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Identification of phytotoxic metabolites of a new *Fusarium* sp. inhibiting germination of *Striga hermonthica* seeds

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Summary. An undescribed species of *Fusarium* was isolated from the parasitic plant *Striga hermonthica* in Sudan. When grown both on liquid and solid media this strain produced toxic compounds causing the total suppression of germination of *Striga* seeds. Purification of the liquid culture led to the isolation and identification of fusaric and dehydrofusaric acid, at 263 and 382 mg l^{-1} respectively. The organic extract of solid culture did not contain those acids nor some other powerful known *Fusarium* toxins, but showed the presence of three main toxic metabolites, having a strong inhibitory activity, whose identification is in progress.

Key words: bioherbicides, weed biocontrol, phytotoxins, parasitic plants.

Introduction

Striga spp. (witchweed) are among the most serious pests of several important food crops including corn, sorghum and pearl millet, in subsaharan Africa. Among recorded species, *S. hermonthica* (Del.) Benth. is considered to be the most important throughout the savanna. In northern Ghana, yield losses in grains such as corn, sorghum and millet due to *S. hermonthica* parasitism are estimated to reach about 80,000 tons per year (Sauerborn, 1991). Heavy infestation can lead to total yield loss and the abandonment of very productive fields (Hamdoun and Babiker, 1988).

Traditional control methods have been tried by various workers on different crops but all have proved to be only partially effective. The use of chemicals is not easy because they can cause injury to the host plant. Other control strategies are hampered by the biological characteristics of *Striga*, which produces large amounts of very small seeds that can remain viable for ten or more years even in the absence of a host. A *Striga* seed germinates only if stimulated by host root exudates; it then produces a tubercle that, if it is near enough to the host roots, attacks them and starts to remove nutrients from the host. In addition, *Striga* plants have a long underground phase, so that when they finally emerge, much of the damage has already been done.

Biological control is considered an attractive approach for suppressing parasitic weeds, and some promising agents have been found. Since the great-

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est damage to the host from *Striga* spp. occurs before the emergence of the parasite, soil-borne pathogens that attack *Striga* seeds by inhibiting their germination could well be valuable tools for *Striga* management. The commonest soil-borne pathogens isolated from diseased *Striga* plants belong to the genus *Fusarium*. *F. nygamai* Burgess and Trimboli has been proposed as a biological control agent, since it strongly reduces the emergence of *S. hermonthica* in pot trials (Abbasher and Sauerborn, 1992). Ciotola *et al*. (1995) found an isolate of *F. oxysporum* (Schlecht) Snyd. & Hans. particularly promising for *S. hermonthica* biocontrol, in that it reduced seed germination and the attachment of the parasite to the host roots.

Since many plant pathogens produce toxic metabolites that are possible natural herbicides (Evidente and Motta, 2001), an alternative strategy would be to look for natural products produced by phytopathogenic fungi that will inhibit the germination of *Striga* seeds.

Members of the genus *Fusarium* produce a range of phytotoxic compounds, such as fusaric acid, fumonisins, beauvericin, enniatin, moniliformin and the trichothecenes. These compounds possess a variety of biological and metabolic effects, including necrosis, chlorosis, growth inhibition, wilting, inhibition of seed germination and effects on calli (Wakulinski, 1989, Van Asch *et al*., 1992), and some *Fusarium* toxins, such as enniatin and fumonisin have been reported to have herbicidal properties (Abbas *et al*., 1991; Hershenhorn *et al*., 1992). A previous study (Capasso *et al.,* 1996) found that four phytotoxins produced by *F. nygamai* isolated from *S. hermonthica* (fusaric and dehydrofusaric acids and their respective methyl esters), strongly inhibited *S. hermonthica* seed germination (Zonno *et al*., 1996). Other toxins, produced both by *Fusarium* species and by other fungal species, caused different levels of inhibition of seed germination, ranging from total inhibition at low concentration to no inhibition (Zonno and Vurro, 1999).

A *Fusarium* strain isolated in 1997 from some heavily witchweed-infested fields in Sudan was found in a preliminary examination to produce a liquid culture filtrate that strongly inhibited *Striga* seed germination and that contained bioactive metabolites (Idris, 1997). It therefore seemed of interest to identify these metabolite(s) inhibiting germination of witchweed seeds.

