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Mycotoxins produced by Fusarium spp. isolated from Striga hermonthica (Del.) Benth. in Sudan

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Abstract
Three isolates of *F. solani* and one isolate of each of F. oxysporum, F. nygamai, F. equiseti, and F. compactum were fully toxicologically characterized. These Fusaria were isolated from naturally infected Striga hermonthica plants collected from sorghum cultivations in central (Gezira), eastern (Gadarif), and southeastern Sudan (Damazin). HPLC/MS or HPLC/DAD system was used for the toxicological characterization of Fusarium spp. using nivalenol, deoxynivalenol, 3-acetyl deoxynivalenol, 15-Acetyl deoxynivalenol, fusarenon X, T2-toxin, HT-2 toxin, diacetoxyscirpenol, zearalenone, fumonisin B (B1 and B2), enniatin B, B1 and A and fusaric acid as toxin standards. The three isolates of *F. solani* produced none of the above toxins. Fusarium oxysporum and *F. nygamai* produced fusaric acid. Fusarium nygamai also produced fumonisns B1, B2, and B3. Fusarium equiseti produced nivalenol, fusarenon X, and diacetoxyscirpenol. Fusarium compactum produced nivalenol. This is the first report of nivalenol production by a strain of F. compactum. Non toxin-production by the three isolates of *F. solani* and production of only fusaric acid by *F. oxysporum* make them toxicologically safe for further evaluations as biocontrol agents for the witchweed.

Keywords: Mycotoxins, Fusarium spp., Striga hermonthica, Sudan

Introduction

Recent observations of sporadic natural Striga death promoted investigations into isolation and identification of the pathogens involved (Abbasher, 1994). Intensive surveys were conducted in Sudan (Abbasher and Sauerborn, 1992), Burkina Faso, Mali and Niger (Abbasher et al., 1998; Ciotola et al., 1995), Ghana (Kroschel et al., 1996; Abbasher et al., 1995), and Nigeria (Marely et al., 1999). Numerous fungi and several bacteria were isolated and identified. Fungi have received more attention than other microorganisms because they can be aggressive and host-specific parasites (Charudattan, 1985; Templeton, 1982). In addition, it is usually simple to grow them under in vitro conditions and large amount of inoculum can be produced relatively quickly and easily. Fusarium spp. and Curvularia spp. were the two most common genera isolated from Striga. Subsequently, Fusarium spp. were the prevalent soil-borne fungi isolated from Striga and being isolated from 90% of the naturally diseased S. hermonthica in northern Ghana (Abbasher et al., 1995), Burkina Faso, Mali and Niger (Abbasher et al., 1998; Yonli et al., 2004). Fusarium spp. have received the most attention for biological control of S. hermonthica ever since the isolation and pathogenicity testing of F. equiseti isolated from Striga by Zummo (1977). One isolate of *F. oxysporum* (M12-4A) from Mali, grown on sorghum straw and incorporated into potted soil, prevented all emergence of S. hermonthica and resulted in a four fold increase in sorghum yield (Ciotola et al., 1995, 2000). In northern Ghana, *F. oxysporum* (Foxy 2) was highly virulent, reducing the emergence of S. hermonthica by 90% and increasing sorghum yield by 26% (Kroschel et al., 1996). Another *F. oxysporum* (4-3-B) isolated from Burkina Faso, was particularly virulent to S. hermonthica among other Fusarium spp. tested (Yonli et al., 2004, 2006). In the Nigerian Savanna, virulence tests for S. hermonthica of several Fusarium spp., led to the selection of another *F. oxysporum* isolate (PSM-197) (Marely et al., 1999). When was grown on sorghum grains and incorporated into soil, this isolate completely inhibited S. hermonthica emergence. In the Sudan an isolate of *F. oxysporum* isolated from S. hermonthica was found less virulent and caused 45% inhibition of S. hermonthica emergence (Idris, 1997).

The mentioned surveys encompassed a substantial portion of Africa infested by S. hermonthica and showed abundance of *F. oxysporum* recovered from Striga plants with vascular wilt symptoms. The

host specificity of most Fusarium spp. is at the genus or species level, leading to the formae specialis taxonomic classification (Nelsson et al., 1981; Lesli and Summerell, 2006). The *F. oxysporum* f. sp. striga isolates tested (M12-4A, Foxy-2, 4-3-B, and PSM-197) were host specific to Striga spp. (Ciotola et al., 1996; Kroschel et al., 1996; Marely et al., 1999; Elzein, 2003; Elzein and Kroschel, 2004b), and non pathogenic on sorghum, pearl millet, maize, rice, fonio, cotton, groundnut, cowpea and okra.

The major constraint to the use of bioherbicides based on Fusarium species is their ability to produce several types of mycotoxins some of which associated with chronic and acute mycotoxicoses and even cancer promoting activities in farm animal and man (D'Mello et al., 1999). Therefore, it is always recommended to evaluate Fusarium species as biocontrol agents to the witchweed for their ability to produce mycotoxins e.g. trichothecenes, fumonisins, and zearalenone which are the major contaminants in cereal grains, animal feed, and forage worldwide (D'Mello et al., 1999).

This study was aiming at: investigation of ability of Fusarium spp. isolated from naturally diseased S. hermonthica to produce toxins usin HPLC-MS. Such investigation would give answer to two main questions:

Do they produce secondary metabolites having effect on the parasite without being toxic to humans and animals? Do these metabolites could be used as bioherbicides instead of using the fungus itself?

