29_2 and 29_3 resulted in
324 a number of peaks with different intensities, indicating the likely presence of impurities
325 within these fractions. Both spectra were rather similar displaying resonances at δ_H 7.77
326 and 7.66 ppm, which may belong to amide protons because no UV-absorption of an
327 aromatic ring to which these protons could belong was seen. Broad multipletts at δ_H 5.53,
328 4.44, and 4.25 ppm may be α-protons of amide or ester bonds. An AB double doublet was
329 seen at 3.77 and 3.61 ppm and a number of protons form multipletts between 2.3 and 1.0.
330 In the 2D $^{1}H\{^{1}H\}$ COSY NMR spectra (Fig. 5a) the AB double doublet showed
331 couplings to a resonance at 1.8 ppm and couplings were seen between the protons at δ_H
332 7.77 and 7.66 ppm. The COSY spectra revealed some further couplings but due to the
333 scarcity and the impurity of the material it was not possible to elucidate the structures of
334 the metabolites.

335 Mass spectrometry with electron, chemical and electrospray ionisation both
336 recorded in the negative and the positive modes were used to determine the molecular
337 mass of the metabolites. It was not possible to determine the molecular weight of the
338 toxin extract(s) from the molecular ion peaks obtained with the different analytical
339 methods. A quasi-molecular ion was found at m/z 536 with the "softest" ionisation
340 method, DCI and ammonia as reagent gas, leading usually to only moderate
341 fragmentations of any molecular ion. However, the other ionisation techniques produced
342 only lower molecular weight ions. This was probably due to the likely presence of
343 different compounds within the toxin fraction (data not shown).

344 Temperature treatments up to 100°C had no effect on partially purified toxins of
345 Cg29. Phytotoxic activity on detached leaves of *D. alata* cv. TDa 92-2 was not affected

346 by thermal treatment of the crude toxin extract at 103 kPa for 15 min. Autoclaving did
347 not destroy the toxin extract.

348 When crude extracts prepared from *C. gloeosporioides* were used to test host
349 selectivity of the extracts, isolates Cg29, Cg33 and Cg66 induced necrotic reactions in
350 cassava, while extracts of Cg25 was necrotic on cassava and cucumber. This indicates
351 that the phytotoxic activity in these extracts is not host selective.

352

353 ***Combined analysis of secondary metabolite and ITS-RFLP data***

354 *Colletotrichum* strains associated with yam anthracnose fell into two ITS-RFLP groups,
355 one comprising the avirulent/weakly virulent fast growing grey (FGG) isolates while the
356 other consisted of the highly virulent slow growing grey (SGG) and the moderately
357 virulent fast growing salmon (FGS) forms (23 isolates) (Fig. 6). ITS products of FGG
358 isolates gave 100 bp and 490 bp fragments with *Alu*I while FGS and SGG isolates
359 produced 190 bp and 380 bp fragments. Similarly, *Hae*III digestion of the ITS products
360 gave 180 bp and 400 bp fragments with FGG isolates, while FGS and SGG isolates
361 produced 140 bp, 170 bp and 280 bp fragments. Marked differences in ITS-RFLP profiles
362 were also observed with *Hha*I. No polymorphism in ITS-RFLP profiles was observed
363 with *Msp*I and *Rsa*I. FGS isolates Cg1, Cg13 and CgS_6 appeared more similar to the SGG
364 isolates in that they also did not produce a metabolite at Rf 0.66. These isolates grouped
365 together with the SGG isolates and formed a cluster seperate from other FGS isolates
366 (Fig. 6).

367

368 ***Biological assays***

The extract from Cg33 induced severe necrosis on TDa 92-2 but caused only mild lesions on TDa 95/00328. Water yam cultivars treated with phytotoxic metabolites showed a differential response to various concentrations of the crude toxin extracts. When the extracts were applied to yam tissue cultures, high concentrations induced necrosis and adversely affected the growth of cultures from nodal cuttings. A 1:25 (v/v) dilution gave the best results for differentiating *D. alata* cultivar response. At this concentration, nodal cuttings of the susceptible cultivar TDa 92-2 died or produced necrotic roots or shoots (data not shown). Although growth of the resistant cultivar TDa 95/00328 was retarded at this dilution, nodal cuttings grew and produced normal roots and shoots. Higher toxin extract concentrations caused bleaching and death of all nodal cuttings and were not useful for differentiating cultivar response to the phytotoxin.

