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Occurrence of Beauvericin in Corn from Croatia

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Summary

The occurrence of beauvericin has been investigated in corn kernel (*Zea mays L.*) samples collected in 1996 (105 samples) and 1997 (104 samples) from 14 corn-producing counties of Croatia. Corn sample extracts were cleaned up by silica gel minicolumns and analyzed for beauvericin by high performance liquid chromatography with UV diode array detector. Higher incidence of positive samples was found in the 1996 crop as compared to the 1997 crop. In particular, 18 samples (17.4 %) of the 1996 crop were found contaminated with a mean beauvericin content of 393 ng/g and the highest level at 1864 ng/g. Only 1 out of 104 samples collected in the 1997 crop was contaminated with 696 ng/g of the toxin. Beauvericin co-occurred with fumonisins B_1 and B_2 and with ochratoxin A in 17 and 4 samples, respectively.

The results of mycological analysis of corn samples for beauvericin producing *Fusarium* species were in agreement with results of chemical analysis. In particular, higher incidence of *Fusarium verticillioides* (Sacc.) Nirenberg (known as *Fusarium moniliforme* Sheldon) (3.7 %) and *Fusarium subglutinans* (Wollenweber & Reinking) Nelson, Toussoun & Marasas (5.3 %) was found in 1996 with respect to 1997 (1.9 % of *F. verticillioides* and 0.4 % of *F. subglutinans*). This is the first report on the occurrence of beauvericin in Croatia.

Key words: beauvericin, fumonisins, ochratoxin A, mycotoxins, *Fusarium*

Introduction

Beauvericin (BEA) is a bioactive cyclodepsipeptide produced primarily by *Fusarium subglutinans* and *Fusarium proliferatum* (Matsushima) Nirenberg which are common pathogens of corn causing stalk and ear rot (*1*). BEA was first isolated as a natural contaminant from Polish corn (*2*), and has been found in corn from Italy (*3*) and Austria (*4*) as well as in the USA, Peru and parts of Africa (*5*). BEA has been shown to be substantially toxic to several mammalian cell lines, which indicates that it may be involved in human and animal pathology through contamination of corn. However, the toxicity of BEA is still under discussion and the role of the toxin in outbreaks of mycotoxicoses is unclear (*4*). The occurrence of BEA in corn produced in neighboring countries of Croatia encouraged us to examine the occurrence of BEA in Croatian corn. In the present study we measured the occurrence of *Fusarium* spp. and BEA in corn kernel samples collected from 14 corn-producing counties of Croatia from the 1996 and 1997 crops. The co-occurrence of BEA with fumonisins (FB_1 and FB_2) and ochratoxin A (OTA) has also been evaluated.

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Materials and Methods

Corn samples (209) were collected randomly from farms (one sample per farm) in 14 counties of Croatia. Whole ear samples (1.5–2 kg/sample) destined for animal and human consumption were collected after being stored in the farm for 2–3 weeks after harvest. Kernels were shucked in the laboratory and 270–320 g of each sample was stored at –20 °C and then dried overnight at 45–50 °C before mycotoxin analyses were performed in February-March 1998.

Mycological analysis

Kernel samples were washed for 10 min in 1 % sodium hypochlorite and rinsed in sterile distilled water for mycological analysis. Two hundred kernels per sample were placed in Petri dishes on sterile wet filter paper. After 10–12 days of incubation at 25 °C the incidence of *Fusarium* spp. was determined. Fungal identification was performed according to Neergaard (*6*). Statistical treatment of data was performed by the Kruskal-Wallis Test (non parametric ANOVA) using Instat software (San Diego, CA, USA).

Beauvericin analysis procedure

Analysis of BEA was performed (from the same sample used in the mycological analysis), according to the procedure reported by Krska *et al.* (*7*). Twenty grams of ground samples were extracted with 100 mL of methanol containing 1 % aqueous NaCl (volume ratio 55:45) by shaking for 60 min with an orbital shaker at approx. 200 rpm. After filtering through Whatman No. 4 filter paper, 50 mL of aliquots were transferred into a separatory funnel and extracted 3 times with 30 mL dichloromethane. The dichloromethane extracts were collected, evaporated to dryness at 70 °C, dissolved and transferred to 4 mL screw-capped vials with 4 x 1 mL chloroform-methanol (volume ratio 3:1). The solution was then evaporated under nitrogen at 50 °C. The residue was dissolved in 1 mL of chloroform and applied to a silica solid-phase extraction (SPE) column (Supelclean LC-Si, 500 mg, Supelco, Bellefonte, PA, USA). The column was preconditioned with 2 x 1 mL of chloroform and after application of the extract, it was washed with 4 x 1 mL of chloroform and 2 x 1 mL of chloroform-methanol (volume ratio 99:1). The BEA was eluted with 2 mL of chloroform-methanol (volume ratio 98:2) and the eluate was evaporated under nitrogen (50 °C) and reconstituted to 250 mL with HPLC mobile phase.