Materials and Methods

Source of Fusarium isolates

Fusarium species used in this study were isolated from naturally infected Striga hermonthica plants collected from sorghum cultivations in central (Gezira), eastern (Gadarif), and southeastern Sudan (Damazin). This included three isolates of *F. solani* and one isolate of each of F. oxysporum, F. nygamai, F. equiseti, and F. compactum (Mohukker, 2009).

Media and cultivation conditions

Hundred ml of each of PDB and SNB (amended with 4g sucrose and 10g corn steep liquor) in 250 ml flask were inoculated using two 9 mm diameter plugs of approximately equal fungal mass. The plugs were obtained from 7-10-days old cultures of each Fusarium isolate on PDA. Three replicates were used for each medium and Fusarium spp. The inoculated flasks were then incubated under stationary conditions in the dark at 25°C for four weeks

Rice media were inoculated using 3 plugs of each Fusarium spp. as described above using 3 replicates. Inoculated flasks were then incubated at room temperature (25-27°C) for four weeks with daily shaking for the first few days to permit the fungi to uniformly penetrate the rice.

Filtration, centrifugation and extraction of the inoculated liquid media

After four weeks incubation, the liquid cultures (PDB; SNB+Succrose and Corn steep ligour) of Fusarium isolates were filtered under suction using a Buchner funnel lined with Whatman filter paper no1. The mycelia were collected from the filter papers after being thoroughly washed with water under suction and kept in small falcon tubes in -20°C. Filtrates were centrifuged at 10,000 rpm for 5 min. at 4°C. The supernatants were further filtered through micropore filters (0.20 µm) and filtrates were then extracted three times using total volume 120-140 ml ethylacetate. 1.5 ml from each extract was transferred to 2ml Eppendorf-tubes and concentrated to dryness at 30°C using Eppendorf concentrator 5301, Germany. The dried metabolites were then stored in – 20 ° C for further use.

Extraction of Fusarium metabolites from rice Cultures

The fungus-invaded rice was transferred to plastic trays (tray/isolate) and allowed to air dry under a ventilated hood. The dry rice was then ground to the consistency of flour using a coffee grinder. Four grams of the ground rice were suspended in 40 ml ethanol/water (80:20, v/v) or Acetonitrile/water (80:20, v/v) in 250 ml Erlenmeyer flasks and extracted using high speed automatic shaker at 150 rpm for 2 h. The supernatants were filtered using Whatman filter paper no. 4 and the filtrates were then stored in – 20 ° C for further use.

Preparation of Fusarium metabolites for HPLC/MS analysis

Two ml MeOH were added to the dry metabolites in Eppendorf-tubes extracted from PDB and SNB media. Then 0.5 ml were pipetted into other 2ml Eppendorf tubes, 0.5 ml of the buffer methanol/water 50% with 1ml acetic acid, pH 4.5 were added and 0.5 ml of n-hexan were also added. The tubes were then

centrifuged at 14000 rpm at 17° C for 10 minutes. After centrifugation, the lower layer, containing metabolites, were sucked using a syringe and directly filtered through 0.20 µm micropore filters into small glass vials for HPLC analysis.

Four ml of the organic filtrates (ethanol/water or acetonitrile/water) were slowly passed through a Bond Elut Mycotoxin Column (Varian, Darmstadt, Germany). Two ml of the elute were evaporated to dryness at 50° C using a speed vacuum and the dried sample was redissolved in 0.5 ml methanol/5 mM ammonium acetate 50/50. Ten µl aliquots were injected into HPLC-mass spectrometry (HPLC/MS) for analysis. HPLC column (4 µm Synergi Fusion-RP 80A; 10 cmX 2 mm dimension) was eluted with water (eluant A) and methanol (eluant B) with linear gradients of 0-1 minute 15% B, 1-18 min. 10-70%B, 18-19 min. 70-98%B, 19-28 min. 98%B, 28-29 min. 15%B, 29-39 min. 15% B at a flow rate of 0.2 ml/min. The effluent from the column was introduced to the mass spectrometer through electrospray ionization interface.

The standards of the Fusarium toxins, Nivalenol, Deoxynivalenol, 3-Acetyl deoxynivalenol, 15-Acetyl deoxynivalenol, Fusarenon X, T2-toxin, HT-2 toxin, Diacetoxyscirpenol, Zearalenone, Fumonisin B (B1 and B2), Enniatin B, B1 and A and fusaric acid were purchased from Sigma.

Assessment of Trichothecense production by the Fusarium solani isolates in YES medium

Yeast Extract Sucrose (YES) medium is a secondary metabolism stimulating medium used by many authors for the production of some of the trichothecense and some other Fusarium mycotoxins (Krikštaponis et al., 2001; Cvetnić et al., 2005). Since the three Fusarium solani isolates did not produce any of the mycotoxins in any of the media used above, YES medium was used in this test to confirm the previous findings.

Inoculation of YES medium

Hundred milimeters of YES medium were suspended into 250 ml Erlenmeyer flasks, cotton stoppered and autoclaved as previously mentioned. Half milimeter spore suspension (3.8; 3.4; 1.5X106 spores/ml) in 20 % glycerine of Fusarium isolates 1, 2, and 4 was added, aseptically, to the sterilized media in flasks. Three replicates were used for each isolate. All inoculated flasks were incubated without shaking at 25°C for four weeks.

