Production of the Mycotoxins Fusaproliferin and Beauvericin by South African Isolates in the *Fusarium* Section *Liseola*

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The production of fusaproliferin (FUS), a recently described mycotoxin, and beauvericin (BEA), a mycotoxin recently reported to co-occur with FUS in *Fusarium*-infected corn, by South African isolates in the *Fusarium* section *Liseola*, was investigated. Five isolates each of *F. verticillioides*, *F. proliferatum*, *F. subglutinans*, and *F. globosum* were cultured on corn kernels. Four each of the five South African isolates of *F. proliferatum* and *F. subglutinans* produced FUS (10−1725 and 330−2630 mg/kg, respectively). BEA was produced by four of the *F. proliferatum* strains (310−1130 mg/kg) and three of the *F. subglutinans* strains (140−700 mg/kg). The isolates of *F. verticillioides* failed to produce significant levels of either of these secondary metabolites. *F. globosum* was a weak producer of both in that one isolate of five produced 25 mg/kg FUS and five out of five produced BEA at levels ranging between 10 and 110 mg/kg. To further characterize these strains, their production of fumonisins B_1, B_2, and B_3, as well as moniliformin, was investigated. Of the four species investigated, fumonisins were produced by all except *F. subglutinans*, which in turn was the only species whose isolates in this study produced moniliformin (four of five isolates, ranging from 155 to 2095 mg/kg). Analysis of visibly *Fusarium*-infected home-grown corn collected in the Transkei region of the Eastern Cape Province of South Africa showed that nine of the ten samples contained low levels of FUS (up to 62 μg/kg), whereas all ten samples showed BEA contamination ranging from 8 to 1734 μg/kg with a mean of 258 μg/kg.

Keywords: *Fusaproliferin; beauvericin; Fusarium; fumonisins; moniliformin; mycotoxins*

INTRODUCTION

The *Fusarium* section *Liseola* contains several toxigenic fungal pathogens that infect corn around the world and that have been implicated in various human and animal mycotoxicoses (Marasas et al., 1984). *Fusarium verticillioides* (Sacc.) Nirenberg (syn. = *F. moniliforme* Sheldon), one of the most prevalent fungi associated with corn (*Zea mays* L.), has been shown to cause nephrosis and hepatosis in sheep and rats and to cause congestive heart failure or hepatic cirrhosis in baboons (Kriek et al., 1981). It is a significant producer of the fumonisin mycotoxins, which are the causative agents of equine leukoencephalomalacia (Kellerman et al., 1990), porcine pulmonary edema (Harrison et al., 1990), and hepatocarcinoma in rats (Gelderblom et al., 1991). In addition, *F. verticillioides* has been implicated in the etiology of human esophageal cancer as a result of its infestation of home-grown corn harvested in the high esophageal cancer prevalence areas of the Transkei region of South Africa (Rheeder et al., 1992). *F. proliferatum* (Matsushima) Nirenberg is an important pathogen of corn in certain areas of the world and is also a significant producer of the fumonisins (Nelson et al., 1992). *F. subglutinans* (Wollenweber & Reinking) Nelson, Toussoun & Marasas occurs on a wide range of hosts around the world, including corn, although it seems to have a lower optimum temperature for growth and hence predominates in relatively cooler areas (Marasas et al., 1984). It has been well characterized as a producer of the toxin moniliformin (MON) (Marasas et al., 1986) but does not appear to be a significant producer of fumonisins in that, of 24 strains previously investigated, none showed significant fumonisin production (Thiel et al., 1991; Nelson et al., 1992). MON is also produced by *F. proliferatum* (Marasas et al., 1986; Logrieco et al., 1995), whereas its reported production by "*F. moniliforme*" has not been clear-cut (Marasas, 1985, 1986; Leslie et al., 1996). Recent work on the taxonomy in the section *Liseola* has utilized mating tests of the sexual stage to distinguish species. This resulted in the identification of various genetically distinct mating populations of the teleomorph *Gibberella fujikuroi* (Sawada) Wollenw. in which mating population A, primarily isolated from corn and producing high levels of fumonisins and low levels or no MON, was identified as *F. verticillioides* (Leslie et al., 1996). In contrast, mating population F, previously also referred to as *F. moniliforme* but now described as *F. thapsinum* Klittich, Leslie, Nelson & Marasas, was primarily isolated from sorghum and produced low levels of fumonisins and relatively high levels of MON. Within this classification, *F. proliferatum* corresponds to the D mating population and *F. subglutinans* isolated predominantly from corn corresponds to the E mating

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population. *F. globosum* Rheeder, Marasas & Nelson is a recently described *Fusarium* species that has been classified in the section *Liseola* and was originally isolated from corn harvested in the Transkei region of South Africa (Rheeder et al., 1996). Of 17 strains investigated, all produced fumonisins but none produced MON when grown in culture on corn kernels (Sydenham et al., 1997).