The HPLC system consisted of an isocratic pump (LKB 2150, Bromma, Sweden) with a Rheodyne 7125 injector connected to a UV diode array detector (Hewlett Packard HP 1040) set at 192 nm connected to a HP 9000 Series 300 computer. The column used was a Supelcosil LC-18 15 cm x 4.6 mm, i.d. 5 μm (Supelco, Bellefonte, PA, USA) with a guard column inlet filter, 0.5 μm x 3 mm diameter (Rheodyne Inc. CA, USA) and a mobile phase acetonitrile-water (volume ratio 90:10) with a flow rate of 1.2 mL/min was used as mobile phase. Twenty microliters were injected for each run and BEA was identified by comparing retention time and UV spectrum with authentic BEA standard purchased from SIGMA, Gallarate, Italy. Quantification was done by comparing the peak of BEA against a calibration curve obtained with standard solutions of BEA. The method used for determination of BEA gave an average recovery of 78 % with a limit of detection (signal-to-noise ratio of 3:1) of 10 ng/g.

Results and Discussion

Mycological analysis

The results of the mycological analysis are reported in Table 1. There was a higher incidence of *Fusarium* spp. infection in 1996 as compared to 1997. The average percentages of corn kernels infected with *F. verticillioides* were 3.7 and 1.9 % in 1996 and 1997, respectively, whereas corn was infected with *F. subglutinans* with an incidence of 5.3 % in 1996 and 0.4 % in 1997. *Fusarium graminearum* Schwabe and *Fusarium poae* (Peck) Wollenw. (*8*,*9*) were also found, but at negligible levels (<0.1 %).

Table 1. Fraction of corn samples infected with *Fusarium verticillioides* and *F. subglutinans* in Croatia

County	*Fusarium verticillioides*		*Fusarium subglutinans*	
	Fraction / %			
	1996	1997	1996	1997
Zagrebačka	3.7	2.0	3.4	0.4
Krapinsko-zagorska	1.0	1.3	4.2	0.0
Sisačko-moslovačka	3.0	0.5	3.7	0.5
Karlovačka	5.5	1.5	6.7	0.0
Varaždinska	2.5	0.2	12.2	3.0
Koprivničko-križevačka	2.3	1.0	3.3	0.0
Bjelovarsko-bilogorska	1.0	1.5	4.5	0.0
Virovitičko-podravska	6.4	2.4	3.8	0.0
Požesko-slavonska	1.5	0.8	4.5	0.0
Brodsko-posavska	2.6	2.0	6.6	0.9
Zadarska	4.7	7.5	4.7	0.0
Osječko-baranjska	6.0	2.0	10.0	0.8
Vukovarsko-srijemska	9.4	2.7	6.6	0.0
Međimurska	2.0	1.0	0.0	0.0
Average percentages	3.7	1.9	5.3	0.4**

**Indicates significant difference ($p<0.01$) with respect to 1996

Occurrence of beauvericin

The data on BEA occurrence are reported in Table 2. Higher incidence of positive samples was found in the 1996 crop year as compared to the 1997 crop. Eighteen out of 105 samples (17 %) of the 1996 crop were found contaminated with BEA with a mean content of positive samples of 393 ng/g and the highest content of 1864 ng/g. Only one out of 104 samples (1 %) of the 1997 crop was contaminated with BEA at 696 ng/g. This is the first survey for BEA in corn produced in Croatia with a significant number of corn samples (209) analyzed. The limited data available in the literature are mainly related to selected corn samples visibly infected with *Fusarium* spp. In particular BEA was found at levels ranging from 8 to 1734 ng/g in 10 visibly *Fusarium*-infected home-grown corn samples from Transkei region in South Africa (*10*). Twenty two out of 42 selected preharvest *Fusarium*-infected corn ears collected in Po-

Table 2. Beauvericin contamination of corn samples collected in 1996 and 1997 from 14 counties in Croatia

County	1996		1997	
	Positive/Total	Means content of positive sample ng/g (range)	Positive/Total	Means content of positive sample ng/g (range)
Zagrebačka	0/7	0	0/5	0
Krapinsko-zagorska	1/8	34	0/3	0
Sisačko-moslavačka	1/8	158	0/7	0
Karlovačka	1/4	1346	0/2	0
Varaždinska	1/4	549	0/5	0
Koprivničko-križevačka	0/7	0	0/5	0
Bjelovarsko-bilogorska	0/2	0	0/2	0
Virovitičko-podravska	2/5	109 (225–322)	0/5	0
Požeško-slavonska	0/2	0	0/6	0
Brodsko-posavska	10/45	90 (13–1864)	1/50	696
Zadarska	0/4	0	0/2	0
Osječko-baranjska	2/4	33 (48–82)	0/6	0
Vukovarsko-srijemska	0/4	0	0/4	0
Međimurska	0/1	0	0/2	0
Total	18/105	393 (13–1864)	1/104	696

Table 3. Occurrence of fumonisins (FB_1 + FB_2), ochratoxin A (OTA) and beauvericin (BEA) in corn samples collected in 1996 and 1997

