CHAPTER 4

Mycoflora and Fumonisin Mycotoxins Associated with Cowpea (*Vigna unguiculata* (L.) Walp) Seeds

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Abstract

Cowpea seed samples from South Africa and Benin were analyzed for seed mycoflora. *Fusarium* species detected were *F. equiseti*, *F. chlamydosporum, F. graminearum, F. proliferatum*, *F. sambucinum*, *F. semitectum* and *F. subglutinans*. Cowpea seed from South Africa and Benin and *F. proliferatum* isolates from Benin, inoculated onto maize patty medium, were analyzed for fumonisin production. Samples were extracted with methanol/water and cleaned-up on strong anion exchange solid phase extraction cartridges. HPLC with pre-column derivatization using *o*-phthaldialdehyde was used for the detection and quantification of fumonisins. Cowpea cultivars from South Africa showed the presence of fumonisin B_1 with concentrations ranging between 0.12 - 0.61 µg/g whilst those from Benin showed no fumonisins. This is believed to be the first report of the natural occurrence of FB_1 on cowpea seed. Fumonisin B_1, B_2 and B_3 were produced by all *F. proliferatum* isolates. Total fumonisin concentrations were between 0.80 - 25.30 µg/g and the highest level of FB_1 detected was 16.86 µg/g.

Keywords: cowpea, fumonisins, FB_1, *Fusarium proliferatum*, mycoflora, *Vigna unguiculata*

INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp.) is a popular, nutritious and important legume crop of many subsistence farmers and rural communities living in less developed countries of tropical and sub-tropical Africa. This indigenous African legume is cultivated as a pulse, vegetable, fodder and cover crop (*1*), providing more than half of the plant protein in human diets in certain areas of the semi-arid tropics (*2*) and is also a valuable source of carbohydrates and minerals (*3*). Cowpea seeds have an average protein content of 23-25% and a carbohydrate content of 50-67% (*3*) and are consumed in various ways. In Nigeria, the seed is consumed after boiling to softness, and seasoned with salt and pepper with palm oil to form a porridge or after frying in oil to form "akara" (*4*). Dry seeds alone or as part of other dishes are popular in southern Africa (*5*).

Unfortunately, fungal contamination often prevails under conditions of relative high humidity and high ambient temperatures (*6-8*) and some of these fungi produce toxic secondary metabolites under these sub-optimum storing conditions. These mycotoxins, when ingested during the consumption of infected seed and other foodstuffs, can lead to serious health complications in animals as well as humans. It is well documented that various legume seeds are prone to fungal infestation and subsequent mycotoxin contamination (*9-14*). However, reports on mycotoxins associated with cowpea seed are scant and mainly refer to *Aspergillus* infection associated with aflatoxin production (*7, 8, 15-17*). Hitokoko et al. (*7*) reported that cowpea seeds inoculated with toxigenic fungi were contaminated with sterigmatocystin, ochratoxin A and T-2 toxin.

Fumonisins, produced primarily by *Fusarium verticillioides* (Sacc.) Nirenberg, *F. proliferatum* (Matsushima) Nirenberg and *F. nygamai* Burgess and Trimboli (*18, 19*), are mycotoxins that have major toxicological significance in animal and human health (*20, 21*). Various analogues of fumonisins have been identified and characterized of which the most abundant are fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) (**Figure 1**) (*19, 21*). FB_1 causes equine leukoencephalomalacia in horses (*22*) and pulmonary edema in pigs (*23*). The incidence of *F. verticillioides* infection on homegrown maize is associated with the high incidence of human oesophageal cancer in Transkei, southern Africa and China (*24, 25*). The International Agency for Research on Cancer (IARC) classified the toxins produced by *F. verticillioides* as being possibly carcinogenic to humans (*26*). Furthermore, fumonisins have been detected in naturally infected mouldy navy beans (*Phaseolus vulgaris* L.) (*12*), and *Fusarium* infected adzuki beans (*Phaseolus angularis* (Willd.) W.F. Wight) and mung bean (*Phaseolus aureus* Roxb.) (*13*) and the phytotoxic activity of fumonisins to various plants has been reported (*27, 28*).

The objectives of this study were to investigate the detection and quantification of the fumonisin mycotoxins in cowpea seeds and to identify the fumonisin-producing *Fusarium* species from cowpea seeds and to investigate their potential for fumonisin production.

