Accumulation of the Mycotoxin Beauvericin in Kernels of Corn Hybrids Inoculated with *Fusarium subglutinans*

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The accumulation of the mycotoxin beauvericin (BEA) after artificial inoculation of corn ears with *Fusarium subglutinans* is reported here for the first time. Ears of nine hybrids have been examined using a trace analytical method capable of detecting BEA down to 0.05 mg/kg (recovery 82.6%). *Fusarium*-damaged kernels contained 6.5−15.8 mg of BEA/kg and healthy looking kernels 0.07−0.13 mg of BEA/kg. The hybrids Mona and Anna, which showed the lowest susceptibility to *Fusarium* ear rot, accumulated the lowest amounts of BEA in the total ear, on average 0.40 and 0.85 mg/kg, respectively. Ears of the remaining hybrids accumulated 1.02−5.77 mg of BEA/kg.

Keywords: *Beauvericin; corn; mycotoxins; susceptibility; ear rot; Fusarium subglutinans*

INTRODUCTION

The *Fusarium* mycotoxin beauvericin (BEA) contains in alternating sequence three D-α-hydroxyisovaleryl and three *N*-methyl-L-phenylalanyl residues (Figure 1). It is structurally similar to the enniatins, differing only in the nature of the *N*-methylamino acid, which are also produced by a number of *Fusarium* species (Grove and Pople, 1980). Both enniatins and BEA have been shown to act as ionophores by increasing the permeability of biological and artificial membranes for alkali cations (Steinrauf, 1985). The only paper dealing with the cytotoxicity of BEA to mammalian cells reports on the substantial toxicity of BEA to several mammalian cell lines (Macchia et al., 1995).

BEA has been found in Poland in corn grain in amounts of 5−60 mg/kg in the first and 1.8−36.9 mg/kg in the second study on its natural occurrence (Logrieco et al., 1993b; Kostecki et al., 1995). Actually, *Fusarium subglutinans*, *Fusarium proliferatum*, and *Fusarium semitectum* (according to the Nelson et al., 1983, nomenclature) are well known to be producers of BEA (Gupta et al., 1991; Logrieco et al., 1993a). Well-documented production of BEA by *F. subglutinans*, a dominating pathogen of corn ears in Poland and other areas with similar moderate climate (e.g., Austria, Canada, Peru, South Africa), encouraged us to examine the accumulation of this metabolite in several corn hybrids under field conditions (Chelkowski et al., 1993; Teich, 1989; Neish et al., 1983; Bottalico et al., 1989; Kriek et al., 1977). However, the detection limits of current analytical methods for BEA in corn are ≥1 mg/kg. Therefore, evaluation of healthy looking kernels, which probably contain only trace amounts of BEA at μg/kg levels, has not been possible so far. The use of high-performance liquid chromatography (HPLC) with a diode array detector (DAD) for optimization of detection wavelength and an optimized sample cleanup enabled us to determine the BEA concentration down to 0.05 mg/kg for the first time.

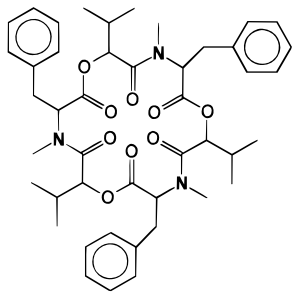

Figure 1. Idealized drawing of BEA structure with its three-dimensional symmetry.

MATERIALS AND METHODS

Preparation of Samples. *F. subglutinans* strain KF 555, which produced 212 mg of BEA/kg under *in vitro* conditions (Nelson et al., 1983), was isolated from a corn ear and has been used to inoculate ears of nine hybrids (Mona, Anna, Smolimag, KLG 2210, Milpa, Buran, SMH 4792, Ela, and Zenit) of low to high susceptibility to infection (Chelkowski et al., 1994). Hybrids were sown in May 1994 in four rows, 40 plants in each row in Radzików (30 km west of Warsaw, Poland). At midsilking stage 10 ears of each hybrid were inoculated at random with two toothpicks overgrown with *F. subglutinans* KF 555 at the tip and the middle part of the ear (Hart, 1982). Ears were manually harvested in the second half of October and transported in paper bags to the laboratory. In the case of *Fusarium* damage the kernels were either rotted and/or mycelium covered. The extent of the disease of each hybrid was determined by visually scoring 10 ears on a 0 (=0% diseased kernels) to 5 (=100%) scale. Kernels were subsequently separated in two fractions: *Fusarium*-damaged kernels (FDK) and healthy looking kernels (HLK). Kernels were ground in a laboratory mill and subjected to BEA analysis.