Materials and methods

Strain

The strain, isolated in Sudan (Idris, 1997) was identified by H.I. Nirenberg, Federal Biological Research Centre for Agriculture and Forestry, Institute for Plant Virology, Microbiology and Biological Safety, Berlin, Germany as an undescribed species in the genus *Fusarium* Section *Liseola* (personal communication). It was grown and stored on PDA dishes.

Production of toxic metabolites

For the production of toxic metabolites, the fungus was grown in both liquid and solid cultures as described below, using spore suspensions of young and actively growing mycelia obtained from fresh cultures grown on PDA medium.

Liquid culture

For the production of toxic metabolites in liquid culture, 1-L Roux bottles containing 200 ml of M-1 D medium (Pinkerton and Strobel, 1976) were used. Bottles were inoculated with actively growing mycelia and the cultures were incubated in static conditions at 25°C for 4 weeks in the dark, then filtered and kept either frozen or lyophilised until extraction or testing for phytotoxic activity.

Solid culture

Solid culture was prepared in 500 ml flasks each containing 100 g rice and 45 ml distilled water. The rice was stored overnight at room conditions, steamed for 20 min, then sterilised for 20 min at 121°C. The flasks were inoculated with pieces of agar containing actively growing mycelia and incubated at 25° C for about 4 weeks. Each strain was grown in duplicate. After harvesting, the rice was dried in a drier chamber, finely ground using a mill and stored at 4°C until use.

Extraction and TLC analysis of metabolites from fungal solid cultures

Dried and ground culture (25 g) was extracted with 100 ml methanol-water (55:45, v:v, 1% NaCl) and filtered through filter paper. The filtrate (50 ml) was de-fatted with *n*-hexane (50 ml, twice) and extracted with methylene chloride (50 ml, three times). Extracts were combined, dried (Na_2SO_4), evaporated under reduced pressure and dissolved in 1 ml methanol for further analysis and assays.

Extracts from solid cultures were analysed by TLC on 0.25 mm silica gel plates (Kieselgel 60 F_{254}, Merck, Darmstadt, Germany) and compared with standards from the major known fungal toxins using toluene:ethyl acetate:formic acid (60:30:10, eluent A). The spots were visualized by spraying either with 20% sulphuric acid in water, or with *p*-anisaldehyde (0.15 ml in methanol:acetic acid:sulphuric acid 7:1:0.5).

Extraction and purification of toxic metabolites from fungal liquid culture filtrates

Lyophilised culture filtrate corresponding to 100 ml was dissolved in 100 ml ultra-pure water, and extracted (4×100 ml) with ethyl acetate under acidic conditions (pH 2, HCOOH 1M). Organic extracts were combined, dried and then evaporated under reduced pressure. Preliminary screening of the organic extract was carried out by TLC on 0.25 mm silica gel plates and on 0.20 mm reversed-phase (Stratocrom, KC-18, Whatman, Cliffort, NJ, USA) using ethyl acetate:methanol:water (85:20:10, eluent B) and acetonitril:water (1:1, eluent C) respectively, and extracts were compared with authentic fusaric acid (Sigma-Aldrich, St. Louis, MO, USA) and 9,10-dehydrofusaric acid (isolated and identified from culture filtrates of *F. nygamai* in a previous work by Capasso *et al*. 1996) and their corresponding methyl esters. TLC spots were visualized by exposure to UV radiation and/or spraying first with 10% H_2SO_4 in methanol, then with 5% phosphomolybdic acid in methanol followed by 10 min heating at 110°C. An aliquot of the organic extract, which by TLC was found to contain both toxic acids, was further analysed by HPLC for quantification, while the remaining part was purified for a biological assay on *Striga* seeds and for chemical characterisation.