Extraction of metabolites from YES media

After four weeks incubation in the dark at 25°C, the liquid cultures (YES) of the three Fusarium isolates were filtered under suction using a Buchner funnel lined with Whatman filter paper no. 1. The mycelia were collected from the filter papers after thoroughly washed with water under suction and kept in small falcon tubes then frozen in -20°C. Filtrates were centrifuged at 10,000 rpm for 10 minutes at 4°C and the supernatants were further filtered through micropore filter (0.20 µm). Hundred and fifty ml of the filtrates of each isolate were extracted using the same volume of ethylacetate (EtOAc) by means of a magnetic stirrer for 10 h. The organic phase was separated from the aqueous phase using a separating funnel.

Detection of Trichothecense in EtOAc extracts

Four ml of the EtOAc extracts was evaporated to dryness in a speed vacuum. The dry sample was reconstituted in 4 ml Acetonitrile/water (80:20, v/v) and slowly passed through a Bond Elut Mycotoxin Column (Varian, Darmstadt, Germany). Two ml of eluate were evaporated to dryness at 50° C under vacuum. The dry sample was reconstituted in 0.5 ml methanol/5mM ammonium acetate 50/50. 10 µl were injected into HPLC/MS system for analysis as previously described in 3.4.4.

Assessment of Fumonisin B production by Fusarium isolates on Rice cultures

Cultures of Fusarium isolates were prepared on rice media as described above.

Extraction of metabolites from rice culture

The fungus-invaded rice was transferred to plastic trays (tray/isolate) and allowed to air dry in a ventilated hood. The rice culture was then ground to the consistency of flour using a coffee grinder. Ten grams of the ground rice culture were suspended in 40 ml of MeOH: 1% acetic acid (99:1) in 100 ml Erlenmyer flasks and extracted using high speed automatic shaker at 150 rpm for 2 h. The supernatants were filtered using Whatman filter paper no. 4 and refiltered using micropore filters (0.2 µm). The filtrates were then stored in – 20 ° C for further use.

Preparation of extracts for Fumonisin B detection by HPLC- MS/MS

For clean up of the sample a small chromabond C18ec column (Machery-Nagel) 500 mg (3 ml)

was used. The cartridge was first activated with 1 ml MeOH and then equilibrated with 3 ml acetic acid 2%. Before application on the column, 3 ml of the extract was evaporated to dryness in a speed vacuum and redissolved in 3 ml of 2% acetic acid. The sample was applied on the column which was fixed on a special collecting chamber connected with an electric pump to speed up the flow rate. The column was then washed with MeOH:water:acetic acid (50:48:2, v/v) and the collected sample was discarded. The cartridge was left to dry and the toxins were eluted twice with 500 µl MeOH: acetic acid, (95:5, v/v). The eluate was evaporated to dryness under vacuum and the dried sample was redissolved in 500 µl 50% MeOH/10 mM acetic acid at pH 5.0.

HPLC-MS/MS conditions

Ten µl of the sample was applied into the HPLC/MS system using a synergi fusion. HPLC column was eluted with 7 mM acetic acid with 5% acetonitrile (eluant A) and methanol with 7 mM acetic acid (eluant B) with linear gradients of 0-1min. 45% B, 1-4.5 min. 45-50% B, 5.5-8.5min. 50-98% B, 8.5-9min. 98-45%B, and 9-19 min. 45%B at a flow rate of 0.2 ml/min.

Detection of Fusaric Acid by HPLC/ DAD and HPLC/MS

Preparation of sample for analysis

Fifty milimeters of PDB containing metabolites of Fuasrium spp. were acidified to pH 2 using HCl 4M, mixed with 50 ml of ethylacetate and extracted in a high speed by mean of a magnetic stirrer for 2 h. The organic phase was separated from the aqueous phase by a separating funnel. One ml of the extract was evaporated to dryness and dissolved in 0.5 ml of MeOH/H2O 50% for direct detection under HPLC/DAD (Diod Aray Detector) or HPLC/MS. The same procedure was used for the preparation of the control which consisted of non-inoculated PDB.

HPLC conditions

The samples were applied to ZIC-HILIC 3.5 µm, 100 x 2.1 mm HPLC-column (Sequant, Haltern, Germany). Subsequently, the column was eluted with acetonitrile/20 mM Amonium acetate 85:15, isocratic at a flow rate of 0.2 ml/min.

Results

Mycotoxin production by Fusarium isolates

Trichothecevse produced by Fusarium spp. are shown in table (1) and Figs 1 and 2. All Fusarium spp. tested did not produce zearalenone. The three isolates of *F. solani* did not produce any of type A and type B trichothecenes in all media. However, F. equiseti produced Nivalenol (NIV), Fusarinon X (FUSX) and Diacetoxyscirpenol (DAS) in different media (Fig. 1 and table 1). F. compactum was found to produce nivalenol only (Fig. 2 and table 1).

Of all Fusarium isolates tested only tow isolates were found to produce FusA. This included *F. oxysporum* and *F. nygamai* (Figs. 3 and 5). On DAD the absorbance of FusA was found to be at 269 nm. On MS, after a calibration curve was established using concentrations of 500, 1000, 2000 ppb (0.5, 1, 2 ppm) (Fig. 4) FusA was detected at a retention time of approximately 3.25 min. at a flow rate of 0.2 ml/min (Fig. 5). The amount of the produced FusA was estimated to be between 500 ppm – 1000 ppm as deduced from the standard.

When investigated for the presence of FB series using HPLC/MS, extracts of rice cultures of *F. nygamai* were found to contain FB1, FB2 and FB3 (Figs.6). Although FB3 was not present in the standard, which contained only FB1 and FB2, it was not difficult to detect its presence depending on its retention time when compared to that of FB1 and FB2. Rice culture extracts of the rest of Fusarium spp. contained no fumonisins.