Chemical Analysis. For spiking experiments different amounts of a 100 mg/L standard solution of BEA (99% purity; Sigma Chemical Co., Deisenhofen, Germany) in acetonitrile (Baker, Groβ Gerau, Germany)−monodistilled water (9 + 1) were added to corn that contained no traces of BEA; 20 g of corn sample was extracted in an Ultra Turrax T25 blender with 100 mL of methanol (analytical grade; Merck, Darmstadt, Germany)−1% aqueous NaCl (Baker) (55 + 45) solution for 3

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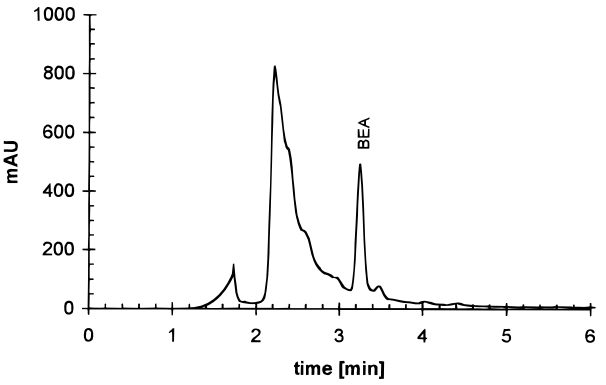

Figure 2. Chromatogram of the extract of hybrid Zenit-FDK containing 8.4 mg of BEA/kg corn.

min and then filtered through Whatman no. 1 filter paper. For low BEA concentrations in corn (up to 3 mg/kg) 50 mL of filtrate was transferred into a separatory funnel. Due to the limited capacity of the silica solid phase extraction column employed during the cleanup procedure, only 5 mL of filtrate reconstituted to 50 mL with extraction solvent was used for analysis of FDK. In the literature a defatting step with *n*-hexane (Merck) (2 × 50 mL) is described here as the next step (Logrieco et al., 1993b). However, the results of our spiking experiments revealed considerable loss of BEA during this defatting step. Therefore, we modified the subsequent cleanup procedure, which enabled us to leave out the extraction with *n*-hexane. Instead, we switched directly to the further extraction step with dichloromethane (analytical grade; Baker) (3 × 30 mL) which is also described in the literature (Logrieco et al., 1993a,b). The dichloromethane extracts were collected, evaporated to dryness on a rotary evaporator (50 °C), dissolved, and transferred to 4 mL screw-capped vials (Supelco, Bellefonte, PA) with 4 × 1 mL of chloroform−methanol (3 + 1). The solution was evaporated under nitrogen with gentle heating (50 °C). The residue was dissolved in 4 × 250 μL chloroform and applied to a silica solid phase extraction (SPE) column (Supelclean LC-Si, 1 mL, Supelco). The column was preconditioned with 2 × 1 mL of chloroform (analytical grade, stabilized with 0.75%, v/v, ethanol; Baker) and after application of the extract washed with 4 × 1 mL of chloroform and 2 × 1 mL of chloroform−methanol (99 + 1) solution. The BEA was eluted with 2 mL of chloroform−methanol (98 + 2). The eluate was evaporated under nitrogen (50 °C) and reconstituted to 250 μL with mobile phase.