Fusaproliferin (FUS) is a recently identified sesterterpene purified from corn cultures of *F. proliferatum* ITEM-1494 (isolated from corn ear rot in northern Italy) using toxicity to brine shrimp larvae (*Artemia salina* L.) as a guide to extract fractionation (Ritieni et al., 1995; Santini et al., 1996). Besides toxicity to brine shrimp (LD_{50} 53.4 μM), it is cytotoxic to the lepidopteran cell line SF-9 and the human nonneoplastic B-lymphocyte cell line IARC/LCL 171 (Logrieco et al., 1996) and produces teratogenic effects in chicken embryos (Ritieni et al., 1997a). FUS was produced by 71 of 72 strains of *F. subglutinans* at levels of 30−1500 mg/kg (Logrieco et al., 1996). Studies on strains of *G. fujikuroi* subdivided into six distinct mating populations (A−F) showed FUS to be produced only by members of the D (*F. proliferatum*) and E (*F. subglutinans*) mating populations at levels of up to 1500 mg/kg (Moretti et al., 1996). Only limited data exist on the natural occurrence of FUS in maize. It has been detected in nine naturally contaminated samples of visibly moldy (mostly *F. proliferatum*) corn ears in Italy at levels as high as 500 mg/kg (Ritieni et al., 1997b). Four *Fusarium*-infected corn and animal feed samples from Iowa in the U.S. have been found to be naturally contaminated with FUS at levels of 0.1−30 mg/kg. Strains of *F. proliferatum* and *F. subglutinans* isolated from these samples produced FUS in culture on corn at levels up to 350 and 1000 mg/kg, respectively (Munkvold et al., 1998).

Beauvericin (BEA) is a cyclic hexadepsipeptide originally reported to be produced by some entomopathogenic fungi such as *Beauveria bassiana* (Balsamo) Vuill. (Hamill et al., 1969). It consists of an alternating sequence of three D-α-hydroxyisovaleryl and three *N*-methyl-L-phenylalanyl residues. It acts as an ionophore and increases the permeability of membranes for alkali cations (Steinrauf, 1985). BEA is an inhibitor of cholesterol acyltransferase (Tomoda et al., 1992) and is highly toxic to insects (Grove and Pople, 1980) and to mammalian cell lines (Macchia et al., 1995). The production of BEA by two species of *Fusarium*, viz., *F. semitectum* Berk. & Rav. and *F. subglutinans*, was reported by Gupta et al. (1991). BEA is produced by several strains of the common corn pathogens *F. subglutinans* and *F. proliferatum* (Logrieco et al., 1993; Moretti et al., 1994; Plattner and Nelson, 1994; Bottalico et al., 1995). Besides BEA, isolates of *F. proliferatum* have been shown to simultaneously produce fumonisin B_1 and FUS (Bottalico et al., 1995; Plattner and Nelson, 1994; Munkvold et al., 1998), and the coproduction of BEA and MON by isolates of *F. subglutinans* has also been reported (Logrieco et al., 1993). More recent results on the production of BEA by the various mating populations (A−F) of *G. fujikuroi* showed it to be produced by all groups except mating population F (*F. thapsinum*), although in mating population A (*F. verticillioides*) only 2 of 29 strains produced trace levels (5 mg/kg) (Moretti et al., 1996). Recent work has indicated that the ability to produce BEA is common in a number of other *Fusarium* species, such as the wheat pathogen *F. poae* (Peck) Wollenw. and *F. sambucinum* Fuckel, which causes storage rot of potatoes (Logrieco et al., 1998). The natural occurrence of BEA was first reported at levels as high as 6 mg/kg in preharvest *F. subglutinans*-infected corn ears collected in Poland (Logrieco et al., 1993). Subsequently, BEA has also been detected in naturally contaminated corn in Italy at levels up to 520 mg/kg in visibly moldy corn ears (Bottalico et al., 1995; Ritieni et al., 1997b), whereas *Fusarium*-infected corn and feed samples submitted for mycotoxin analysis in Iowa had levels up to 3 mg/kg (Munkvold et al., 1998).