MATERIALS AND METHODS

Seed samples. Fumonisins were determined in cowpea seeds received from the Agricultural Research Council – Grain Crops Institute in Potchefstroom, South Africa consisting of four cultivars (Bechwana White, Glenda, Iron Grey and Rhino) and seed samples collected in street markets, Kpodjiguégué, Ghebami and Tawa, from Benin, western Africa. Potential fumonisin-producing *Fusarium* species were isolated from the Benin cowpea seed samples.

Isolation and identification of seed-borne fungi. One hundred seeds from each sample of the South African cultivars and two hundred seeds from each of the Benin samples were chosen randomly. The seeds were surface disinfected using 1% sodium hypochlorite for 1 min and rinsed three times in sterile distilled water. Fifty seeds from each South African cultivar were not surface disinfected. Thereafter, the seeds of the South African samples and Benin samples were directly plated out onto malt extract agar (MEA) and potato dextrose agar (PDA), respectively, (five seeds per plate, one seed in the center and one seed in each quadrant). The Petri dishes were incubated at ± 25 °C for 5-7 days with 12 h light/dark cycles after which the seeds were examined for fungal growth. The fungi were transferred to PDA plates for growth and fungal genera and species were identified with the aid of various references (*29-32*) and recorded.

Maize patty media. *Fusarium proliferatum* isolates from the cowpea seed samples were grown on maize patty medium in duplicate based on the method by Alberts et al. (*33*). These isolates (MRC 8275, 8276, 8277 and 8278) were deposited in the culture collection of the PROMEC Unit, Medical Research Council, Tygerberg, South Africa. Maize patty media was prepared in 90 mm Pyrex Petri dishes by adding 25 g finely ground maize kernels / 25 g water. The Petri dishes were autoclaved for 1 h at 121 °C and 120 kPa on two consecutive days. *F. proliferatum* suspensions were prepared in 100 mL sterile distilled water from cultures grown for 7–9 days. The maize patty media were inoculated with 1 mL of the suspension and the cultures were incubated at 25 °C for ± 21 days or until all the media were completely colonized by the fungus.

Sample extraction and clean-up. The samples included the inoculated maize patty cultures and ± 50 g of cowpea seeds of each of the four South African cultivars and the three Benin samples. After incubation, the maize patty cultures were allowed to dry overnight at ± 40 °C. The maize patty cultures

and cowpea seeds were ground into a fine meal using a laboratory grinder. The sample extraction and clean-up was carried out according to the method described by Sydenham et al. (*34*). After the addition of 100 mL methanol/water (70/30) (v/v) to 20 g of the fine meal, the samples were homogenized for 3 min at 5000 rpm with an Ultra-Turrex homogenizer (Jankel-Kunkel, Ika-Werk, Germany). The homogenized samples were centrifuged for 10 min at 4000 rpm and the supernatant filtered through Whatman No. 4 filter paper. The pH of the filtrate was adjusted to 5.8–6.5 with 0.1 M sodium hydroxide.

Clean-up of the filtrates were carried out on Chromabond strong anion exchange (SAX) cartridges (Machery-Nagel, Dűren) attached to a solid phase extraction (SPE) vacuum manifold. Prior to loading 10 mL of the filtrate, the SAX cartridges were preconditioned by washing them successively with 5 mL methanol and 5 mL methanol/water (70/30) (v/v), whilst maintaining a flow rate of 1 mL/min. After loading, the cartridges were washed with 5 mL methanol/water (70/30) (v/v) and 3 mL methanol. This was followed by elution with 10 mL methanol/acetic acid (1/99) (v/v) at a flow rate of 1 mL/min and the eluate collected in vials. Eluates were evaporated to dryness in vials on a Reacti-Therm Heating module with a Reacti-Vap Evaporator (Pierce, Rockford, Illinois) at ± 50 ºC under nitrogen gas. The collection vials were washed with methanol and evaporated to dryness. The dry residues were stored at 4 ºC until analyzed.

Fumonisin analyses. The fumonisin analyses were performed at the PROMEC Unit, Medical Research Council, Tygerberg, South Africa, utilizing high performance liquid chromatography (HPLC) on a 150 x 4.6 mm Ultracarb 5 ODS (20) column (Phenomenex) with *o*-phthaldialdehyde (OPA) pre-column derivatization and fluorescence detection with a model 474 scanning fluorescence detector (Waters Corp., Milford) at 335 nm (excitation) and 440 nm (emission). Fumonisin standards were purified as described previously by Cawood et al. (*35*). OPA (225 µL) was added to 25 µL of the combined standard (FB_1, FB_2 and FB_3) and 10 µL injected, whilst 150 µL OPA was added to 100 µL sample (redissolved in 200 µL methanol) and 50 µL was injected. The mobile phase was methanol: 0.1 M sodium dihydrogen phosphate (NaH_2PO_4) (77/23) adjusted to pH 3.35 with *ortho*-phosphoric acid and run at a flow rate of 1 mL/min.