Resistance screening of the seven yam genotypes using diluted toxin extracts of *C. gloeosporioides* isolate Cg25 showed that *D. rotundata* was most tolerant to the crude toxin extract while there was no obvious difference in the response of *D. alata* and *D. dumetorum* to the phytotoxin. *Dioscorea rotundata* cv. TDr 93-1 appeared highly resistant to treatments with the crude toxin extract (Table 3). *D. alata* cv. TDa 95/00328, which showed tolerance to crude toxin extract of FGS isolate Cg33 (zero mortality of nodal cuttings), was found to be highly sensitive to the crude extract of SGG isolate Cg25 (70% mortality). In fact, its response did not differ from that of the anthracnose susceptible cv. TDa 92-2 (80% mortality). *D. alata* cvs. TDa 87/01091 and TDa 85/00257 previously showed resistance to Cg25 in a whole plant assay but were sensitive to crude toxin extract of the isolate (1:25 dilution) with 60% and 50% mortality of nodal cuttings, respectively.

Discussion

Phytotoxin production has been reported for several *Colletotrichum* species and the chemical structure has been elucidated for toxins such as colletotrichins (Gohbara *et al*. 1978), colletopyrone (Gohbara *et al*. 1976), aspergillomarasmins (Ballio *et al*. 1969) and ferricrocin (Ohra *et al*. 1995). In agreement with the findings of this study, these toxins were not host-specific. Ahousou (1989, Moura-Costa *et al*. 1993) and Alleyne (2001) attributed the phytotoxicity of a yam anthracnose-associated toxin to glycoprotein-type compounds. The purification and characterization of a highly toxic fraction in this study suggested the presence of a low molecular weight peptide. Many avirulence determinants are proteins or peptides, although in some cases it is clear that the products of some avirulence genes are involved in the synthesis of low molecular weight secondary metabolites that can be recognized by plants (Kobayashi *et al*. 1990; Smith *et al*. 1993). Further purification and chemical analyses are warranted to elucidate the structure of the crude toxin fractions identified in this study.

Evidence is emerging suggesting that the genomes of plant pathogenic fungi are rich in genes that are likely to be involved in the synthesis of secondary metabolites such as nonribosomal peptide synthases and polyketide synthases, whereas saprophytes appear to be deficient in such genes (Yoder & Turgeon 2001). This study demonstrated the utility of metabolic profiling in differentiating pathogenic FGS and SGG isolates from the non-pathogenic FGG form of *C. gloeosporioides* associated with yam anthracnose disease. The former had ITS-RFLP and secondary metabolite profiles that differed

markedly from the latter, with strong bootstrap support for separate clustering of the two groups (Fig. 6) (Abang *et al*. 2002). The highly toxic fraction 13 (Fig. 3) was detected in FGS and SGG strains, which are virulent on yam, but was not detected in FGG strains. FGG isolates have been isolated from symptomless leaves of yam (Abang *et al*. 2001), and it has been suggested that the association of this group of isolates with yam may be simply endophytic (Abang *et al*. 2002). The apparent inability of these isolates to produce particular toxic metabolites in this study may, at least in part, explain their endophytic nature (Tan & Zou, 2001), and suggests that toxins play a role in the pathogenesis of *C. gloeosporioides* strains infecting yam.

The HPTLC profiles of the secondary metabolites clearly distinguished FGS from SGG isolates (Fig. 1), which supports our previous finding that these strains represent two genetically distinct populations of *C. gloeosporioides* on yam (Abang *et al*. 2005). A few FGS isolates appeared related to SGG isolates in that they also failed to produce a metabolite at Rf 0.66 under UV light. The diversity in metabolite profile among FGS isolates is probably a reflection of their high genetic diversity (Abang *et al.* 2006), with a wide range of genes and regulatory molecules influencing metabolite production in nature and in culture (Frisad *et al*. 2008).

Alleyne (2001) found that *C. gloeosporioides* toxin extract induced electrolyte leakage in yam cell suspension cultures, a mechanism that may explain the toxicity of the toxins in this study. Autoclaving did not destroy the crude toxin extracts, hence the toxin extract can be considered to be thermostable. Our results are in agreement with those of Jayasinghe & Fernando (2000) who extracted a thermostable toxin from *C. acutatum* infecting rubber. Sharma & Sharma (1969) reported that crude toxin produced by *C.*

438 *gloeosporioides* causing citrus die-back was thermolabile, suggesting that the causative
439 agent(s) for the phytotoxicity they reported are different from those reported here.
440 Although the structure of two compounds purified in this study could not be elucidated,
441 the isolation protocols described and the characteristic information about their HPLC
442 chromatograms and NMR spectra may facilitate the identification of these compounds in
443 future studies.