HPLC detection of FA and DFA in liquid culture filtrates of *Fusarium* species

The HPLC system employed was a Shimadzu (Kyoto, Japan) consisting of an LC-10 AD *VP* pump and a SPD-10 AV *VP* UV spectrophotometric detector. The separations were performed using a Macherey-Nagel (Düren, Germany) high-density reverse-phase C18 100-5 Nucleosil column (250×4.6 i.d. mm, 5 μm). The mobile phases employed were methanol (eluent D), HPLC-grade water (eluent E) and 1% dipotassium hydrogen phosphate in water adjusted to pH 7.4 with concentrated phosphoric acid (eluent F). Elution was initially with D:E:F (50:10:40) which was transformed according to a linear gradient over 20 min to D:E:F (75:10:15); the initial conditions were restored according to a linear gradient for 5 min, and the column was then re-equilibrated under these conditions for 10 min before the next run was commenced. The flow rate was 1 ml min^{-1}, and 20 ml aliquots of organic extract (3 mg ml^{-1}) was dissolved in methanol then injected for analysis. Detection was at 268 nm where FA and DFA showed a common absorption maximum.

Seed germination assay

Striga hermonthica seeds collected from infested fields in Sudan in 1999 were supplied by Rut Babiker, Agricultural Research Corporation, Wad Medani, Sudan. The seeds were surface-sterilised by immersion for 5 min in a 1% sodium hypochlorite solution containing a few drops of Tween 80. After rinsing with sterile water on filter paper in a Buchner funnel the seeds were dried and stored in vials at room temperature. For *Striga* seed preconditioning, two layers of moistened glass-fibre filters were cut into disks with an 8 mm diameter cork borer and placed in Petri dishes. The sterilised and dried seeds were sprinkled on the discs (approximately 25–40 seeds per disc). Petri dishes were kept in an incubator at 30°C for 10–14 days in the dark before the germination assays.

For these assays, three discs per Petri dish were placed on large filters moistened with 2 ml of toxin solution in three replicate Petri dishes per toxin. The assay solution contained GR24 (5–10 ppm) (supplied by Barry Cohen, Weizmann Institute of Science, Rehovot, Israel) and an extract of the pure compounds dissolved at the desired concentration. Controls without toxins were prepared using the same procedure. Dishes were then kept at 30°C in the dark. After 36 hours, germinated seeds were counted and percent germination was determined. Each measurement was repeated three times. Data were subjected to analysis of variance. For multiple mean comparisons, the Student-Newman-Keuls test was performed.

In vitro assay

For the assessment of phytotoxicity of the culture filtrate and of the FA a system in plastic bags

was adapted (Amsellem *et al*., 2001). Transparent plastic sheets (A4 size) were used. A window (approximately 13×25 cm) was cut on one side of the plastic bag, and a sheet of microfibre glass filter (Whatman GF/A, 15×23 cm) was put inside. The microfibre was moistened with distilled water. Witchweed seeds (25 mg) were dispersed homogeneously on the filter surface. Sorghum seedlings previously grown in greenhouses in pots with vermiculite were transferred to the plastic bags at the second-leaf-stage, leaving the shoot and leaves outside the bag and placing the roots on the glass fibre. Three plants were placed in each plastic bag. The cut was sealed with tape. Bags were placed vertically in a paper folder to keep the roots and seeds in darkness, and to allow host plant growth and witchweed seed germination. After 2 weeks culture filtrate or FA solution at different concentrations (between 10^{-4} to 10^{-6} M) was added (10 ml). Plants were observed daily for seed germination for two weeks.

Results and discussion

The main metabolites produced by the fungus in liquid culture were fusaric and dehydrofusaric acid, as shown by chromatographic purification of the acidic organic extract and the identification of the acids with TLC, HPLC and NMR and MS spectroscopy. The organic extract (62 mg) was purified by preparative TLC on 0.50 mm silica gel plates using cloroform-*iso*-propanol (97:3), where two main UV-absorbing bands were obtained. The residues of 22.6 mg and 31.9 mg were homogenous compounds having R_f 0.40, 0.33 by TLC analysis on silica gel (eluent B) and R_f 0.30, 0.51 by TLC on reversed phase (eluent C), corresponding to those of authentic samples of FA and DFA respectively. ^{1}H NMR (recorded in tetradeuteromethanol at 400 MHz on a Bruker spectrometer, Gloucestershire, UK) and ES MS (recorded on a API 100 LC-MS, Perkin-Elmer, Wellesley, MA, USA) spectroscopic data of the two purified substances were identical with those of standards recorded under the same conditions and described in a previous work (Capasso *et al*. 1996).