Table 1 mycotoxins produced by *Fusarium* species

Fusarium spp.	**Mycotoxin(s)**
F. solani	Not detected
F. oxysporum	Fusaric acid
F. nygamai	Fusaric acid; Fumonisin B1, B2 and B3
F. equiseti	Nivalenol; Fusarenone X; Diacetoxyscirpenol
F. compactum	Nivalenol

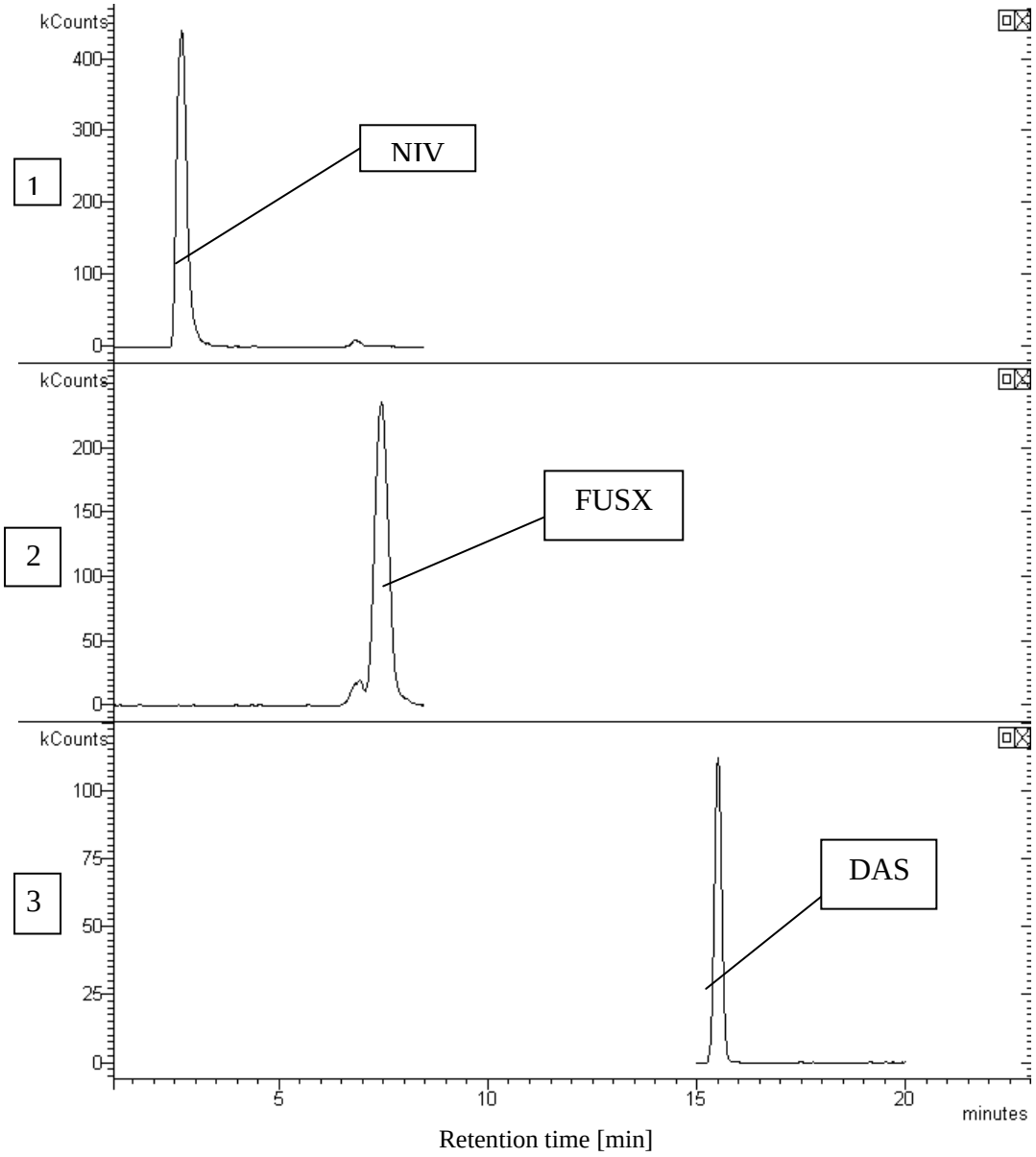

Figure 1 HPLC chromatograms of 1) Nivalenol (NIV) 2) Fusarenone x (FUSX) and 3) Diacetoxyscirpenol (DAS) produced by *F. equiseti*