444 Several authors have evaluated phytotoxins as factors in the pathogenesis of
445 *Colletotrichum*, and also determined whether the toxin is host-specific (Alleyne 2001;
446 Jayasankar *et al*. 1999; Yoshida *et al*. 2000). Amusa *et al*. (1993) found that the toxin
447 extracted from *C. gloeosporioides*-infected leaves induced necrotic lesions similar to
448 those induced by the pathogen. Extracts from the pathogen in infected leaves and in
449 culture had similar properties. Although the findings of this study are not directly
450 comparable with those of Amusa *et al*. (1993), they confirm the fungal origin of the
451 phytotoxin, as also reported by Alleyne (2001). Because nodal cuttings are the source
452 plant materials for yam multiplication *in vitro* (Ng & Ng 1994), and since tissue
453 organization apparently has no effect on the expression of resistance in yam to
454 anthracnose (Moura-Costa & Mantell 1993; Moura-Costa *et al*. 1993), nodal cutting
455 cultures were used to test the response of yam genotypes to *C. gloeosporioides*
456 phytotoxin. Yam species and cultivars differed in their response to *C. gloeosporioides*
457 toxin, which agrees with earlier findings that yam tissue cultures can be used for early
458 screening of yam for anthracnose resistance in the absence of the pathogen (Alleyne
459 2001; Moura-Costa *et al*. 1993). TDa 95/00328 showed tolerance to crude toxin extract
460 of FGS isolate Cg33 (zero mortality of nodal cuttings) but was found to be highly

461 sensitive to crude extract of SGG isolate Cg25 (70% mortality) of the same
462 concentration, indicating that the highly virulent nature of SGG isolates may be related to
463 the higher phytoxicity of their toxins. The sensitivity of TDa 87/01091 and TDa 85/00257
464 to crude extract of isolate Cg25, in spite of their resistance in a whole plant assay
465 (Mignouna *et al.* 2001), may be due to the high concentration of crude toxin extract used.
466 The dilution of 1:25 was found optimum for differentiating *D. alata* host response based
467 on crude toxin extract of Cg33. This concentration may have been inappropriate for the
468 more phytotoxic extracts of highly virulent SGG isolates such as Cg25. The wider range
469 of cultivars and a larger number of crude extracts (from different isolates) and extract
470 concentrations need to be evaluated in order to develop standardized screening protocols
471 for anthracnose resistance screening *in vitro*.

472 Tissue culture techniques are advantageous in the development of novel
473 disease-resistant germplasm because they allow large-scale screening of germplasm in
474 limited space and time, and the study of host-pathogen interactions in a controlled
475 environment (Daub 1986). The ability to use pathogenesis-related pathotoxins in the
476 selection of anthracnose-resistant genotypes is expected to have a profound impact on
477 breeding schemes for crops with long growth cycles such as yam. Yam breeding
478 programs active in areas such as India and the Caribbeans where particular strains or
479 pathotypes (e.g. the SGG form of *C. gloeosporioides*) have not been reported could use
480 the toxic activity to screen pre-emptively for resistance and thus avoid the danger of
481 international exchange of fungal cultures.

482 Engineered toxin detoxification represents a promising approach for resistance
483 that may be applicable to a disease such as anthracnose in which pathogenesis-related

pathotoxins are believed to be involved (Soledade & Pedras 2004). This study demonstrated the applicability of the phytotoxin to differentiate between pathogenic and non-pathogenic forms of *Colletotrichum gloeosporioides* on yam. The use of the toxic activity may prove to be a beneficial tool for future studies on chemotaxonomy, host-pathogen interaction, and on the nature of anthracnose resistance in yam.

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621 **Figure legends**

622 **Fig. 1**. HPTLC chromatogram of *Colletotrichum gloeosporioides* isolates showing a single
623 band detected with UV light (366 nm) in the extract from FGG (left) and FGS isolates
624 (right). Note the absence of the band in SGG isolates Cg14, Cg25, Cg26, Cg29, Cg66 and
625 Cg219. K = extract from uninoculated Czapek-Dox yeast medium.

626 **Fig. 2.** HPTLC chromatogram of toxin extracts from different forms of *Colletotrichum*
627 *gloeosporioides* showing a para-anisaldehyde stained band ($R_f = 0.08$) that was detected
628 in the SGG and FGS isolates, but absent in FGG isolates.