We have applied a recently developed liquid chromatography−mass spectrometry (LC−MS) analytical method for the simultaneous determination of FUS and BEA to investigate their production by South African strains of *F. subglutinans*, *F. proliferatum*, *F. verticillioides*, and *F. globosum* in culture on corn kernels. Together with determination of MON and fumonisins, these results further aid in clarifying the toxigenic differences within the *Fusarium* section *Liseola*. Ten moldy home-grown corn samples recently collected in the Transkei region of the Eastern Cape Province of South Africa were analyzed to establish the natural occurrence of FUS and BEA toxins in South Africa.

MATERIALS AND METHODS

CAUTION. The fumonisins are known carcinogens. Consequently fungal cultures and solvent extracts should be handled with care.

Reagents. BEA was purchased from Sigma (St. Louis, MO). FUS was obtained from Dr. A. Ritieni, (University of Naples "Federico II", Italy). Fumonisins B_1, B_2, and B_3 (FB_1, FB_2, and FB_3) and MON were isolated and purified at PROMEC, Medical Research Council (MRC), Tygerberg, South Africa, according to the methods of Cawood et al. (1991) and Steyn et al. (1978), respectively. All other reagents and solvents were analytical grade from Merck (Darmstadt, Germany).

***Fusarium* Isolates and Culture Material.** The *Fusarium* strains used in this study are listed in Table 1 and were obtained from the culture collection of PROMEC. The isolates of *F. verticillioides* and *F. subglutinans* were obtained from home-grown corn, and those of *F. globosum* were from naturally infected field trial corn in the Transkei region of the Eastern Cape Province, South Africa. The isolates of *F. proliferatum* were obtained from asparagus grown in South Africa. Lyophilized conidia of each strain were suspended in sterile water and used to inoculate moistened yellow corn kernels (400 g of kernels and 400 mL water) in 2-L glass fruit jars previously autoclaved at 121 °C for 1 h on each of two consecutive days. Cultures were incubated in the dark at 25 °C for 21 days, dried overnight at 45 °C, ground to a fine meal in a laboratory mill, and stored at 4 °C until analyzed for FUS, BEA, MON, FB_1, FB_2, and FB_3. A sample of the corn used as culture medium was also ground to a meal and analyzed for the presence of these toxins.

Naturally Contaminated Corn. Samples of visibly *Fusarium*-infected home-grown corn were hand selected from storage cribs at households in the Centane district of the Transkei region of the Eastern Cape Province, South Africa, during August, 1997.

Chemical Analyses. FUS and BEA were determined in culture material and naturally contaminated corn using a newly developed LC−MS method based on reversed-phase HPLC separation of the toxins with subsequent electrospray ionization (ESI) MS detection of selected toxin ions (Sewram et al., 1999). Briefly, the toxins were extracted from the fungal cultures and naturally contaminated corn samples (20 g) by homogenization with methanol (100 mL). An aliquot of each extract (5 mL for fungal culture, 30 mL for naturally contaminated) was evaporated to dryness at 55 °C. Prior to analysis, the residue was redissolved in HPLC mobile phase (water/

Table 1. Production of the Mycotoxins Fusaproliferin, Beauvericin, Moniliformin, and Fumonisins B_1, B_2, and B_3 by South African Isolates of *Fusarium* Species in the Section *Liseola*

species	strain (MRC[a] no.)	mycotoxin concentration (mg/kg)					
		fusaproliferin	beauvericin	moniliformin	fumonisin B_1	fumonisin B_2	fumonisin B_3
F. verticillioides	826	nd[b]	nd	nd	1520	480	230
	1069	nd	nd	nd	295	35	40
	4315	nd	nd	nd	1880	395	195
	4319	nd	nd	nd	3285	800	555
	4321	tr[c]	nd	nd	1605	270	335
F. proliferatum	7137	10	310	nd	395	70	45
	7138	940	1130	nd	1010	275	105
	7139	1725	575	nd	1210	355	70
	7140	895	455	nd	1035	370	75
	7141	nd	nd	nd	155	15	10
F. subglutinans	115	1250	tr	2095	nd	nd	nd
	1077	2630	370	180	tr	nd	nd
	1084	330	700	155	tr	nd	nd
	1093	540	140	205	tr	nd	nd
	1097	nd	nd	nd	nd	nd	nd
F. globosum	6646	nd	110	nd	170	tr	10
	6647	nd	75	nd	200	tr	10
	6648	25	60	nd	185	tr	10
	6651	nd	100	nd	225	tr	15
	6655	nd	10	nd	170	tr	10
control corn		nd	nd	nd	nd	nd	nd