RESULTS AND DISCUSSION

The incidence of fungi isolated from each cultivar obtained in South Africa was higher in the untreated seeds than in the surface disinfected seeds (**Table 1**). In both the untreated and the surface

disinfected seeds the highest infection was reported in the Iron Grey and Rhino cultivars. *Aspergillus* and *Phoma* species were present in all the cultivars and in both surface disinfected and untreated seeds. *Aspergillus glaucus* Link ex Gray was the most abundant *Aspergillus* species, occurring in three of the cultivars, followed by *A. flavus* Link ex. Fries and *A. niger* van Tieghem. Hitokoko et al. (*7*) reported *A. glaucus*, *Penicillium* and *Alternaria* species to be present in cowpea seed. Esuruoso (*6*) observed various fungi to be associated with 81 samples of cowpea seed in western Nigeria. These included *A. flavus*, *A. niger*, *A. ochraceus* Wilhelm, *Penicillium digitatum* Sacc., *Rhizopus arrhizus* Fischer, *Chaetomium*, *Cladosporium*, *Curvularia* and *Macrophomina* species. In the present study *Chaetomium* and *Cladosporium* species were isolated from two and three samples, respectively. Other fungal genera isolated from these samples included *Penicillium* and *Trichothecium* species. The most abundant fungi from cowpea seeds from India were *F. verticillioides*, *F. oxysporum* Schecht ex. Fries, *Colletotrichum gleosporiodes* Penz. and Sacc., *A. niger* and *Penicillium* sp. (*36*). Similarly, Ushamalini et al. (*1*) reported that *Macrophomina phaseolina* (Tassi.) Goid., *F. oxysporum, Alternaria alternata* (Fr.:Fr.) Keissler, *A. flavus, A. niger* and *Penicillium* sp. were isolated from seeds of different districts in Tamil Nadu, India. Cowpea samples analyzed by Seenappa et al. (*8*) were invariably infected by *Aspergillus* and subsequently contaminated by aflatoxin. In a study by El-Kady et al. (*17*) two of three cowpea seed samples artificially infected by *A. flavus* produced aflatoxins.

In the present study six *Fusarium* species were isolated, of which *F. equiseti* (Corda) Sacc. was the most abundant. Of these *Fusarium* species four were present in the Rhino, two in the Bechwana White and one in the Glenda cultivar. *Fusarium* species producing high concentrations of mycotoxins other than fumonisins, which include *F. equiseti, F. sambucinum* Fuckel and *F. subglutinans* (Wollenw. and Reink.) Nelson, Toussoun, and Marasas (*37*), were isolated.

FB_1 was detected in all four samples of the South African cultivars, while FB_2 and FB_3 were not detected. The Rhino cultivar had the highest average concentration of FB_1 (0.61 µg/g) followed by Glenda, Bechwana White and Iron Grey with low levels of 0.16, 0.13 and 0.12 µg/g, respectively. Even though the most important fumonisin-producing species are *F. verticillioides* and *F. proliferatum*, neither of these two species was isolated from the South African cowpea seed samples. No fumonisins, however, were detected in the Benin seed samples, which could be attributed to conditions being unfavorable for fumonisin production in these samples. Tseng et al. (*12*) detected FB_1 levels of 0.5 µg/g and 1.1 µg/g in naturally infected mouldy navy beans from Ontario, Canada. *Fusarium* species isolated from these beans included *F. avenaceum* (Fr.) Sacc., *F. culmorum* (W.G. Smith) Sacc., *F. graminearum* Schwabe, *F. verticillioides, F. oxysporum*, and *F. solani* (Mart.) Appel and Wollenw. emend. Snyd. and Hans. However, the *Fusarium* species responsible for FB_1 production was not

identified. Furthermore, Tseng & Tu (*13*) investigated the presence of FB_1 in adzuki and mung beans from Ontario, Canada. It was found that the adzuki and mung bean samples contained average concentrations of 261 and 230 µg/g of FB_1, respectively. Identified *Fusarium* species isolated from mouldy beans included *F. avenaceum, F. culmorum, F. equiseti, F. graminearum, F. verticillioides, F. oxysporum, F. solani* and *F. sporotrichioides* Sherb. In an attempt to identify the *Fusarium* sp. responsible for FB_1 production, the beans were inoculated with *F. graminearum*, and analyzed for FB_1 and FB_2 and the results proved to be negative. The authors suggested that FB_1 found in diseased adzuki and mung beans was due to *F. verticillioides* infection (*13*).