	1996			1997		
	FB_1+FB_2	OTA	BEA	FB_1+FB_2	OTA	BEA
Positive/Total	102/105	10/105	18/105	97/104	36/104	1/104
Fraction / %	(97 %)	(10 %)	(17 %)	(93 %)	(35 %)	(1 %)
Mean content of positive samples/(ng/g) (range)	645 (12–11661)	37.87 (0.36–224)	393 (13–1864)	134 (12–2524)	57.13 (0.26–614)	696
Overall mean content / (ng/g)	621	3.61	67.3	125	19.77	6.7

land and Italy were also contaminated with BEA at levels ranging from 5 000 to 520 000 ng/g (*2,3,11*). Lower levels of BEA, ranging from 100 to 300 ng/g, were found in 5 out of 8 corn and feed samples in Iowa (*5*). *Fusarium verticillioides, F. proliferatum* and *F. subglutinans* were found to be associated with BEA in corn and feed in Iowa (*5*), whereas *F. verticillioides* and *F. proliferatum* were found to be associated with BEA in Italian maize (*3,11*). Only *F. subglutinans* was detected in BEA contaminated maize collected in Poland (*2*). In our study we have found that *F. verticillioides* and *F. subglutinans* associated with BEA contamination and their incidence in the two years were positively correlated with the incidence of samples found contaminated with BEA.

In Table 3, the data on the occurrence of BEA was compared with those on the occurrence of FBs and OTA in the same corn samples (*9*). From Table 3, it is evident that the incidence of fumonisin contamination was high in both years being 97 and 93 % in 1996 and 1997, respectively. Of the 18 samples positive for BEA in 1996, 16 were positive for FB_1 + FB_2, and 3 were positive for OTA. Of the 10 samples positive for OTA in 1996, 9 were positive for FB_1 + FB_2, while of the 36 samples positive for OTA in 1997, 34 samples were positive for FB_1 + FB_2 and 1 was positive for BEA. However, 35 % of positive samples from 1996 and 66 % of positive samples from 1997 were contaminated by fumonisins at levels lower than 100 ng/g, a level considered not dangerous for human and animal health. The incidence of OTA was lower in 1996 (10 %) than in 1997 (35 %), with the highest level at 224 ng/g and 614 ng/g, respectively. Within the 19 samples found contaminated with BEA, 4 samples (3 collected in 1996 and in 1 collected in 1997) were also contaminated with fumonisins and OTA whereas the co-occurrence of BEA with FBs was found in 13 samples collected in 1996. This is the first report on the co-occurrence of BEA with FBs and OTA. Co-occurrence of BEA and FBs had already been reported in Italy and South Africa (*3,10*). The co-occurrence of beauvericin with fumonisins and ochratoxin A suggests an increased toxicological risk for human and animal health, although no conclusion can be drawn before the assessment of BEA toxicity.

Conclusion

This is the first report on natural occurrence of beauvericin (BEA) in Croatia, and also the first report on the co-occurrence of beauvericin with fumonisins (FB1+ FB2) and ochratoxin A (OTA). Co-occurrence of BEA and fumonisins (FB1+FB2) have already been reported in Italy and South Africa (*3,10*). The co-occurrence of BEA with FB1, FB2 and OTA suggests an increased toxicological risk for human and animal health, although no conclusion can be drawn before the assessment of BEA toxicity.

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Raširenost bovericina u hrvatskom kukuruzu

Sažetak

Učestalost bovericina analizirana je u uzorcima kukuruza skupljenim tijekom 1996. (105 uzoraka) i 1997. godine (104 uzorka) iz 14 županija Republike Hrvatske. Ekstrakt uzoraka kukuruza pročišćen je na silicij-gelskim minikolonama i analiziran na bovericin s tekućinskim kromatografom velike učinkovitosti (HPLC) s UV-diodnim detektorom. Učestalija pojava pozitivnih uzoraka nađena je godine 1996. u usporedbi s 1997. godinom. Godine 1996. nađeno je 18 uzoraka (17,4 %) kontaminiranih bovericinom sa srednjom vrijednošću pozitivnih uzoraka od 393 ng/g, odnosno s najvećom količinom od 1864 ng/g. Samo 1 od 104 skupljena uzorka godine 1997. bio je onečišćen s 696 ng/g toksina. Bovericin je nađen zajedno s fumonizinom B_1 i B_2 u 17 uzoraka, a s okratoksinom A samo u 4 uzorka. Mikološkom analizom utvrđene *Fusarium* vrste slažu se s kemijskim analizama na mikotoksine. Veća pojava *Fusarium verticillioides* (Sacc.) Nirenberg (poznat i kao *Fusarium moniliforme* Sheldon) (3,7 %) i *F. subglutinans* (Wollenweber & Reinking) Nelson, Toussoun & Marasas (5,3 %) nađena je godine 1996. u usporedbi s 1997. godinom kada je ustanovljeno (1,9 %) *F. verticillioides* i (0,4 %) *F. subglutinans*. Ovo je prvo izvješće o prisutnosti bovericina u Republici Hrvatskoj.