629 **Fig. 3.** HPLC chromatogram of toxin extracts from (a) either SGG isolate Cg25 or FGS
630 isolate Cg29 and (b) FGG isolate Cg19. Note the absence of peaks for fraction 12
631 (compound A) and fraction 13 (compound B) in the extract from Cg19.

632 **Fig. 4.** HPTLC chromatograms showing relative positions of a toxin fraction of
633 *Colletotrichum gloeosporioides* isolate Cg29 in the crude and HPLC-purified extract. (a)
634 fraction 13 in crude extract (b) fraction 13 as HPLC-purified fraction (compound B)

635 **Fig. 5.** 2D $^{1}H\{^{1}H\}$ COSY NMR spectra of two *Colletotrichum gloeosporioides* isolate
636 Cg29 toxin fractions. (a) fraction 12 (compound A) (b) fraction 13 (compound B)

637 **Fig. 6.** UPGMA phenogram of *Colletotrichum gloeosporioides* isolates based on
638 combined rDNA ITS-RFLP and secondary metabolite profile data. Numbers on branches
639 are bootstrap values in percent based on 1000 permutations. The isolates are designated
640 by alphanumerics indicating the isolate name as in Table 1 (CgS3, Cg10, etc.), followed
641 by the morphotype and virulence phenotype (e.g. FGS for the moderately virulent fast-
642 growing salmon type), the ITS-RFLP group (1 for group 1 and 2 for group 2), and then
643 the chemotype (A, B, or C) based on their secondary metabolite production.

644

645 Table 1. Designation, morphotype, and rDNA ITS group, and chemotype of slow
646 growing grey (SGG), fast growing salmon (FGS), and fast growing grey (FGG) forms of
647 *Colletotrichum gloeosporioides* based their secondary metabolite profile in high
648 performance thin layer chromatography (HPTLC)[a]

			HPTLC		Chemotype
Isolate	Morphotype	rDNA ITS group	UV (360 nm)	Anis-aldehyde staining	
Cg1	FGS	1	-	+	B
Cg2	FGS	1	+	+	A
CgS2	FGS	1	+	+	A
CgS3	FGS	1	+	+	A
CgS5	FGS	1	+	+	A
CgS6	FGS	1	-	+	B
Cg9	FGS	1	+	+	A
Cg10	FGS	1	+	+	A
Cg11	FGS	1	+	+	A
Cg12	FGS	1	+	+	A
Cg13	FGS	1	-	+	B
Cg14	SGG	1	-	+	B
Cg15	FGG	2	+	-	C
Cg16	FGG	2	+	-	C
Cg17	FGS	1	+	+	A

Cg18	FGG	2	+	-	C
Cg19	FGG	2	+	-	C
Cg20	FGS	1	+	+	A
Cg21	FGS	1	+	+	A
Cg22	FGS	1	+	+	A
Cg25	SGG	1	-	+	B
Cg26	SGG	1	-	+	B
Cg29	FGS	1	+	+	A
Cg33	FGS	1	+	+	A
Cg35	FGS	1	+	+	A
Cg66	SGG	1	-	+	B
Cg219	SGG	1	-	+	B

[a]Based on previous morphotype and rDNA ITS analysis (Abang *et al.* 2002)

Table 2**.** Reported reactions of seven yam genotypes to *Colletotrichum gloeosporioides* isolates Cg25 and Cg33.

Genotype	**Host response (Cg25)**	**Host response (Cg33)**	**Source**
TDa 87/01091	Resistant	Resistant	Mignouna *et al*. (2001)
TDa 85/00257	Resistant	Resistant	Mignouna *et al*. (2001)
TDa 95/00328	Susceptible	Resistant	Mignouna *et al*. (2001)
TDa 92-2	Susceptible	Susceptible	Mignouna *et al*. (2001)
TDr 87/00211	nt[1]	nt[1]	Akem & Asiedu (1994), Green (1998)
TDr 93-1	nt[1]	nt[1]	Akem & Asiedu (1994), Green (1998)
D. dumetorum cv. Yelisofa	nt[2]	nt[2]	Akem & Asiedu (1994)

[1]nt = not tested for resistance to Cg25 and Cg33 but species is generally resistant in the field

[2]nt = not tested for resistance to Cg25 and Cg33 but species is generally susceptible in the field

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677 Table 3. Survival *in vitro* of ten nodal cuttings in yam multiplication medium amended

678 with *Colletotrichum gloeosporioides* phytotoxin. Phytotoxin was extracted from isolate

