Short Communication

TOXIGENIC POTENTIAL OF *FUSARIUM* SPECIES ISOLATED FROM NON-HARVESTED MAIZE*

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The objective of this study was to determine the frequencies of distribution and the toxigenic potential of *Fusarium* species isolated from non-harvested maize left in field over winter in 1999 (N=56) and 2003 (N=56) in northern Croatia. Zearalenone and trichothecenes (DON, DAS, T-2) were isolated and detected using multitoxin extraction and TLC method. Modified TLC method was applied to detect fumonisin B_1. *Fusarium* species were the most frequent fungi found in maize with 78.6 % in 1999 and 85.0 % in 2003. Among fusaria *F. verticillioides* was dominant species found in 12.5 % (1999) and 35.7 % (2003) of maize samples. Other determined fusaria were *F. graminearum* (8.9 % in 2003), *F. poae* and *F. sporotrichoides* (2.0 % to 3.6 %), *F. tricinctum* and *F. tabacinum* (2.0 % in 1999). Production of FB_1 was established for all *F. verticillioides* (7/7) isolated in 1999 in concentration range from 280 mg L^{-1} to 918 mg L^{-1}, and for 11 of 20 strains found in 2003 (48 mg L^{-1} to 400 mg L^{-1}). Three strains also produced zearalenone: one strain in 1999 produced 3.80 mg L^{-1} and 2 strains in 2003 produced 20.0 mg L^{-1} and 70.0 mg L^{-1}. In addition, four strains of *F. graminearum* isolated in 2003 produced higher amounts of zearalenone (60.0 mg L^{-1} to 180.0 mg L^{-1}). T-2 production was found in *F. tricinctum* (1.55 mg L^{-1}) isolated in 1999.

KEY WORDS: *fumonisin B_1, trichothecenes, zearalenone*

During growth in the fields, maize and other cereals are exposed to mycoflora. Substrate moisture (>20 %), air temperature and relative humidity (< 90 %) provide "field fungi" excellent environmental conditions for development (1). The most frequent "field fungi" are *Fusarium* species, which can colonise the straw, grain and ear before the harvest. They spoil the maize, decrease its quality, and produce mycotoxins. Members of the *Fusarium* genera are potent producers of trichothecenes (deoxynivalenol-DON, diacetoxiscyrpenol-DAS, T-2), zearalenone (ZEN) and fumonisin B_1 (FB_1). Trichothecenes are associated with various animal toxicoses, including feed refusal, vomiting, diarrhoea, skin inflammation, haemorrhagic syndrome in internal organs, cellular damage of the bone marrow, thymus and spleen (immunosuppression) and disturbance of the nervous system. In addition, T-2 toxin and DON have been implicated in alimentary toxic aleukia in humans who had eaten contaminated grain (2). Zearalenone is uterotrophic and estrogenic, usually causing vulvovaginitis, vaginal or rectal prolapse, loss of pregnancy and infertility in domestic animals, particularly swine, and there is a possible impact on human health (2). Fumonisin B_1 causes liver and kidney toxicity and carcinogenicity, immunosuppression, pulmonary oedema and neurotoxicity. Home-grown maize contaminated with FB_1 has been associated with oesophageal cancer in humans in Africa, China and the USA (3). The production of mycotoxins can begin in preharvest-infected maize standing in the field and can continued in postharvest and stored products. Some strains of *Fusarium* species can produce several toxic metabolites (multitoxigenic

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strains) under the field conditions, and these may accumulate in infected plants. The occurrence of toxigenic fusaria and their mycotoxins, particularly in maize and other cereals as components of food and feed products, is often associated with economic loss and poses a serious problem for animal and human health (4). The objective of this study was to determine the distribution and toxigenic potential of *Fusarium* species isolated from non-harvested maize left in field over winter in northern Croatia.