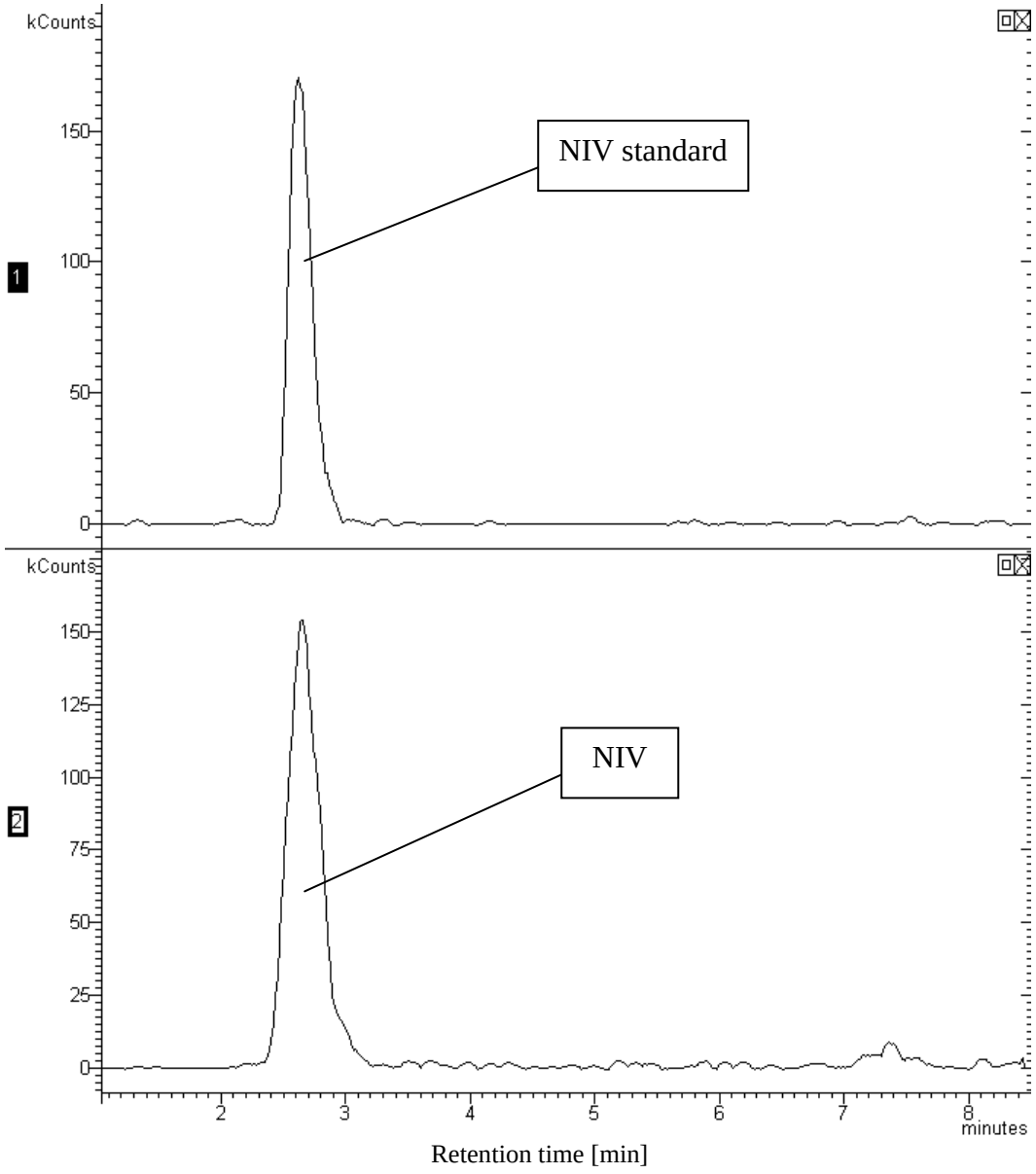

Figure 2 HPLC/MS chromatograms of Nivalenol 1) Nivalenol standard 2) Nivalenol produced by F. compactum

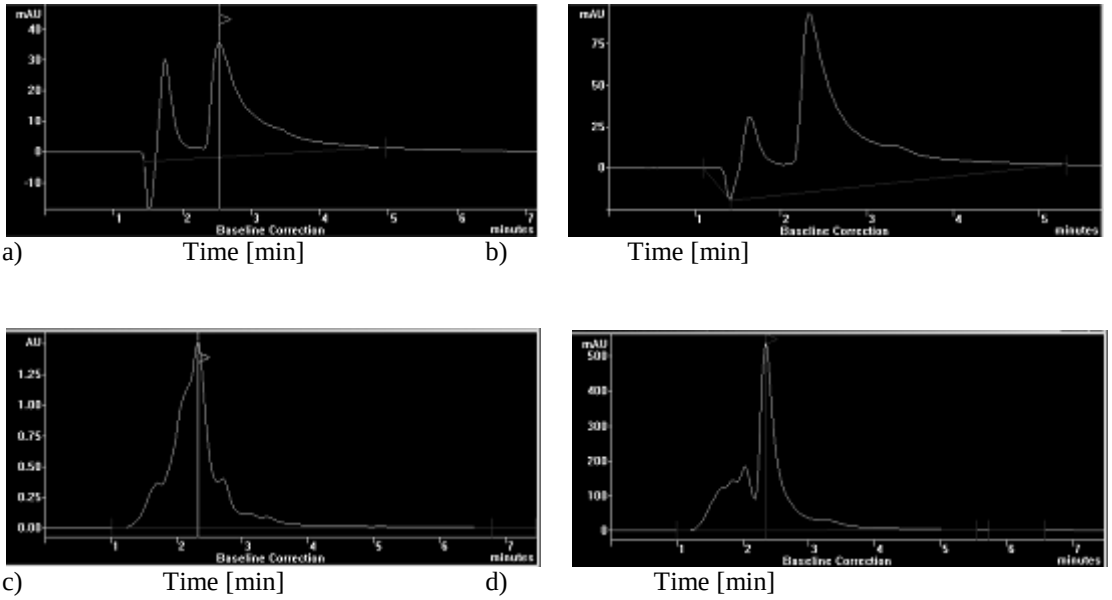

Figure 3 DAD chromatograms of fusaric acid (FUSA) a) and b) FUSA standard c) FUSA produced by *F. oxysporum* d) FUSA produced by *F. nygamai*

