Natural Occurrence of Beauvericin in Preharvest *Fusarium subglutinans* Infected Corn Ears in Poland†

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Fourteen samples of preharvest infected corn ears, collected in 1990–1991 from different Polish cornfields, were found to be highly infected by *Fusarium subglutinans*. This species was recently shown to produce in culture beauvericin (BEA), a toxin mostly known for its insecticidal properties. Chemical analysis by high-performance liquid chromatography and high-performance thin-layer chromatography revealed the occurrence in the naturally infected corn samples of BEA (up to 60 mg/kg). When cultured on autoclaved corn kernels for 4 weeks at 25 °C 8 of the 10 assayed strains of *F. subglutinans* produced BEA (up to 200 mg/kg), whereas 9 strains also produced moniliformin (up to 500 mg/kg). Cultural extracts containing the highest amounts of BEA proved to be toxic in *Artemia salina* bioassay. The natural occurrence of BEA in corn is reported here for the first time.

Fusarium subglutinans (Wollenw. & Reinking) Nelson, Tousson & Marasas [*Gibberella subglutinans* (Edwards) Nelson, Tousson & Marasas], a member of the *Liseola* Wollenw. section, is considered one of the species infecting corn, causing stalk and ear rot, mostly in temperate regions (Booth, 1971; Marasas et al., 1979; Burgess et al., 1981; Maric, 1981).

In recent years this disease has acquired increased importance also from the mycotoxicological point of view, due to the formation of several mycotoxins in infected corn. *F. subglutinans*, together with *Fusarium moniliforme* Sheldon, has been associated with human esophageal cancer in Transkei, southern Africa (Marasas et al., 1981), and various strains of the fungus are known to be highly toxic in animals (Kriek et al., 1977; Rabie et al., 1982; Abbas et al., 1984; Marasas et al., 1984). Moniliformin is the major toxin produced by this species, although not all isolates are able to produce it (Rabie et al., 1982; Marasas et al., 1984; Chelkowski et al., 1990), and no clear evidence exists of its direct involvement in mycotoxicoses (Marasas et al., 1984). Moniliformin has already been found as a natural contaminant of corn, and its occurrence was correlated with heavy tissue contamination by *F. subglutinans* (Thiel et al., 1982). Recently, beauvericin (BEA), a cyclodepsipeptide toxin (Figure 1), has been produced *in vitro* by strains of *F. subglutinans* isolated from insects (Gupta et al., 1991) and from corn (Logrieco et al., 1993). Although the toxicity of beauvericin has not been sufficiently investigated, there are reports on its cytotoxic (Vey et al., 1973) and insecticidal properties (Grove and Pople, 1980).

In Poland, *F. subglutinans* has been reported for many years as the dominating species affecting corn ears (Chelkowski, 1989), and moniliformin (M) was commonly found as a contaminant in corn kernels infected by this species (Sharman et al., 1991). The purpose of this study was to investigate the presence of BEA in preharvest corn samples collected in Poland in 2 years and mainly infected by *F. subglutinans*. In addition, tests were conducted to assess the capability of the isolates to synthesize *in vitro* BEA and M, as well as the toxicity of the cultural extract toward *Artemia salina* bioassay.

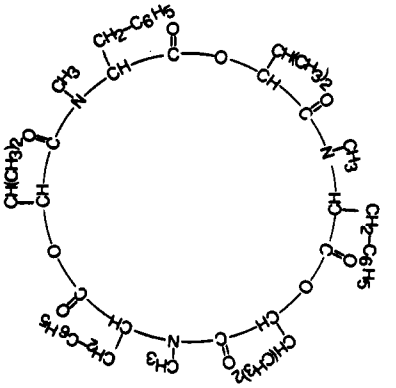

Figure 1. Chemical structure of beauvericin.

MATERIALS AND METHODS

Source of Samples. During the 1990–1991 harvest season, 14 samples of corn ears visibly damaged by *Fusarium* were collected at random from cornfields around Warsaw, Poland. One hundred kernels for each sample were surface sterilized for 1 min in 3% NaOCl, rinsed twice in sterile water, placed on plates (five kernels per plate) containing a modified pentachloronitrobenzene medium selective for *Fusarium* (Nash and Snyder, 1962; Nelson et al., 1983), and incubated in the dark at 25 °C for 1 week. The developed *Fusarium* colonies were transferred to a potato sucrose agar (PSA) plate and incubated at 25 °C for 10–14 days under fluorescent and black light lamps (12-h photoperiod). Single-conidium cultures were maintained on PSA and carnation leaf agar (CLA) (Nelson et al., 1983) under the conditions described above and identified according to the classification system of Nelson et al. (1983). To preserve the cultures for fermentation studies, mycelia and conidia from wild strains grown on CLA were transferred aseptically in 1 mL of sterile 18% glycerol and frozen at −75 °C. The isolates were

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deposited, with accessioned numbers (ITEM), at the collection of the Istituto Tossine e Micotossine da Parassiti Vegetali, Bari, Italy.