[a] MRC: Medical Research Council, Tygerberg, South Africa Culture Collection. [b] Nd: not detected (<2 mg/kg). [c] Tr: trace (<5 mg/kg).

acetonitrile, 34:66, containing 0.1% formic acid). FUS was identified and quantified in the MS−MS mode through collision-induced dissociation of its dehydrated protonated molecular ion (*m*/*z* 427) in which the product ions at *m*/*z* 367 and *m*/*z* 349 were monitored. BEA was identified and quantified by MS detection of its protonated molecular ion (*m*/*z* 784).

FB_1, FB_2, and FB_3 were determined according to the method of Sydenham et al. (1996). Briefly, a portion of each fungal culture was extracted with methanol/water and cleaned up on strong anion exchange (SAX) solid-phase extraction cartridges, prior to HPLC separation and fluorescence detection of preformed *o*-phthaldialdehyde (OPA) derivatives. MON levels were determined by the method of Scott and Lawrence (1987). Cultures were extracted with acetonitrile/water, defatted with *n*-hexane, and partitioned on reversed-phase (C_{18}) silica cartridges. The purified extracts were separated by ion-pair HPLC. Quantitation was performed by UV detection at 229 nm, and UV spectra, collected by diode-array detection between 200 and 350 nm, were used for confirmatory purposes.

RESULTS AND DISCUSSION

Results of the determination of FUS and BEA in cultures of 20 *Fusarium* isolates, together with those of MON and fumonisins are summarized in Table 1. Four of each of the five *F. proliferatum* and *F. subglutinans* strains were producers of FUS. Those strains producing FUS were also coproducers of BEA, except for *F. subglutinans* MRC 115, which contained only trace (<5 mg/kg) amounts of BEA. The presence of a low level (<5 mg/kg) of mycotoxin contamination in corn cultures of fungal strains makes the unequivocal assignment of toxin-producing ability difficult because of the possible natural distribution of background levels of toxin contamination in the corn used for the preparation of the cultures. The analysis of the corn used as culture medium for the *Fusarium* strains in this study, however, showed natural contamination by each of the six toxins to be below 2 mg/kg. The isolates of *F. verticillioides* examined in this study produced neither FUS nor BEA. The newly described species *F. globosum* was a weak producer of FUS and BEA in that only one of the five isolates (MRC 6648) produced FUS at a level of 25 mg/kg; all strains investigated produced BEA at levels between 10 and 110 mg/kg.

The production of FUS at a level of 1250 mg/kg by MRC 115 observed in this study agrees well with a previous report that *F. subglutinans* MRC 115 cultured on corn kernels produced FUS at levels between 1100 and 1300 mg/kg (Logrieco et al., 1996). This study reports some of the highest levels of production yet reported for FUS in that *F. proliferatum* MRC 7139 produced 1725 mg/kg and *F. subglutinans* MRC 1077 produced at a level of 2630 mg/kg. Previous studies have reported maximum production of FUS at levels of 1500 mg/kg by *F. proliferatum* (Moretti et al., 1996) and *F. subglutinans* (Logrieco et al., 1996). The highest BEA producer reported to date is *F. oxysporum* Schlecht. emend. Snyd. & Hans. KF-1230 from the *Fusarium* section *Elegans* isolated from a corn stalk in Poland with production at the level of 3200 mg/kg (Logrieco et al., 1998). Within the section *Liseola*, *F. proliferatum* has been reported to produce at levels of up to 1100 mg/kg (Plattner and Nelson, 1994), *F. subglutinans* produced at levels of up to 300 mg/kg (Logrieco et al., 1998), and *F. anthophilum* (A. Braun) Wollenw. KF-391 (NRRL 13286) isolated from sugarcane in India produced BEA at 1300 mg/kg (Logrieco et al., 1998). Clearly, within their respective species, *F. proliferatum* MRC 7138 (BEA production of 1130 mg/kg) and *F. subglutinans* MRC 1084 (BEA production of 700 mg/kg) are significant producers of BEA.