The HPLC method used was applied to evaluate and quantify the content of both acids in the organic extract. The acidic organic extract of the liquid culture filtrates revealed both toxic metabolites. Figure 1 is an example of an HPLC profile of the methanol soluble organic extract where FA and DFA peaks are detected because their retention times coincide with those of the standards. As shown in the figure, with this chromatographic method DFA and FA were eluted separately and in sequence, DFA at R*t* 6.20±0.40 and FA at R*t* 8.30±0.40 min. Recovery experiments confirmed the values of both acids. Yields of 50% from the standards as determined by HPLC, when added to liquid culture filtrates and then extracted with the method described before and in agreement with that used by Amalfitano *et al*. (2002), gave nearly complete recovery of both substances with a standard deviation error not exceeding 5%. In the standard conditions used, FA was 26.3 mg 100 ml^{-1} liquid culture filtrate and DFA 38.2 mg 100 ml^{-1} liquid culture filtrate.

The very strong inhibition of seed germination with the liquid culture filtrate in the biological assay seemed to be mainly due to the very high levels of the two main compounds in the filtrate. The pure compounds assayed at different concentrations caused the inhibition of germination (up to 10^{-6} M, Fig. 2), comparable that of the toxins in the culture filtrate. These results are consistent with Zonno *et al*. (1996), who obtained very strong inhibition of seed germination with a strain of *Fusarium nygamai* that proved to be a very good producer of FA, DFA and also their methyl esters. Both TLC and HPLC analysis showed that this strain did not produce the methyl ester of FA or of DFA.

Experiments to confirm the occurrence of active metabolites in liquid cultures other than FA and DFA are in progress, although apparently it is these two metabolites that are responsible for the high toxicity of the culture filtrate.

The plastic bag proved to be a useful means to evaluate the activity of metabolites both on *Striga* seeds and on *Sorghum* plants, allowing direct observation of the plant-parasite system during the underground phase. Plastic bags could be very useful for preliminary observation of pathogens, chemicals or stimulants applied to the plant. In the plastic bag assay all the FA solutions (from 10^{-4} to 10^{-6} M) as well as the culture filtrate were toxic to *S. hermonthica* seeds, and strongly inhibited their germination (data not shown). However, FA at the highest concentration and the liquid culture caused

necrotic symptoms on sorghum leaves, while FA at the lowest concentration (10^{-6} M) had no effect on the plants, though still strongly reducing seed germination and attachment (data not shown).

The organic extract obtained from solid culture was highly active in the bioassay on stimulated seeds. When the organic extract was compared by means of TLC on silica gel (eluent A) with some of the most common fungal toxins produced by *Fusarium* species (beauvericin, diacetoxyscirpenol, deoxynivalenol, enniatin, fusarenone X, T-2 toxin, HT-2 toxin, moniliformin, neosolaniol, nivalenol and α-zearalenol) it showed compounds with a TLC behaviour different from that of standards having