Fermentation Conditions. Ten single-conidium isolates belonging to *F. subglutinans* were cultured on corn. In particular, 50 g of yellow corn kernels var. Plata, brought overnight to about 45% moisture in 250-mL Erlenmeyer flasks, were autoclaved for 30 min at 120 °C and inoculated with 2 mL of water suspension containing approximately 10^7 conidia/mL. The cultures were incubated at 25 °C in the dark for 4 weeks. The harvested culture material was dried in a forced drauft oven at 60 °C for 48 h, finely ground, and stored at 4 °C until use. Control cornmeal was produced in the same way, except that it was not inoculated.

Toxin Extraction. A 20-g sample of each corn sample (14 infected corn ear samples and 12 inoculated corn cultures) was extracted in a blender with 100 ml of MeOH–1% aqueous NaCl (55:45) for 3 min and filtered through filter paper (Whatman No. 1), and 50 mL of the filtrate was transferred into a separatory funnel and defatted twice using 50 mL of *n*-hexane. The upper *n*-hexane layer was discarded and the methanol layer extracted with CH_2Cl_2 (3 × 30 mL). The CH_2Cl_2 extracts were collected, evaporated to dryness, dissolved in 1 mL of methanol, and then analyzed and bioassayed for BEA toxin. The extraction of M was done according to the methods previously described (Bottalico et al., 1989).

Chemical Analyses. Beauvericin was identified and quantitated by means of high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC). In the latter case, HPTLC precoated silica gel 60 F_{254} plates (10 × 20 cm, thickness 0.25 mm, E. Merck, Darmstadt) were spotted with 20 μL of MeOH extracts and with 0.5, 1, 3, and 5 μL of standard solution of BEA in methanol (1:1 μg/μL). The plates were developed [developing systems: toluene–acetone (75:25), chloroform–2-propanol (95:5), and ethyl acetate–hexane (80:20)] for about 8 cm, air-dried, and then observed under 365- and 254-nm UV light. The spots quenching fluorescence were marked, and the plates were colored with iodine vapors. Beauvericin appeared as a brownish spot which did not quench fluorescence. Its R_f value was about 0.5 in toluene–acetone and about 0.4 in both chloroform–2-propanol and ethyl acetate–hexane. The detection limit for BEA using this method was 3 mg/kg of corn culture. HPLC was carried out using a Knauer Model 64 pump (Berlin, Germany) using an Rp18 Bio-Rad RSC 5 μm, 250 × 4.6 mm column, and a Knauer UV detector, set at 225 nm using acetonitrile and water (85:15 v/v) as an eluent, with a flow rate of 1.3 mL/min under a pressure 5.5 MPa. The corn extract containing BEA was prepurified on a silica column eluted with $CHCl_3$/iPrOH (95:5 v/v); 20 μL, corresponding to an extract of 200 mg of corn, was analyzed under the conditions described above. Quantification by HPLC procedures was done by comparison of the peak height of BEA (retention time about 7 min) against a calibration curve of the peak height obtained with authentic standard. The detection limit in pure BEA samples was 1 mg/kg. Furthermore, there is a linear correlation between sample concentration and the areas of the peaks in the HPLC profile up to 520 μg/μL of BEA. The qualitative analysis of BEA was then confirmed by the co-injection of the extract with different BEA standards. To confirm BEA occurrence, sample M-1612 was analyzed by ^1H NMR spectra and by low-resolution electronic impact mass spectrometry (*m/z* 784).

The identification and quantitative analyses of M were done by TLC according to the methods previously described (Bottalico et al., 1989). Toxin reference standards were purchased from Sigma Chemical Co. (St. Louis, MO).

Bioassay. The toxicity of culture extracts was tested using brine shrimp (*A. salina* L.) according to the procedure of Bottalico et al. (1989).