679 Cg25 and used at a dilution of 1:25.

Cultivar	**% dead nodal cuttings**	**% surviving plantlets with roots**	**% surviving plantlets with shoots**
TDa 87/01091	60	40	30
TDa 85/00257	50	50	50
TDa 95/00328	70	30	30
TDa 92-2	80	20	10
TDr 87/00211	40	60	60
TDr 93-1	0	100	100
D. dumetorum cv. Yelisofa	50	50	0

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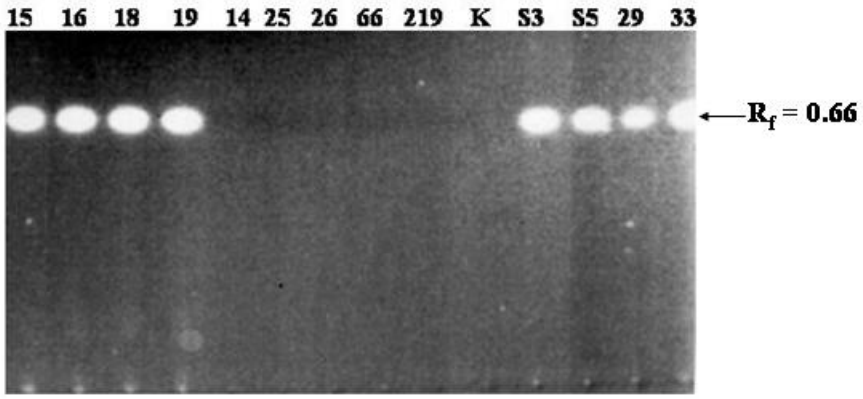
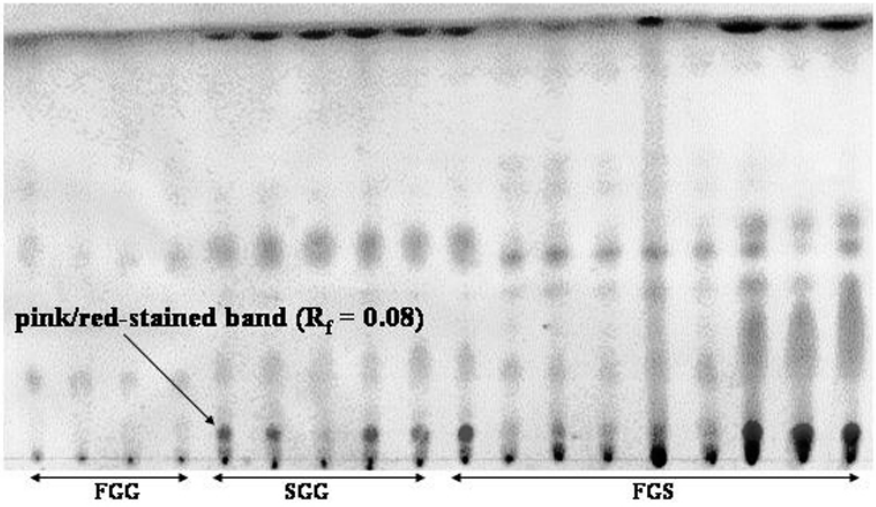


Fig. 1. HPTLC chromatogram of *Colletotrichum gloeosporioides* isolates showing a single band detected with UV light (366 nm) in the extract from FGG (left) and FGS isolates (right). Note the absence of the band in SGG isolates Cg14, Cg25, Cg26, Cg66, and Cg219. K = extract from uninoculated Czapek-Dox yeast medium.



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694 **Fig. 2.** HPTLC chromatogram of toxin extracts from different forms of *Colletotrichum*

695 *gloeosporioides* showing a para-anisaldehyde stained band (R_f = 0.08) that was detected

696 in the SGG and FGS isolates, but absent in FGG isolates.