MATERIALS AND METHODS

Mycological analysis

A total of 112 non-harvested maize samples was collected in northern Croatia after the winter 1999 (N=56) and 2003 (N=56). Maize kernels were treated with an antibiotic solution of penicillin and streptomycin (20:40). Five grains of each sample were plated on 2 % Sabouraud glucose agar (SGA) and 5 grains on humid sterile filter-paper. The plates were incubated 4-7 days at (25±2) °C. *Fusarium* species were identified on the basis of morphological macroscopic and microscopic characteristics on potato-dextrose agar (PDA) and synthetic nutrient agar (SNA) according to an established key (5, 6).

Biosynthesis of mycotoxins

Mycotoxin (ZEN, DON, DAS, T-2, FB_1) production capacity was examined *in vitro* on yeast-extract sucrose (YES) broth (composition: yeast extract 20 g, sucrose 40 g, distilled water 1000 g, pH 5.8±0.2). One milliliter suspension of conidia (10^6 mL^{-1}) of each *Fusarium* strain was inoculated on 50 mL of YES and incubated at (25±2) °C for 10 days with daily shaking.

Extraction and thin layer chromatography detection (TLC) of mycotoxins

Isolation and detection of ZEN were performed using multitoxin extraction and semiquantifiaction TLC method by *Balzer et al*. (7). Trichothecenes (DON, DAS, T-2) were isolated and detected using a modified multitoxin extraction and TLC method (8). Briefly, *Fusarium* cultures (50 mL) were homogenised with 50 mL acetonitrile:water (9:1) for 10 minutes. From each filtrated sample, 50 mL was treated with *n*-hexane (2x25 mL) to remove the lipids. Saturated solution of $NaHCO_3$ (25 mL) and water (25 mL) were added into samples (pH=8-9) and extracted with chloroform (25 mL). Lower chloroform fraction was treated with 1 N NaOH (2x10 mL). Chloroform fraction was washed with 25 mL of water, and lower phase (containing trichothecenes) was filtered through anhydrous Na_2SO_4, evaporated to dryness and redissolved in 0.2 mL of chloroform for TLC analysis. Upper alkaline fraction (NaOH) was acidified with 10 mL of 1.667 mol L^{-1} H_3PO_4 and extracted with 2x20 mL of chloroform. Lower chloroform phase (contains ZEN) was filtered through anhydrous Na_2SO_4, evaporated and redissolved in 0.2 mL of chloroform for TLC analysis. Extracts were spotted on silica gel H plates along with standard solutions and plates were developed in toluene:ethylacetate: formic acid (5:4:1). After air-drying, the plates were sprayed with 50 % H_2SO_4 in ethanol and heated 10 min at 120 °C for visualization of trichothecenes. Fluorescence intensities of toxin spots and standards were compared under the UV light (366 nm).

Fumonisin B_1 was isolated and detected according to established method by *Pepeljnjak et al*. (9). Briefly, *F. vertocillioides* (syn. *moniliforme*) cultures growing on YES (50 mL) were homogenised with 50 mL acetonitrile-water (9:1) for 10 min and then filtered. The filtrate (50 mL) was extracted with *n*-hexane (2x25 mL). Upper hexane phase was discarded and water-soluble phase, adjusted to pH 8-9 with 25 mL of saturated $NaHCO_3$, was then shaken with 25 mL of chloroform for subsequent purification. Upper water-soluble phase was partially evaporated at 80 °C and then concentrated in vacuum by lyophilisation. Lyophilisate was dissolved in acetonitrile:water (1:1) and analysed with FB_1 commercial sample on silica gel GF_{254}. The plates were developed in acetonitrile: toluene:water (93:5:2). FB_1 was visualised under the UV light (366 nm).