Discussion

In these results, the three isolates of *F. solani* did not produce any of the mycotoxins used as standards in this study which included Nivalenol, Deoxynivalenol, 3-Acetyl deoxynivalenol, 15-Acetyl deoxynivalenol, Fusarenon X, T2-toxin, HT-2 toxin, Diacetoxyscirpenol, Zearalenone, Fumonisin B (B1 and B2), Enniatin B, B1, and Fusaric acid. This is in addition to neosolaniol and Monoacetoxyscirpenol which were not available as standards but they could, however, be easily identified by their retention time and transition in comparison with those of other trichothecenes and as well as the literature. The inability to produce these toxins by the isolates of *F. solani* was consistant in all types of culture-media used including the YES-medium which is used by some workers for the production of trichothecenes in particular and some other Fusarium toxins (Krikštaponis et al., 2001; Cvetnić et al., 2005). These findings are in contrast to those of Sugimoto et al. (2002) who isolated trichothecenes acuminatin, neosolaniol, 8-acetylneosolaniol, and tetra-acetoxy T-2 tetrol (neosolaniol diacetate) from *F. solani* (SUD96), isolated from infected Striga plants in the Sudan. Such differences in mycotoxin production between different strains of the same species especially when collected from different locations are, however, expected (ApSimon et al., 1990).

These findings of being non-producers of the major harmful Fusarium toxins raise the chance of these three isolates of *F. solani* to be used in pathogenicity tests for evaluation of their status as biocontrol canididates for the control of the parasitic weed S. hermonthica. This is especially interesting when considering the promising results obtained by other workers when testing *F. solani* for the biocontrol of Striga sp. in vitro as well as in vivo (Meister and Eplee, 1971; Kroschel et al., 1996; Idris, 1997, Ahmed et al., 2001). In addition, *F. solani* f. sp. cucurbitae after being extensively tested for commercial use was applied for the control of the weed Texas gourd (Cucurbita texana Gray) (Weidemann, 1988; Weidemann and Templeton, 1988).

Fusarium nygamai and *F. oxysporum* were found to produce fusaric acid in relatively large amounts ranged between 500- 1000 ppm as deduced from the concentration of the pure standard and its calibration curve. Production of fusaric acid by *F. nygamai* in this study is in