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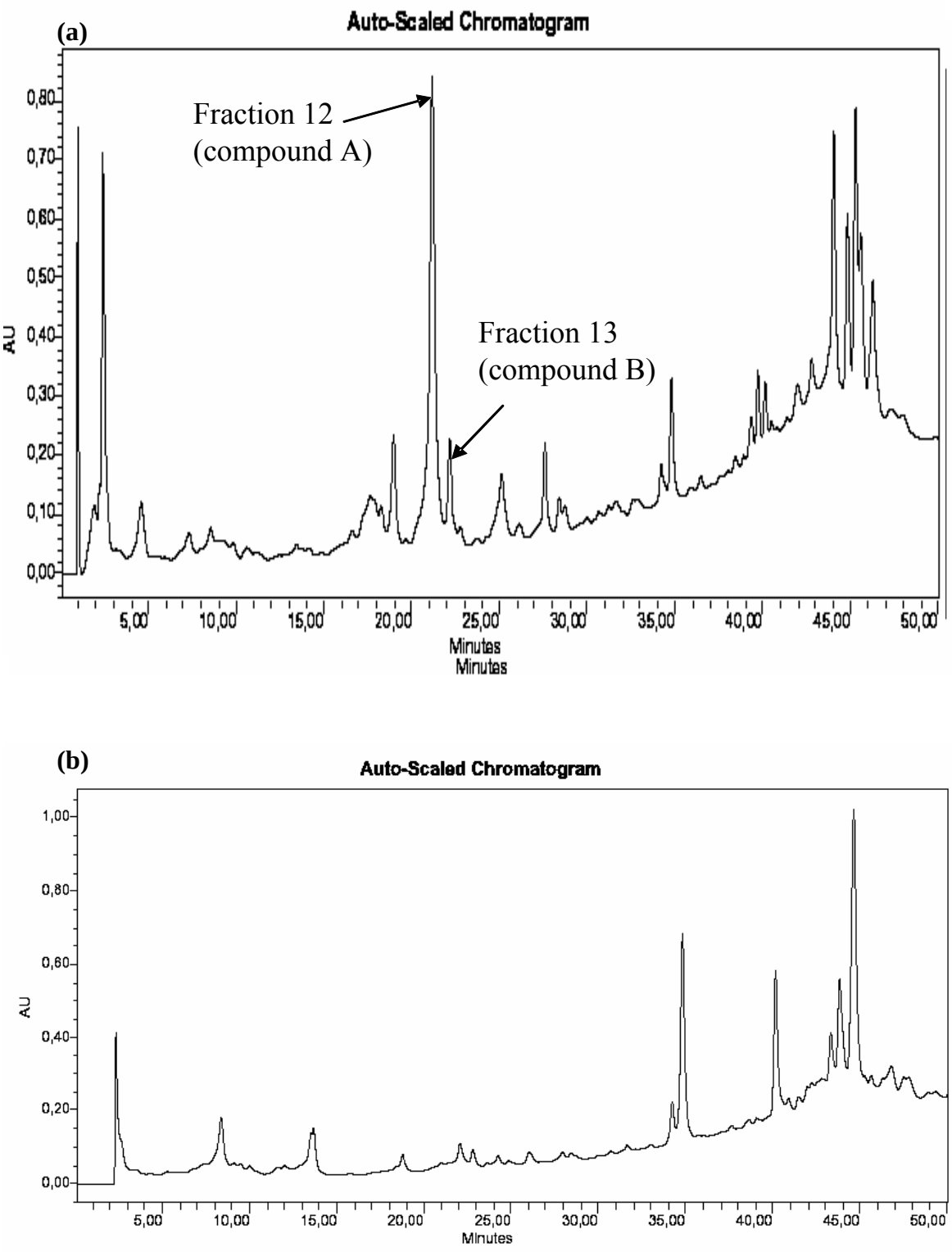


Fig. 3. HPLC chromatogram of toxin extracts from isolates representing different forms of *Colletotrichum gloeosporioides*. (a) Extract obtained from either Cg25 (SGG) or Cg29 (FGS). (b) Extract from Cg19 (FGG). Note the absence of peaks for compounds A and B above.