RESULTS

The most common fungi found in maize were the *Fusarium* species with the frequency of 78.6 % in 1999 and 85.0 % in 2003. Other identified fungi were of the following genera: *Alternaria*, *Cladosporium*, *Penicillium*, *Trichoderma*, *Nigrospora*, *Aspergillus* and *Absidia* (Figure 1). These fungi were detected in higher frequencies in 1999 (3.6 % to 30.0 %) than in 2003 (3.6 % to 12.5 %). Samples collected in 2003 were also highly contaminated with yeasts and antracoid bacteria. Among fusaria, *F. verticillioides* (syn. *moniliforme*) was the dominant species

found in 12.5 % (1999) and 35.7 % (2003) of maize samples (Figure 2). Other identified fusaria were *F. graminearum* (8.9 % in 2003), *F. poae* and *F. sporotrichoides* (2.0 % to 3.6 %), *F. tricinctum* and *F. tabacinum* (2.0 % in 1999). Table 1 shows mycotoxin production of *Fusarium* species on YES broth. The production of FB_1 was established for all *F. verticillioides* strains (7/7) isolated in 1999 in the concentration range of 280 mg L^{-1} to 918 mg L^{-1}, and for 11 out of 20 strains found in 2003 (48 mg L^{-1} to 400 mg L^{-1}). Three strains were found to be multitoxigenic, produced both FB_1 and ZEN. One strain in 1999 produced 3.80 mg L^{-1} and two strains in 2003 produced 20.0 mg L^{-1} and 70.0 mg L^{-1} of ZEN. Four strains of *F. graminearum* isolated in 2003 produced higher amounts of ZEN (60.0 mg L^{-1} to 180.0 mg L^{-1}) than *F. verticillioides*. T-2 production was found in 1/1 strain of *F. tricinctum* (1.55 mg L^{-1}) isolated in 1999. Other isolated fusaria were not found to produce any of the analysed mycotoxins.

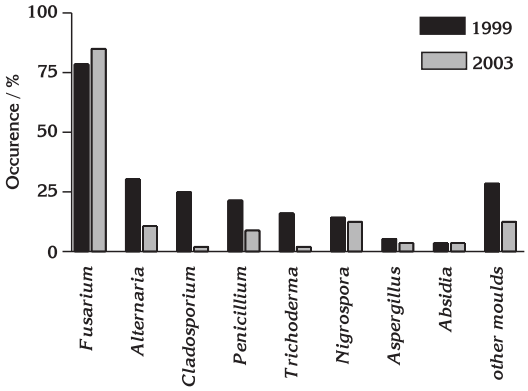

Figure 1 *Mycological contamination of non-harvested maize collected in 1999 and 2003. Occurrence (%) of fungal genera in the total number of maize samples.*

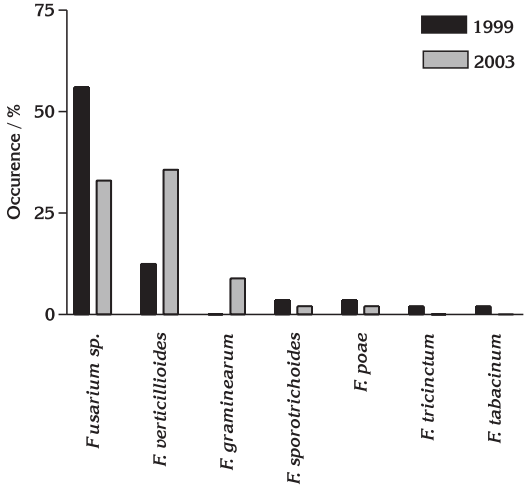

Figure 2 *Occurrence of Fusarium species in non-harvested maize samples collected in 1999 and 2003. Occurrence (%) of Fusarium species in the total number of maize samples.*