The incidence of fungal infection of the cowpea seed of the three Benin market samples is presented in **Table 2**. The fungi isolated from these seeds differ quite substantially from those isolated from the South African cultivars. The highest percentage infection of the cowpea seeds was found in Kpodjiguégué, followed by Tawa and then Gbehami. *A. flavus* was detected in the Tawa and Gbehami samples and a large percentage of *Lasiodiplodia theobromae* (Patouillard) Griffon et Maublanc was detected in the Kpodjiguégué sample. Other fungal genera detected included *Curvularia, Penicillium* and *Mucor* species. The total percentage of *Fusarium* isolates was relatively low and included *F. equiseti* (2.5%), *F. semitectum* Berkeley & Ravenel (1.5%), *F. subglutinans* (0.5%) and *F. proliferatum* (2%). *F. equiseti*, *F. semitectum* and *F. subglutinans* were also isolated from the South African cowpea seed samples in this study as well as cowpeas from Nigeria and India in other studies (*6, 36*). However, in contrast to previous studies on cowpea seeds (*6, 36, 38*) *F. oxysporum, F. solani* and *F. verticillioides* were not detected in these samples. Esuruoso (*6*) recorded *F. verticillioides* infection on most of the 81 cowpea seed samples analyzed. In this study, four *F. proliferatum* isolates were detected (two each from Kpodjiguégué and Gbehami). *F. proliferatum* is a primary producer of fumonisins (*39*), and therefore these four were grown on maize patty medium in duplicate and analyzed for fumonisin production.

The data shown in **Table 3** represents the mean concentration of fumonisin production by the *F. proliferatum* isolates from the Benin cowpea samples. The highest concentration of FB_1 was produced by Gbehami Isolate 2 with a mean of 16.86 µg/g for the two replicates. In previous studies *F. proliferatum* isolated from various other cereals produced higher fumonisin levels than the current isolates (*18, 39*). Thiel et al. (*18*) found that *F. proliferatum* isolates from sorghum and maize produced 20 - 660 µg FB_1/g and 65 - 450 µg FB_2/g, respectively. *Fusarium proliferatum* maize cultures produced 1 670 - 2 790 µg FB_1/g and 150 - 320 µg FB_2/g, respectively (*39*). As far as the authors are aware this is the first report of the natural occurrence of FB_1 in cowpea seeds and this is also the first study that has shown that *F. proliferatum* isolates from cowpea seed has the potential to produce fumonisin

mycotoxins. *F. verticillioides*, a major fumonisin producing fungus, has been isolated from cowpea seed (*6, 36*). Therefore, studies are needed to confirm whether *F. verticillioides* is associated with cowpea seed and whether it produces fumonisins in cowpea seed. Although the fumonisin levels shown in this study are relatively low, further screening for fumonisins in cowpea seeds intended for human consumption and animal feed is warranted.

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Table 1. Fungi isolated from four cultivars of cowpea seeds obtained in South Africa

Fungus	Incidence (%)							
	Cowpea cultivars							
	Glenda		Bechwana White		Rhino		Iron Grey	
	SD[a]	UT[b]	SD	UT	SD	UT	SD	UT
Aspergillus flavus	4	10	-	26	-	-	-	2
A. glaucus	-	4	-	-	8	8	40	68
A. niger	-	18	-	14	-	-	4	2
Chaetomium sp.	2	2	-	-	-	-	2	2
Cladosporium sp.	-	18	-	14	-	-	2	-
Diplodia sp.	-	4	-	-	-	-	-	-
Fusarium chlamydosporum	-	-	-	2	-	-	-	-
F. equiseti	-	2	-	-	2	10	-	-
F. graminearum	-	-	-	-	-	2	-	-
F. sambucinum	-	-	-	-	-	2	-	-
F. scirpi	-	-	-	-	6	-	-	-
F. subglutinans	-	-	-	2	-	-	-	-
Penicillium sp.	-	4	-	-	-	32	-	16
Phoma sp.	2	14	4	28	52	36	2	-
Trichothecium roseum	-	2	-	2	-	-	-	2
Other	-	10	-	4	-	4	2	6
Total fungi	8	88	4	92	68	94	52	98