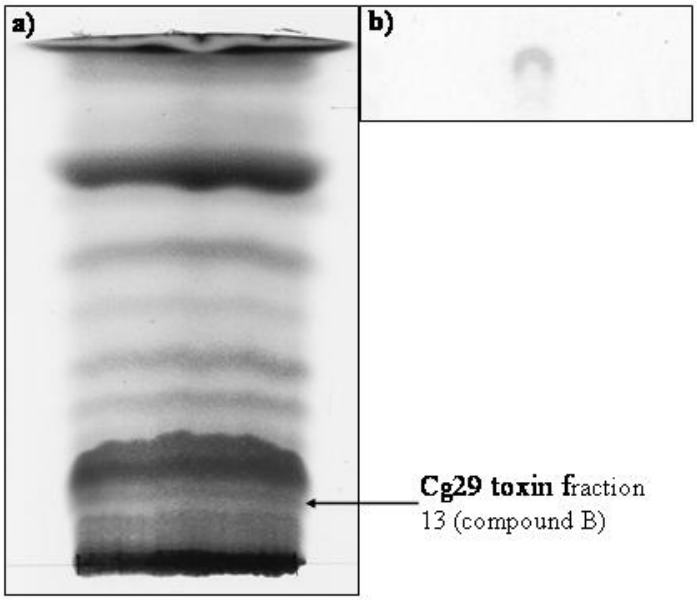
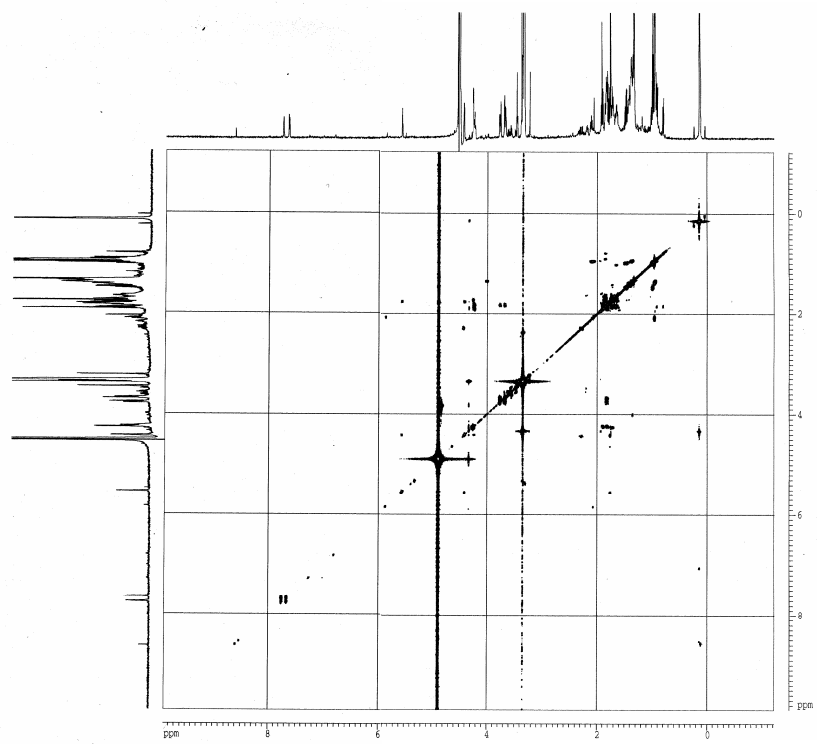
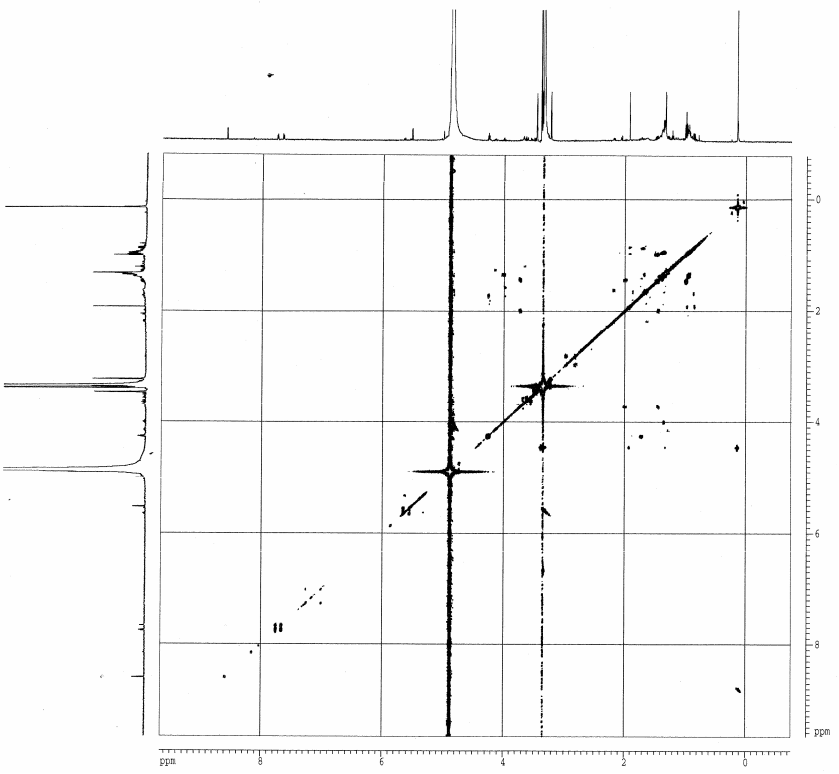