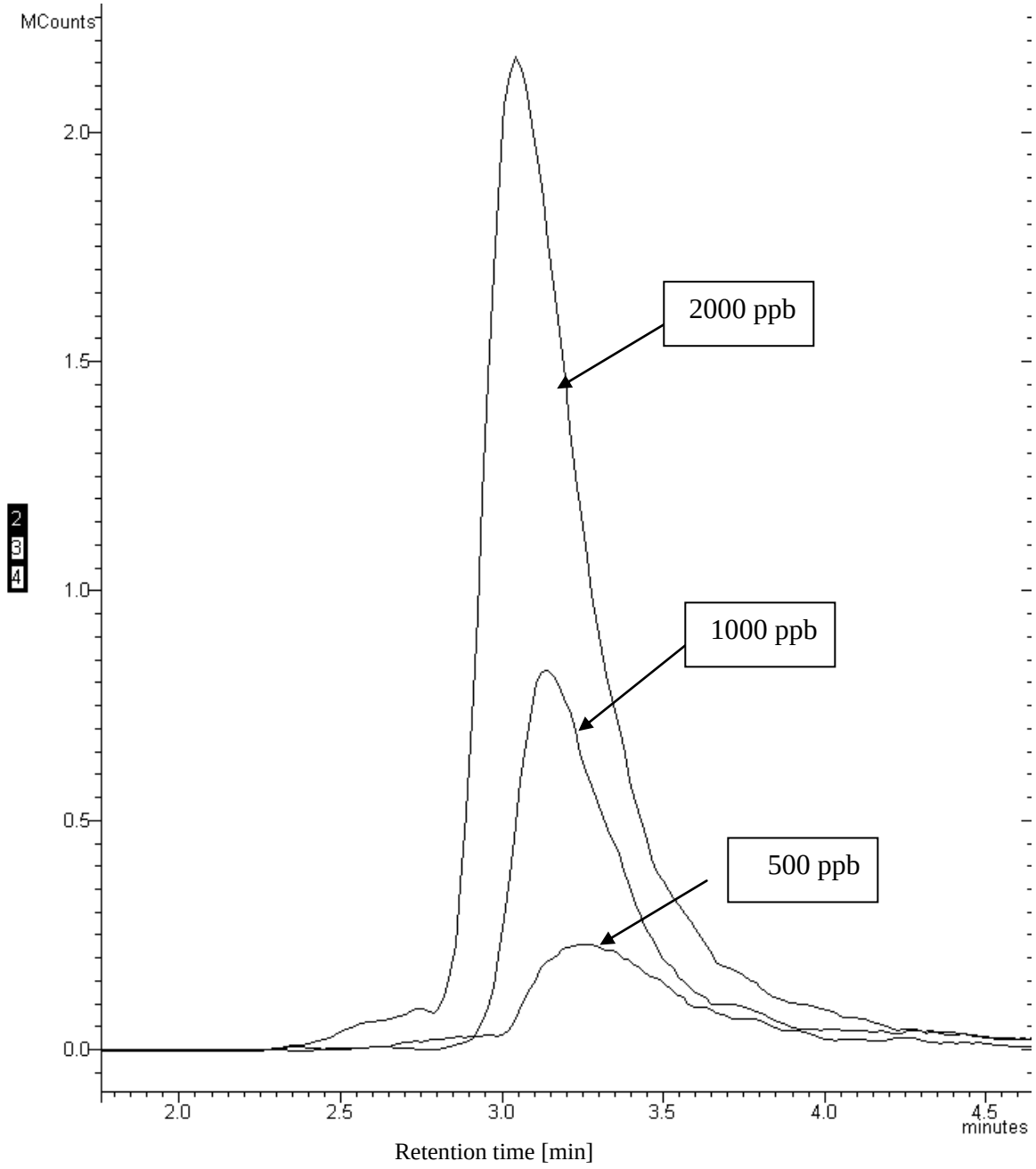

Figure 4 HPLC/MS chromatograms of calibration curve for fusaric acid standard

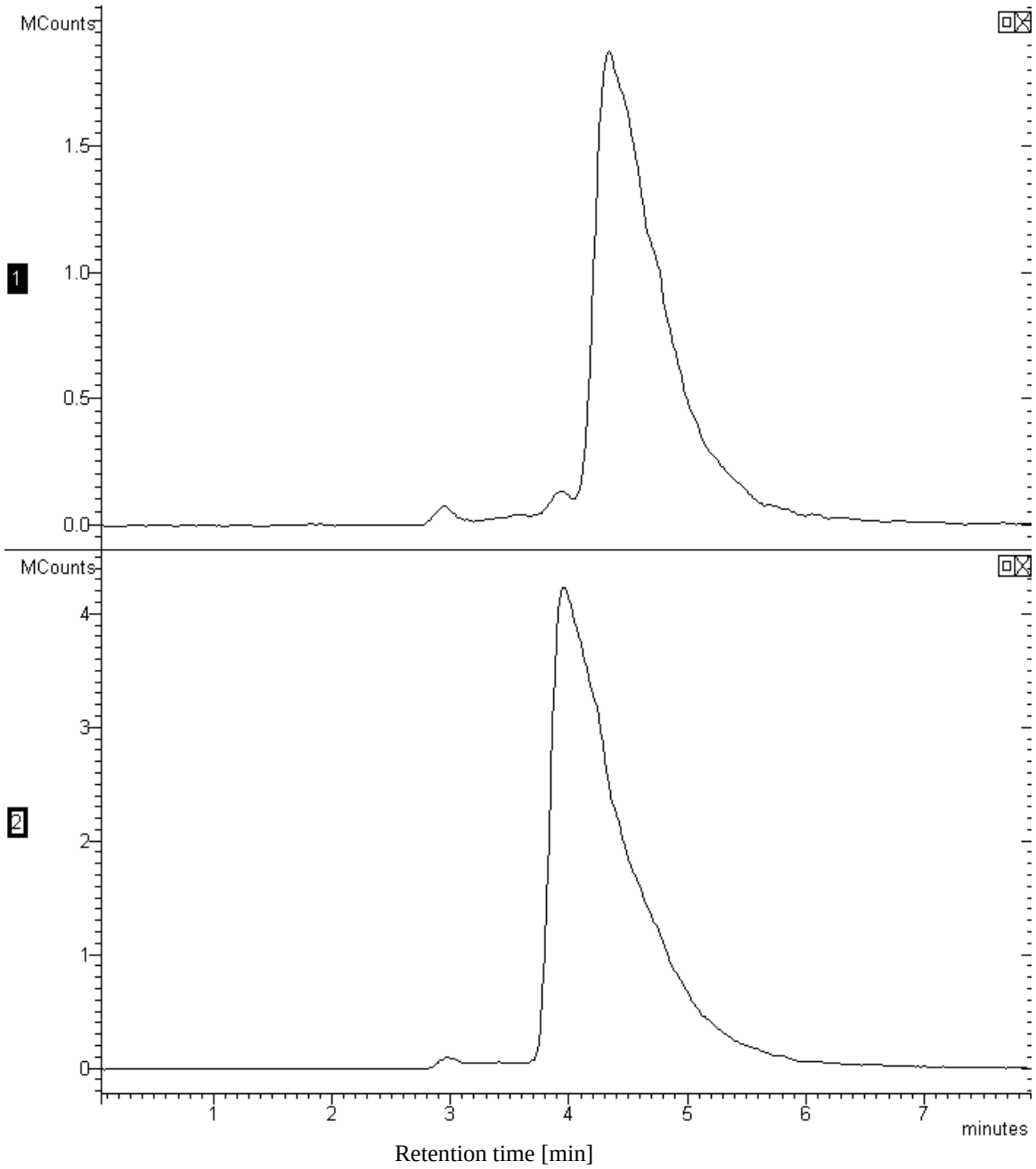

Figure 5: HPLC/MS chromatograms of fusaric acid 1] FUSA produced by *Fusarium nygamai* 2] FUSA produced by F. oxysporum