Analysis of these fungal cultures for MON and fumonisins confirmed that isolates of *F. proliferatum*, besides producing FUS and BEA, were all good producers of fumonisins (FB_1, FB_2, and FB_3). The isolates analyzed in this study did not produce MON. However, previous studies on *F. proliferatum* isolates from corn have shown MON production in 12 of 26 isolates (Logrieco et al., 1995), and a further eight isolates from a variety of sources all produced MON (Marasas et al., 1986). Conversely, the strains of *F. subglutinans* which were also producers of FUS and BEA produced MON but not fumonisins. In contrast, all isolates of *F. verticillioides* were good producers of fumonisins (FB_1, FB_2, and FB_3) but not of the other mycotoxins. This study on South African isolates in the section *Liseola* reveals an overall pattern in the production of secondary metabolites during fungal culture. Previous studies on isolates from Europe and the U.S. have noted a similar pattern of toxin production (Moretti et al., 1996). *F. globosum* is a

Table 2. Fusaproliferin and Beauvericin Levels in 10 Visibly *Fusarium*-Infected Home-Grown Corn Samples from Transkei Region, South Africa

sample designation	fusaproliferin (μg/kg)	beauvericin (μg/kg)
D2B	60	11
D4B	9	8
D6B	62	49
D8B	nd[a]	8
D10B	16	15
F1B	7	1734
F3B	17	193
F4B	33	172
F6B	40	239
F10B	50	151
mean ± s.d.	33[b] ± 22	258 ± 526

[a] Nd: not detected (<1 μg/kg). [b] Mean of positive samples.

recently described species, and its profile of toxin production has previously been restricted to a report of fumonisin but not MON production (Sydenham et al., 1997). This study confirms these previous results as to its production of fumonisins at relatively low levels in comparison to that of *F. verticillioides* and *F. proliferatum* and shows further for the first time that *F. globosum* is also a weak producer of FUS and BEA. As noted by Sydenham et al. (1997), *F. globosum* produces higher levels of FB_3 than FB_2 in that only trace amounts (<5 mg/kg) of the latter were detected in both studies.

Subsequent studies on the possible natural occurrence of FUS and BEA were performed on naturally contaminated home-grown corn from the Transkei region, as both *F. subglutinans* and *F. globosum* are fungal contaminants of Transkeian corn. Table 2 shows the levels of FUS and BEA determined in visibly *Fusarium*-infected home-grown corn in the Transkei region and confirms the natural presence of these toxins in moldy corn from this region. Although the levels of FUS were all relatively low (maximum 62 μg/kg), BEA occurred in all samples, being as high as 1734 μg/kg in one sample. Nevertheless, these levels of FUS and BEA are far lower than those reported to occur in preharvest corn ears with *Fusarium* rot collected in Italy (maxima of 500 and 520 mg/kg, respectively) (Ritieni et al., 1997b) or in corn and animal feed from Iowa (maxima of 30 and 3.0 mg/kg, respectively) (Munkvold et al., 1998). Previously, levels of BEA up to 60 mg/kg were reported in *F. subglutinans*-infected corn ears in Poland (Logrieco et al., 1993), and BEA and FUS levels up to 3 and 8 mg/kg, respectively, have been detected in samples of preharvest corn ear rot in Slovakia (Moretti et al., 1998).

Studies on moldy corn samples collected on various occasions have shown Transkeian corn to contain a wide range of *Fusarium* toxins. Thiel et al. (1982) reported the co-occurrence of MON with deoxynivalenol and zearalenone, mycotoxins produced by *F. graminearum* Schwabe, in a sample of moldy corn ears from the Transkei region, and later analysis of the same sample showed the presence of the mutagenic mycotoxin fusarin C (Gelderblom et al., 1984). Analysis of further samples of moldy corn collected in both the high and low esophageal cancer incidence areas of the Transkei region again showed the co-occurrence of MON, deoxynivalenol, and zearalenone, as well as of nivalenol and fumonisins (FB_1 and FB_2) (Sydenham et al., 1990). The presence of the fumonisins but not the other *Fusarium* toxins correlated with the incidence of esophageal cancer in the Transkei (Sydenham et al., 1990; Rheeder et al., 1992). Moldy corn in the Transkei, which is separated from visibly "healthy" corn by the inhabitants themselves, is generally used for the preparation of home-brewed beer but could be consumed as part of the staple corn-based diet of this region once the visibly healthy corn is no longer available (Rheeder et al., 1992). This present study has now shown that moldy home-grown Transkeian corn also contains the mycotoxins FUS and BEA and raises further concerns over the health implications of this fungal and mycotoxin contamination. Consumption of this moldy corn, co-contaminated with several *Fusarium* toxins whose presence is potentially detrimental to human health, may be an important vehicle of mycotoxin exposure in Africa.

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