Fig. 4. HPTLC chromatograms showing relative positions of a toxin fraction of *Colletotrichum gloeosporioides* isolate Cg29 in the crude and HPLC-purified extract. (a) fraction 13 in crude extract (b) fraction 13 as HPLC-purified fraction (compound B)

(a)



(b)



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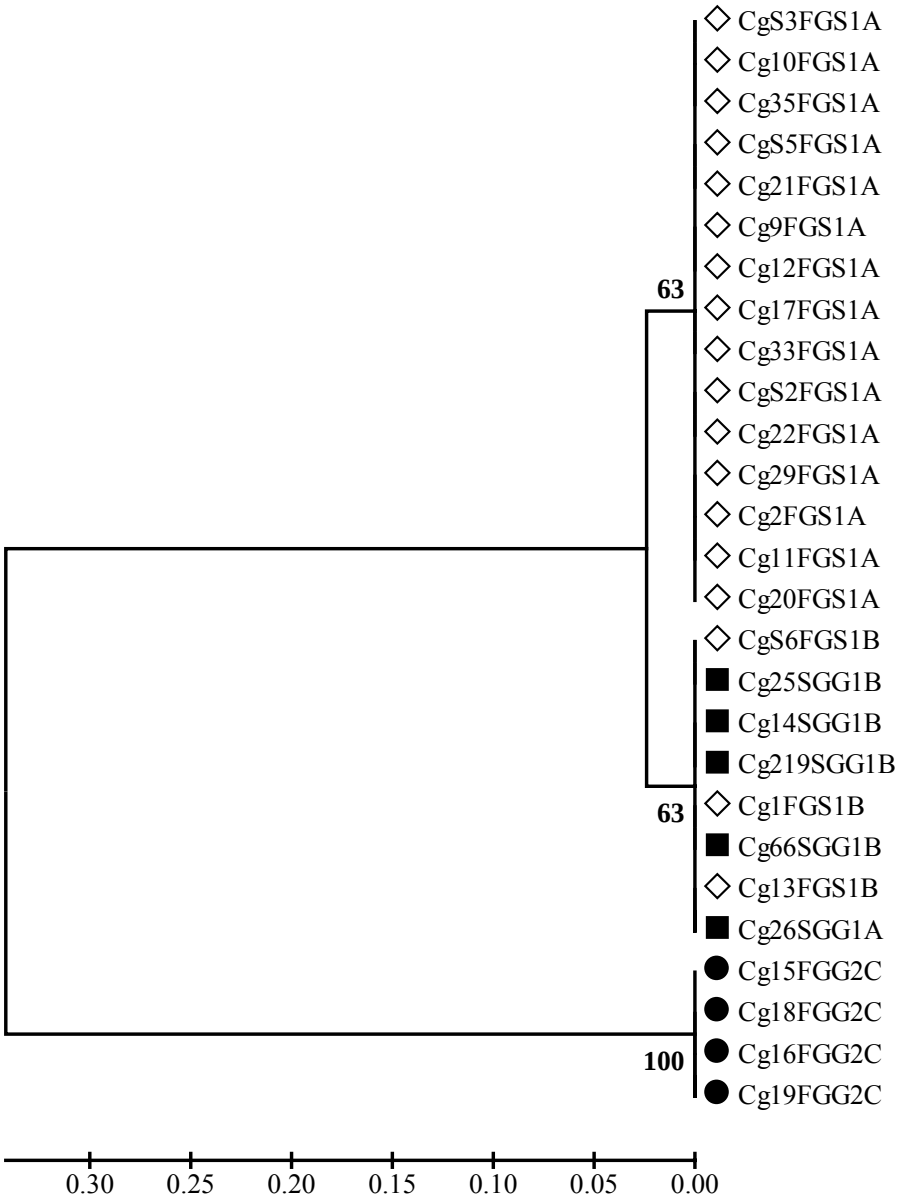

Fig. 6. UPGMA phenogram of *Colletotrichum gloeosporioides* isolates based on combined rDNA ITS-RFLP and secondary metabolite profile data. Numbers on branches are bootstrap values in percent based on 1000 permutations. The isolates are designated by alphanumerics indicating the isolate name as in Table 1 (CgS3, Cg10, etc.), followed by the morphotype and virulence phenotype (e.g. FGS for the moderately virulent fast-growing salmon type), the ITS-RFLP group (1 for group 1 and 2 for group 2), and then the chemotype (A, B, or C) based on their secondary metabolite production.