An HPLC apparatus equipped with a DAD (HP 1090, Series II, Hewlett Packard) was employed in this work. The DAD system enabled us to find the optimal absorption wavelength for measurements of BEA and to control the purity of the peaks. The column was a reversed phase Vydac C_{18} (250 × 4.6 mm i.d.). Acetonitrile−water (90 + 10) was used as mobile phase under isocratic conditions, with a flow rate of 1.2 mL/min. The detection wavelength used was 192 nm. Spectra were recorded after subtracting the solvent absorption. Injections were made in duplicate. The injection volume was 20 μL.

Identification of BEA was achieved by comparing the retention times and spectral data (obtained by DAD) with those of the standard obtained from Sigma.

RESULTS AND DISCUSSION

The use of the DAD revealed that maximum absorption of BEA occurs at 192 nm. Since 225 nm is the wavelength most frequently found in the literature, we measured BEA simultaneously at 192 and 225 nm in preliminary experiments. The investigations showed the dramatic increase in sensitivity for 192 nm by a factor of up to 14.6 when compared to 225 nm. In addition, excellent linear correlation between sample concentration and the height of the peaks in the HPLC profile from 1 to 100 μg/mL BEA standard ($r = 0.9998$) could be achieved at this wavelength. Spiking experiments at four concentration levels (100, 500, 1000, and 2000 μg/kg) were carried out for the determination of the mean recovery of the method. The mean recovery for BEA for the concentration range 100−2000 μg/kg was 82.6% for $n = 8$ ($s = 5.0\%$). The detection limit for BEA using the method described in this work was 0.05 mg of BEA/kg of corn.

Table 1. BEA Content in *Fusarium*-Damaged Kernels (FDK) and Healthy Looking Kernels (HLK), Percentage of FDK on Ear, and Average BEA Content in Total Ear Calculated from Percentage of FDK

hybrid	BEA in FDK (mg/kg)	% FDK	av BEA content in total ear (mg/kg)	BEA in HLK (mg/kg)
Ela	8.57	41.76	3.58	ND[a]
SMH 4792	15.82	36.50	5.77	0.13
Milpa	14.12	19.67	2.78	<0.05
Anna	6.53	6.05	0.40	ND
Mona	14.12	6.01	0.85	0.12
Smolimag	14.75	17.21	2.54	<0.05
Zenit	8.40	22.36	1.88	0.07
Buran	7.92	14.84	1.18	0.10
KLG 2210	9.47	10.81	1.02	<0.05

[a] ND, not detected.

The HPLC chromatogram obtained for the measurement of 8.4 mg/kg BEA in hybrid Zenit-FDK (Figure 2) shows the well-separated BEA peak due to the appropriate sample cleanup. Table 1 shows the results of the inoculation experiments. Nearly the total amount of BEA has been present in the FDK fraction of the ears, which consisted of shriveled and light kernels, covered with fungal mycelium. Kernels without such symptoms (HLK) contained only low amounts of BEA (up to 0.13 mg/kg). The applied analytical method proved to be very sensitive and effective to detect such low amounts of the metabolite in corn kernels.

Highly susceptible hybrids, with 20−42% of FDK, contained on average 10 times higher amounts of BEA in the total ear (up to 5.77 mg/kg). However, the average content of BEA in the total ear depends mainly on the percentage of FDK and not as much on the BEA concentration in FDK. Susceptibility to *Fusarium* ear rot is a very undesirable trait in commercial corn hybrids. In susceptible hybrids the accumulation of mycotoxins is usually 1 order of magnitude higher as compared to hybrids of low susceptibility.

This is the first report on accumulation of BEA after artificial inoculation of corn ears with *F. subglutinans*. Besides the high concentration of BEA found in the samples investigated, significant concentrations of moniliformin may also be expected in the same samples (Kostecki et al., 1995). Taking into consideration the well-proven toxicity of *F. subglutinans* cultures, using biological tests in various laboratories (Abbas et al., 1988; Kriek et al., 1977) further investigations to evaluate the susceptibility of breeding material to accumulation of the species' metabolites in corn seem to be important.

ABBREVIATIONS USED

BEA, beauvericin; HPLC, high-performance liquid chromatography; FDK, *Fusarium*-damaged kernels; HLK, healthy looking kernels; DAD, diode array detector.

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