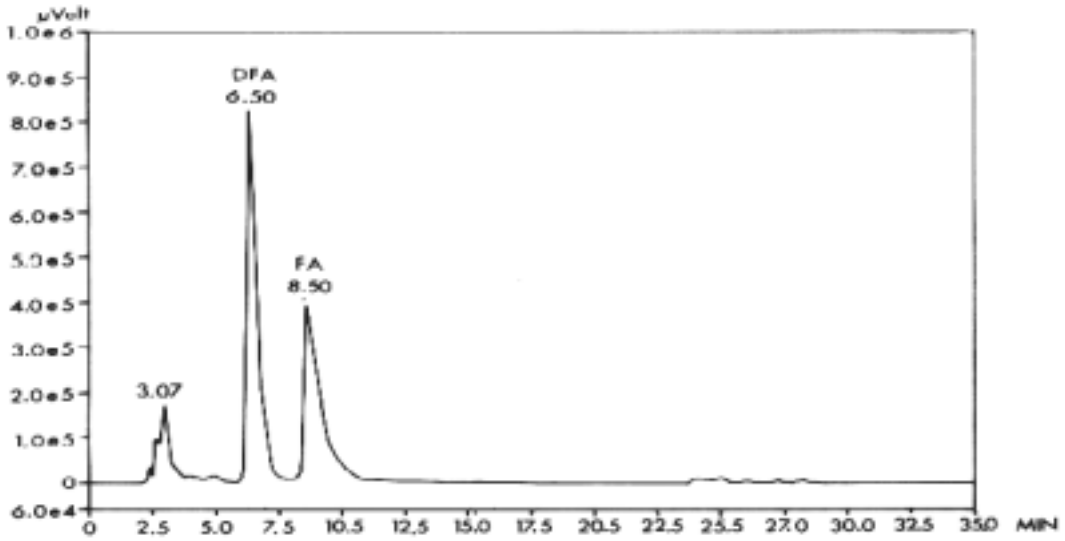

Fig. 1. HPLC pattern of methanol-soluble acidic organic extract of liquid culture filtrate; FA, fusaric acid; DFA, 9,10-dehydrofusaric acid.

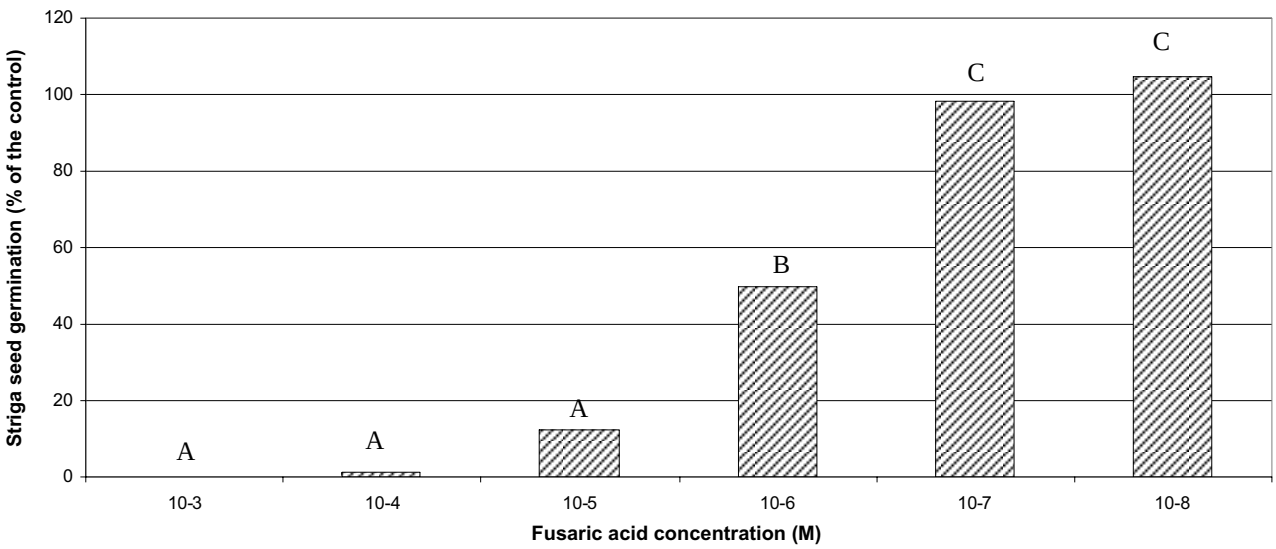

Fig. 2. Effect of fusaric acid on *Striga hermonthica* seed germination.

strong activity against *Striga* seed germination (data not shown). Studies are in progress to purify, identify and characterise the main active compound(s) chemically and biologically.

This is the first report on the production of toxic metabolites by this new species of the section *Liseola*, which is not yet described (Nirenberg, personal communication).

The possible practical use of these metabolites as natural herbicides remains to be ascertained. For this they would have to be shown either to have a lower impact on the environment or to be safer in application than the synthetic compounds. It is worthy of note that FA and DFA had a much stronger inhibiting effect on *Striga* than on *Orobanche* seeds (Zonno and Vurro, 2002), suggesting that these metabolites could be considered in an integrated control strategy against *S. hermonthica,* since, unlike some other *Fusarium* toxins which effectively inhibited germination but also had high mammalian toxicity, FA proved only moderately toxic to animals (intraperitoneal LD_{50} 100 mg kg^{-1} of body weight in mice) (Ueno and Ueno, 1978). Since they are highly active at very low doses, these inhibitors should be evaluated for their use in the soils, having regard also to the possibility of water translocation, physical and microbial degradation, and fixation or absorption by soil particles.

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