RESULTS AND DISCUSSION

All 14 corn ear samples collected in 1990–1991 around Warsaw, Poland, proved to be highly infected by *F. subglutinans* (100% in all samples) and to a lesser extent by *Fusarium poae* (Peck) Wollenw. (present only in 6 samples up to 22% of infected kernels in sample M-1611). These data confirm the importance of *F. subglutinans* as a main causal agent of corn ear rot in Poland. In previous papers, cobs of maize with ear rot symptoms randomly collected at four localities in Poland in 1985–1990 were found to be mostly contaminated by *F. subglutinans*, while *F. poae* was isolated only in some years at a low percentage (Chelkowski, 1989; Chelkowski and Lew, 1992). These findings further support the evidence that *F. subglutinans* and *F. poae* are species distributed as causal agents of corn ear rot mostly in temperate geographical areas (Booth, 1971; Francis and Burgess, 1975; Marasas et al., 1979; Gilbertson et al., 1985). The complete absence of *F. moniliforme*, a species commonly associated with maize plants (Booth, 1971), in the naturally infected corn ears was quite unexpected; nevertheless, this evidence is supported by some previous studies indicating that *F. moniliforme* has a geographical distribution or ecological niche different from that of *F. subglutinans* (Booth, 1971; Francis and Burgess, 1975; Marasas et al., 1979).

Table I. Occurrence of Beauvericin, Moniliformin, and *Fusarium* Species in Preharvest Infected Corn Ears in Poland

year/sample	*Fusarium* spp.[a] (%)	mycotoxins[b] (mg/kg) BEA	M[c]
1990			
M-1608	*F. subglutinans* (100)	60	204
M-1609	*F. subglutinans* (100) *F. poae* (18)	ND	17
M-1611	*F. subglutinans* (100) *F. poae* (22)	10	39
M-1612	*F. subglutinans* (100)	40	170
M-1615	*F. subglutinans* (100)	10	35
M-1617	*F. subglutinans* (100)	10	99
M-1621	*F. subglutinans* (100)	10	81
M-1622	*F. subglutinans* (100) *F. poae* (16)	10	–
M-1658	*F. subglutinans* (100) *F. poae* (4)	20	425
1991			
M-1727	*F. subglutinans* (100)	15	–
M-1738	*F. subglutinans* (100)	30	–
M-1739	*F. subglutinans* (100)	5	–
M-1756	*F. subglutinans* (100) *F. poae* (2)	5	–
M-1758	*F. subglutinans* (100) *F. poae* (6)	20	–

[a] Percentages are based on 100 kernels per sample. [b] BEA, beauvericin; M, moniliformin; ND, not detected; –, not determined. [c] Data from Lew et al. (1993).

The chemical analysis of the 14 samples of corn ears gave positive results for the occurrence of BEA (Table I). In particular, 8 of 9 samples collected in 1990 and all 5 samples collected 1991 contained BEA (10–60 and 5–30 mg/kg, respectively). Besides HPTLC analysis, further evidence for the presence of BEA was obtained by HPLC analysis of extracts. Figure 2A shows the chromatogram of BEA standard, where one well-resolved peak may be seen at the retention time of 9.5 min. Figure 2B shows the chromatogram obtained from an extract of sample M-1608 spiked with BEA, and Figure 2C shows only the extract of sample M-1608 added at a higher concentration. Most of the samples assayed in 1990 proved also to be contaminated by M (Lew et al., 1993), and the amount of M was 3.4–21 times greater than BEA (samples M-1608 and M-1658, respectively) (Table I). The amounts of toxins found in the samples, in particular BEA, could be due to both different colonization of *F. subglutinans* in corn tissues and different capabilities of isolates to produce toxins. Isolates from slightly contaminated samples were both toxin producers and nonproducers (e.g., sample M-1609 and isolates ITEM-1520 and ITEM-1553) (Table II).

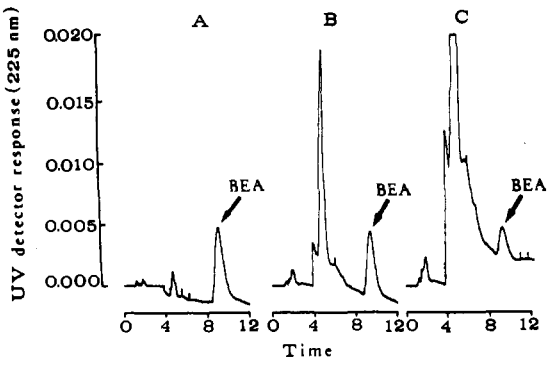

Figure 2. HPLC chromatogram of (A) beauvericin (BEA) standard, (B) extract of sample M-1608 spiked with BEA, and (C) only the extract of sample M-1608.

Table II. Beauvericin and Moniliformin Production by *F. subglutinans* Isolates from Infected Corn Ears in Poland

isolate (ITEM)	maize sample	mycotoxins produced[a] (mg/kg) BEA	M	% mortality of *A. salina* larvae in 24 h[b]
1518	M-1612	ND	60	0
1519	M-1617	100	200	89
1520	M-1608	100	ND	80
1521	M-1609	100	120	91
1548	M-1612	100	85	96
1549	M-1612	100	250	97
1550	M-1617	160	500	100
1551	M-1617	200	415	100
1552	M-1617	40	165	57
1553	M-1609	ND	165	95

[a] Isolates were grown on autoclaved corn kernels at 25 °C for 4 weeks. ND, not detected. [b] Four replicates per isolate.