DISCUSSION

Fungi in the genus *Fusarium* are the most common contaminants of maize and other cereals. *Fusarium* species can survive well on maize crop residues, which remain after the harvest. Fungal structures, like mycelium, chlamydospores (*F. graminearum*) and thickened hyphae (*F. verticillioides*), can survive unfavourable environmental conditions for a longer period (10). Maize crop residues as well as non-harvested plants can be a source of inoculum for infection of soil, seed, root, stalk or silk of plants. During the plant maturation infected maize kernels become shrivelled and discoloured with a white, pink or light brown appearance due to the *Fusarium* mycelial growth (10). The fusaria frequently isolated from maize in Mediterranean countries are widespread *F. verticillioides* (syn. *moniliforme*) and *F. graminearum* (4), which corresponds to our findings. Less frequently isolated species are *F. equiseti*, *F. poae*, *F. sporotrichoides*, *F. acuminatum*, *F. solani* and *F. oxysporum* (4). Under the field conditions,

Table 1 *Toxigenic potential of Fusarium species isolated from non-harvested maize in Croatia*

Fusarium Species	1999		2003	
	N of toxigenic strains/total	Range of mycotoxin concentration / mg L^{-1}	N of toxigenic strains/total	Range of mycotoxin concentration / mg L^{-1}
F. verticillioides	7/7 1/7	FB_1 280-918 ZEN 3.80	11/20 2/20	FB_1 48-400 ZEN 20-70
F. graminearum	Nd	Nd	4/5	ZEN 60-180
F. tricinctum	1/1	T-2 1.55	Nd	Nd

Nd-not detected; FB_1 – fumonisin B_1; ZEN - zearalenone

substrate moisture, air temperature oscillation and relative humidity, fusaria can produce and accumulate mycotoxins. *Fusarium* species commonly isolated from Mediterranean cereals produce high amounts of FB_1 and ZEN *in vitro* on different media. A study of FB_1 production by 28 strains of *F. verticillioides* isolated from cereals in Italy, Spain and France revealed fumonisinotoxigenic potential for all strains with maximum produced concentration of 4100 mg kg^{-1} (11). The highest FB_1 production was found for *F. verticillioides* strains isolated from maize (average 1300 mg kg^{-1}). Similar maximum amount of FB_1 (4200 mg kg^{-1}) was produced by *F. verticillioides* isolated from Spanish maize-based animal feed (12). *Logrieco et al*. (13) reported FB_1 production in 26/26 *F. verticilliodes* maize isolates with the maximum concentration of 2250 mg kg^{-1}. Our earlier research showed all *F. verticillioides* strains (66/66) isolated from home-grown maize in north Croatia were able to produce FB_1 in relatively high concentrations (181 mg L^{-1} to 1320 mg L^{-1}). The highest number of FB_1 producer strains was isolated from non-harvested maize collected in the region of endemic nephropathy (11 strains) and Vinkovci (11 strains) (14). These data corroborate our finding of fumonisin producing *F. verticillioides* strains (7/7) isolated in 1999 (280 mg L^{-1} to 918 mg L^{-1}). On the other hand, in 2003 we found less FB_1-producing strains (11/20) which produced significantly smaller amounts of FB_1 (48 mg L^{-1} to 400 mg L^{-1}). The reasons may be unfavourable environmental conditions (temperature under the zero, frequent snowing, long period of coldness), maize genotype and contamination with yeasts and antracoid bacteria that can have some influence on the fusaria mycotoxin-producing ability. In this study we also found 3 strains of *F. verticillioides* that were multitoxigenic and produced both FB_1 and ZEN (20 mg L^{-1} to 70 mg L^{-1}). There are few reports on ZEN production by *F. moniliforme* (reviewed by *Marasas et al*., see ref. 15). These ZEN producing strains were isolated from maize in Italy, USA, and India (4.5 mg kg^{-1}, 7.7 mg kg^{-1}, and 0.8 mg L^{-1}, respectively, the first two being biosynthesised on autoclaved maize and the third on Richard's solution), and one strain was isolated from barley in the United Kingdom (15 mg kg^{-1}, biosynthesised on autoclaved rice). In addition, positive uterotrophic response was reported in mice and rats with *F. moniliforme* isolates that could be related to ZEN or to giberelins (15). In a study of ZEN production on maize kernels by 15 strains of *F. graminearum* isolated from cereals in Italy, all assayed strains produced high concentrations of ZEN (up to 1500 mg kg^{-1}). *Fusarium graminearum* strains isolated from cereals in other Mediterranean countries produced ZEN in the range of 6 mg kg^{-1} to 60 mg kg^{-1} (16), which is closer to our findings of ZEN production by *F. graminearum* (60 mg kg^{-1} to 180 mg kg^{-1}). High frequency and relatively high levels of fumonisins and ZEN in maize were reported in Croatia. *Jurjević et al*. (17) reported high frequency of fumonisins (FB_1+FB_2) in maize from endemic and non-endemic regions (95 %) in Croatia in a range from 0.012 mg kg^{-1} to 11.903 mg kg^{-1}. Higher amounts of ZEN (up to 19.9 mg kg^{-1}) were found in cereals and beans in 1991 and from 1992 to 1995 (wartime in Croatia) (18, 19). In this study, only one strain of *F. tricinctum* was found in maize samples in 1999, which produced low amounts of T-2 toxin (1.55 mg L^{-1}), compared to the previous findings of T-2 toxin in cereals (0.2 mg kg^{-1} to 20.5 mg kg^{-1}) in Croatia including the war time (20).