[a] surface disinfected seeds

[b] untreated seeds

Table 2. Fungi isolated from cowpea seeds obtained from three localities in Benin

Fungus	Incidence (%)		
	Cowpea samples		
	Kpodjiguégué	Tawa	Gbehami
Aspergillus flavus	-	1.5	5.5
Chaetomium sp.	-	-	1.5
Curvularia sp.	0.5	4	0.5
Fusarium equiseti	1	-	1.5
F. proliferatum	1	-	1
F. semitectum	1	-	0.5
F. subglutinans	-	0.5	-
Lasiodiplodia theobromae	34	3	-
Mucor sp.	-	5	2.5
Penicillium chrysogenum	1	0.5	-
Other	32	17.5	2
Total fungi	70.5	32	15

Table 3. Fumonisin production by *Fusarium proliferatum* isolates grown on maize patty medium

Isolates	Fumonisin concentration (µg/g)			
	FB_1	FB_2	FB_3	Total Fumonisins
Kpodjiguégué Isolate 1	9.33 ± 5.26[a]	1.54 ± 0.52	0.76 ± 0.40	11.62 ± 6.18
Gbehami Isolate 1	0.67 ± 0.55	0.11 ± 0.09	0.03 ± 0.04	0.80 ± 0.68
Kpodjiguégué Isolate 2	2.93 ± 0.45	0.65 ± 0.04	0.20 ± 0.02	3.77 ± 0.52
Gbehami Isolate 2	16.86 ± 3.97	6.61 ± 2.28	1.83 ± 0.16	25.30 ± 6.09

[a] mean ± standard deviation of two replicates

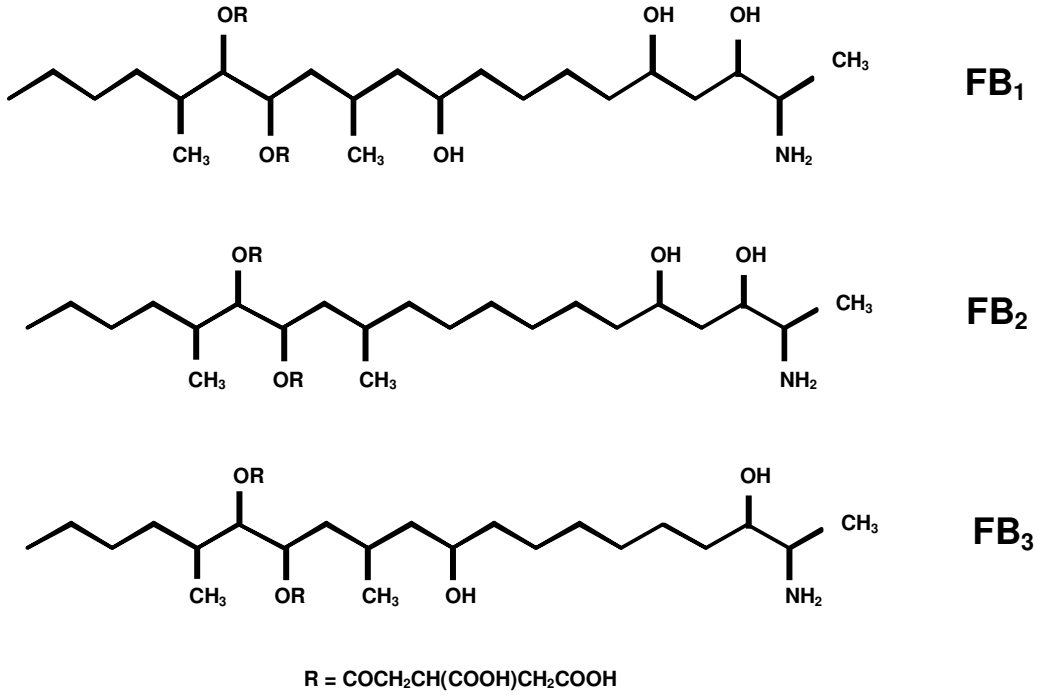

Figure 1. Structures of fumonisin B_1, B_2 and B_3