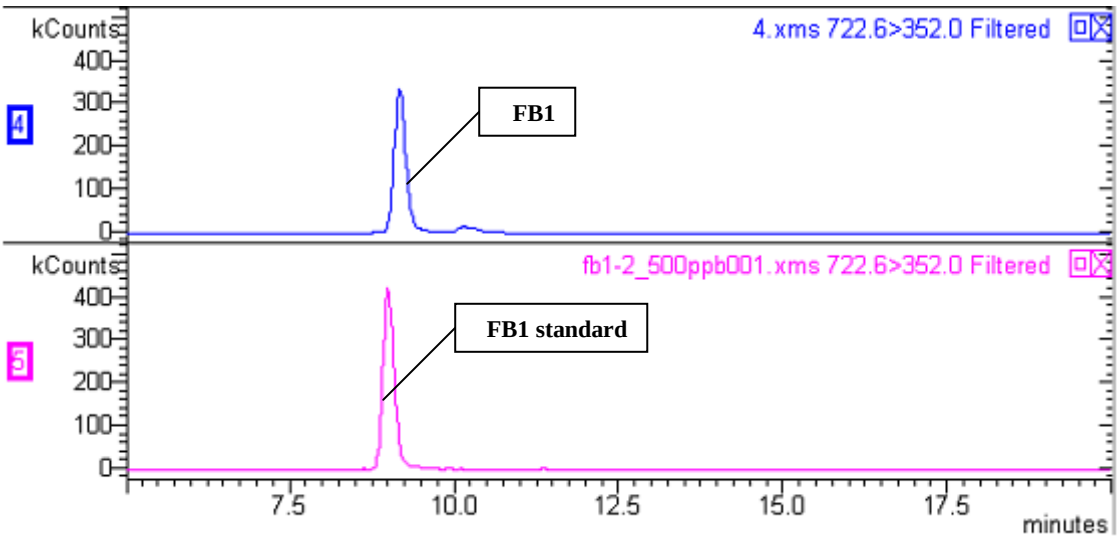

Figure 6 HPLC/MS chromatograms of FB1, FB2, and FB3 produced by *F. nygamai* and of FB1, FB2 standard

agreement with the findings of other workers who reported production of fusaric and 9,10-dehydrofusaric acid and their corresponding methyl esters by strains of *F. nygamai* isolated from diseased S. hermonthica in the Sudan (Zonno et al., 1996; Capasso et al., 1996; Amalfitano et al., 2002). Production of fusaric acid by *F. oxysporum* in this study is also consistent with findings of other researcher of fusaric acid being widely produced by strains of *F. oxysporum* isolated from the parasitic weeds Striga (Ciotola et al., 1996; Savard et al., 1997) and Orabanche (Dor et al., 2007) as well as from other sources (Bacon et al., 1996; Notz et al., 2002). No toxin other than FusA was found to be produced in any of the used culture-media by *F. oxysporum* isolated in this study. Since fusaric acid is moderately toxic to humans and animals, [it can only become very toxic in synergicy with other important mycotoxins e.g. trichothecenes (Smith and Sousadias, 1993)], this isolate of *F. oxysporum*

could safely be further evaluated for its pathogenicity to S. hermonthica plants. Although its involvement in plant pathogenicity is not clearly established (Kuzniak, 2001), fusaric acid could be involved in fungal pathogenicity by decreasing plant cell viability (Gapillout et al., 1996; Kuzniak, 2001; Bouizgarne et al., 2006). Fusaric acid seems also to play a role in the competition between Fusarium spp. and other microorganisms in the plant rhizosphere. In this context, Bacon et al. (2006) reported that at specific concentrations FusA was toxic to most strains of the Gram-positive biocontrol bacterial endophyte Bacillus mojavensis. The effects of these concentrations on other Bacillus species varied in that fusaric acid was either bacteriocidal or bacteriostatic to most species. In addition, FusA produced by some strains of *F. oxysporum* repressed the production of 2,4-diacetylphloroglucinol, a key factor in the antimicrobial activity of the Gram-negative biocontrol strain Pseudomonas fluorescens CHA0 (Notz et al., 2002). These findings support the idea that production of FusA by Fusarium spp. increases their competitive ability in their natural habitats.

Fusarium equiseti in this study was found to produce nivalenol, fusarenon x (type B trichothecenes), and diacetoxyscirpenol (type A trichothecenes) mostly in rice cultures. These finding are in an agreement with the reports of Marasas et al., (1984), Bosch and Mirocha (1992), respectively. F. equiseti as a soil-borne fungus is generally considered to be secondary colonizer of plant tissues. On its own, however, it has been shown to cause seedling diseases of muskmelon and other melons in California (Adams et al., 1987). Therefore, its involvement in the apparent natural death of S. hermonthica can only by approved by pathogenicity tests after its toxilogical status was clarified.

Fusarium compactum in this study was found to produce nivalenol. This was the case in three different independent replications. Nivalenol, however, has not been reported before to be produced by strains of F. compactum, this is the first report of this kind. F. compactum in this study was identified following the keys of Gerlach and Nirenberg (1982), and Burgess et. al. (1994) according to conidiophores, macro, micro conidia and chlamydospores shape and production. Identification was confirmed by Dr. Nirenberg at the University of Berlin, Germany in a written note as well as during a personal communication by the author of this study. Production of toxin that is not normally produced by strains of specific Fusarium species is not uncommon among species of this fungus. Similar to this is the production of fumonisins by strains of F. oxysporum. Namely *F. oxysporum* var. redolens which was reported to produce fumonisins B-series (Abbas et al., 1995) and *F. oxysporum* strain O-1890 which was found to produce fumonisins C-series (Seo et al., 1996). Why these strains of *F. oxysporum* do produce fumonisins and other strains don't is, however, not clear. The phenomenon of production of nivalenol by F. compactum in this study can be clarified in the future by first confirming the identity of the fungus using DNA sequence data and then by investigating the presence of genes responsible for the production of this toxin in the genome of the fungus since primers for this purpose are now available (Lee et al., 2002).

F. nygamai isolated from S. hermonthica in this study was found to produce FB1, FB2, and FB3 in solid rice medium. This finding supports previous findings of strains of *F. nygamai* being able to produce different types of fumonisins (Thiel et al., 1991a; Nelson et al., 1994; Musser and Plattner, 1997; Magnoli et al., 1999; Kroschel and Elzein, 2004). Very recently, delivery systems have been developed for Fusarium Abuharaz and *F. nygamai* e and their performance as biocontrol agents to be used under Sudanese field conditions was evaluated (Zahran et al., 2008). However, production of FB series by *F. nygamai* makes its use as biocontrol agent questionable. This powerful phytotoxin against Striga is also a carcinogenic mycotoxin.

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