When it comes to the mycotoxicological risk, maize contamination by *Fusarium* species is of the greatest concern because of their toxigenic potential, presence of multitoxigenic strains, accumulation of produced mycotoxins in grains intended for foods and feeds, and their possible synergistic action in biological systems.

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Sažetak

TOKSINOGENOST VRSTA *FUSARIUM* IZOLIRANIH S NEOBRANOG KUKURUZA

Cilj rada bio je utvrđivanje učestalosti i toksinogenosti *Fusarium* vrsta izoliranih s neobranog kukuruza koji je prezimio u polju tijekom 1999. (N=56) i 2003. (N=56) na području sjeverne Hrvatske. Izolacija i detekcija zearalenona (ZEN) i trihotecena (DON, DAS, T-2) provedena je multitoksinskom ekstrakcijom i TLC-metodom. Modificirana ekstrakcija i TLC-metoda iskorištena je za određivanje fumonizina B_1. *Fusarium* vrste izolirane su s učestalošću 78,6 % iz uzoraka skupljenih u 1999. te 85 % iz uzoraka skupljenih u 2003. Među fuzarijama dominirala je vrsta *F. verticillioides* s učestalošću 12,5 % (1999.) i 35,7 % (2003.) u ukupnom broju uzoraka kukuruza. Ostale identificirane vrste fuzarija bile su *F. graminearum* (8,9 %; 2003.), *F. poae* i *F. sporotrichoides* (2,0 % do 3,6 %), *F. tricinctum* i *F. tabacinum* (2,0 %; 1999.). Fumonizinotoksinogenost je dokazana u svih sojeva *F. verticillioides* (7/7) izoliranih 1999. u rasponu koncentracija od 280 mg L^{-1} do 918 mg L^{-1} te u 55 % sojeva izoliranih 2003. (48 mg L^{-1} do 400 mg L^{-1}). 11 % sojeva su osim FB_1 tvorili i ZEN; 3,7 % iz 1999. produciralo je ZEN u koncentraciji 3,80 mg L^{-1}, dok je 7,4 % sojeva iz 2003. tvorilo ZEN u višim koncentracijama (20,0 mg L^{-1} i 70,0 mg L^{-1}). Tvorba ZEN utvrđena je za 4 od 5 izoliranih sojeva *F. graminearum* (2003.) u nešto višim koncentracijama (60,0 mg L^{-1} do 180,0 mg L^{-1}). T-2 toksin producirao je jedini nađeni soj *F. tricinctum* (1999.) u koncentraciji 1,55 mg L^{-1}.

KLJUČNE RIJEČI: *fumonizin B_1, kukuruz, trihoteceni, zearalenon*

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