As far as we are aware, this is the first study on the natural occurrence of BEA in corn. Beauvericin is a cyclohexadepsipeptide and consists of three D-α-hydroxyisovaleric acid residues linked alternatively to *N*-methyl-L-phenylalanine to give an 18-membered cyclic skeleton (Figure 1). The cyclodepsipeptides, mostly enniatins, have antibiotic (Shemyakin et al., 1963), insecticidal (Grove and Pople, 1980), and phytotoxic (Gäumann et al., 1960) activities, which appear to be related to their ionophoric properties and to the capability of forming complexes with alkali metal cations (Gorneva et al., 1976). Biological properties of the cyclodepsipeptides appear to be associated with their ability to affect ion transport across membranes (Shemyakin et al., 1965). Regarding BEA, since it was previously isolated mostly from entomopathogenic fungi, its toxicity has only been studied in insect bioassays (Vey et al., 1973; Grove and Pople, 1980). The finding of BEA, together with M in corn ear samples from fields before harvesting, suggests the need for more investigations on the toxicity of BEA also toward animals and plants and its possible synergistic activity with M, a metabolite already largely studied for its activity (Cole et al., 1973).

The results of the toxin analysis of autoclaved corn inoculated with 10 *F. subglutinans* isolates are summarized in Table II. In particular, 7 of the 10 tested strains produced BEA and M with 40–200 and 60–500 mg/kg, respectively; 2 strains produced only M (ITEM-1518 and -1553) and 1 only BEA (ITEM-1520). Except for one isolate (ITEM-1520), all produced more M than BEA. The presence of BEA in the cultural extracts was confirmed by HPLC analysis. Figure 3A shows the chromatogram obtained from cultural extract of strain ITEM 1551 and Figure 3B the same extract spiked with BEA. Overall, the brine shrimp toxicity of the methanolic extracts from

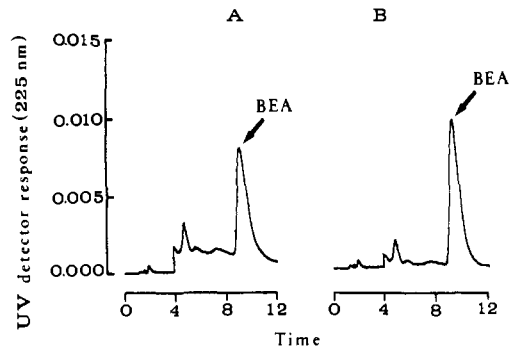

Figure 3. HPLC chromatogram of (A) cultural extract of strain ITEM-1551 and (B) the same extract spiked with BEA.

the cultures of *F. subglutinans* isolates was related to the levels of BEA produced, except in one isolate (ITEM-1553). *A. salina* is already considered to be a reliable animal test toward BEA (Hamill et al., 1969).

Previously, only strains of *F. subglutinans* isolated from insects in India and from corn in Peru proved to be able to produce BEA (Gupta et al., 1991; Logrieco et al., 1993). Consequently, this is also the first report of the production of BEA together with M by European isolates of *F. subglutinans*. Although limited surveys are available, it appears that the ability to produce BEA together with M is fairly widespread among isolates of *F. subglutinans* and that not all strains are able to produce BEA. Concerning the M production by *F. subglutinans*, Marasas et al. (1979) suggested the presence of different M-producing strains with different geographical diffusions. Because most of the isolates of *F. subglutinans* produced BEA and M, it would be interesting to analyze the BEA production by toxigenic, high-M-producing strains of *F. subglutinans* (Marasas et al., 1984; Kriek et al., 1977; Rabie et al., 1982) and other isolates often involved as food contaminants in some natural mycotoxicoses, such as the human esophageal cancer risk in South Africa (Marasas, 1981) and the heart disease called Kashan disease in some regions of China (Zhang, 1988).

The natural occurrence of BEA and the capability of several isolates of *F. subglutinans* from corn to synthesize this toxin led us to suspect that it could be a significant food and feed contaminant. Thus, further studies are needed to assess its toxicity toward corn plants and animals as well as its environmental occurrence.

ABBREVIATIONS USED

BEA, beauvericin; M, moniliformin; ITEM, Istituto Tossine e Micotossine; TLC, thin-layer chromatography; HPTLC, high-performance thin-layer chromatography; HPLC, high-performance liquid chromatography.

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