

FUSARIUM* SPP. CONTAMINATION OF WHEAT, MAIZE, SOYBEAN, AND PEA IN CROATIA

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From 2002 to 2008, 203 samples of wheat, maize, soybean, and pea were analysed for the presence of *Fusarium* species. Contamination with *Fusarium* spp., expressed as the percentage of seeds with *Fusarium* colonies, ranged from 5 % to 69 % for wheat, from 25 % to 100 % for maize, from 4 % to 17 % for soybean, and from 3 % to 17 % for pea. 187 isolates were collected and the following 19 species determined: *F. graminearum*, *F. poae*, *F. avenaceum*, *F. verticillioides*, *F. sporotrichioides*, *F. heterosporum*, *F. crookwellense*, *F. tricinctum*, *F. semitectum*, *F. oxysporum*, *F. proliferatum*, *F. solani*, *F. equiseti*, *F. pseudograminearum*, *F. chlamydosporum*, *F. sambucinum*, *F. compactum*, *F. scirpi*, and *F. culmorum*. Dominant species were *F. graminearum* on wheat (27 % of isolates), *F. verticillioides* on maize (83 % of isolates), *F. sporotrichioides* on soybean (34 % of isolates), and *F. proliferatum* on pea (29 % of isolates). Among species identified, *F. heterosporum*, *F. crookwellense*, *F. pseudograminearum*, *F. sambucinum*, and *F. compactum* have been reported for the first time in Croatia.

KEY WORDS: *feed, food, fungi, mycotoxins, species identification*

The genus *Fusarium* comprises more than 80 species of fungi, which differ considerably in their biology, ecology, and economic significance (1). *Fusarium* species can parasitise plants, vertebrates, insects, humans, or even other fungi, but most of them have a strong saprophytic ability and can develop on dead organic matter. Regardless of their ecological and nutritional behaviour, a great number of *Fusarium* species can colonise nearly all plant parts, including seed.

Different *Fusarium* species can produce a wide range of secondary metabolites, many of which are toxic to humans or animals (2). These mycotoxins produced on plants during vegetation can enter into

food or feed and can cause different adverse effects on human or animal health.

Over the last twenty years, research on the genus *Fusarium* has intensified, especially with the *Fusarium* head blight (FHB) epidemics on wheat, deoxinivalenol (DON) contamination issue (3, 4), and the discovery of fumonisins, cancerogenic mycotoxins that are frequent in maize (5, 6). Interdisciplinary efforts made to solve the global problem of food and feed contamination with *Fusarium* mycotoxins have placed the genus *Fusarium* in the focus of interest of researchers and experts in mycology, agronomy, plant pathology, food technology, toxicology, and animal health.

Our Department of Plant Pathology (Zagreb University Faculty of Agriculture) had conducted mycological analysis of seed and grain of many crops from 2002 to 2008. This analysis revealed the presence of *Fusarium* species on wheat, barley, oat, triticale,

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maize, tobacco, bean, pea, soybean, lupin, vetch, alfalfa, clover, flax, beet, spinach and lettuce seed, and grain. The level of contamination and population structure of *Fusarium* spp. in these substrates on grain could predict the presence of certain *Fusarium* mycotoxins (7-9). This paper presents the results of a six-year analysis of wheat, maize, soybean, and pea grain contamination with *Fusarium* species.

MATERIALS AND METHODS

Sample origin

Samples of maize (*Zea mays*), wheat (*Triticum aestivum*), soybean (*Glycine max*), and pea (*Pisum sativum*) originated from different locations in Croatia and were collected from 2002 to 2008. Grain was sampled from fungicide efficacy trials, cultivars, or hybrid selection trials, or was sent to the Department of Plant Pathology for seed health analysis by individual people and companies. Altogether 116 wheat samples, 48 maize samples, 32 soybean samples, and 7 pea samples were analysed.

Fusarium spp. quantification on grain

One hundred seeds were prepared per each sample. Wheat and maize kernels were placed on double wet blotters, left for 24 hours at 20 °C to 22 °C, then frozen at -20 °C for additional 24 hours, and placed in an incubator at 21 °C for 7 to 10 days. After the incubation, each seed was checked for the presence of fungi using stereomicroscope and light microscope. *Fusarium* colonies were identified by the conidia developed on grain or characteristic carmine red mycelia. If yellow or white sterile mycelial mass resembling *Fusarium* colony occurred on grain, fragments of mycelia were transferred to potato dextrose agar (PDA), incubated for 5 to 10 days, and then identified in culture. Contamination of sample with *Fusarium* spp. was expressed as the percentage of grains on which *Fusarium* colonies were determined. To prevent *Rhizopus stolonifer* or other fast-growing zygomycetous fungi to emerge from the kernels and impede detection of *Fusarium* spp. mycelia, the grain was surface-sterilised in 1 % NaOCl solution for one to three minutes, rinsed twice in sterile water, dried for one day and then placed on the blotter. One sample of maize, six samples of soybean, and two samples of pea were treated this way.

Isolation and identification of Fusarium species

Fusarium species on grain were identified on three samples of wheat from 2003, four samples of maize from 2005, five samples of soybean from 2008, and five samples of pea from 2007 and 2008. One hundred seeds were used for each sample. Wheat and maize kernels were incubated as described for the quantification of *Fusarium* spp. on grain. Soybean and pea grain was placed on *Fusarium*-selective medium (MBA) prepared according to Vujanović et al. (10). After 10 to 14 days, each *Fusarium* (or *Fusarium*-resembling) colony was transferred to PDA. Cultures on PDA were incubated at 21 °C under near-UV light, exchanging 12-hour light and dark cycles. Conidia developed in the cultures were suspended in sterile water and suspensions poured over water agar (WA) in Petri dishes. Twenty-four hours later, germinating spores were cut off from WA plates and placed on PDA. The obtained single-spore isolates were compared macroscopically, denoted, and cultivated on carnation leaf agar (CLA). The isolates were identified by the morphology of macroconidia and other morphological features according to instructions by Nelson et al. (11) and Balmas et al. (12). 2007 and 2008 isolates which were at first identified as *F. graminum*, *F. equiseti*, and *F. graminearum* were re-identified according to Leslie and Summerell (1). Identification was revised for two *F. graminum* isolates into *F. heterosporum*, one *F. equiseti* into *F. compactum*, and three *F. graminearum* into *F. pseudograminearum*. All isolates have been stored on WA at 4 °C and are available at the Department of Plant Pathology.

RESULTS

Fusarium species were found in all analysed samples (Table 1). *Fusarium* spp. contamination of grain ranged from 3 % (pea) to 100 % (maize). The range of *Fusarium* spp. contamination was 5 % to 69 % on wheat grain, 25 % to 100 % on maize grain, 4 % to 17 % on soybean grain and 3 % to 17 % on pea grain. Among plant species analysed, the highest percentage of *Fusarium* spp. contamination was determined on maize grain. Taking into account all 48 samples, average contamination with *Fusarium* spp. on maize grain was 77 %. Average *Fusarium* spp. contamination on all samples of wheat grain was 32 %, on soybean grain 8 %, and on pea grain 8 %.

Table 1 *Fusarium* spp. contamination (% of grain contaminated) of wheat, maize, soybean, and pea grain in different years

Year	Number of samples analysed	Range of <i>Fusarium</i> spp. contamination	Average <i>Fusarium</i> spp. contamination
Wheat			
2002	80	15 to 61	38
2003	30	12 to 69	35
2007	3	5 to 17	10
2008	3	28 to 61	46
Maize			
2005	44	71 to 100	91
2007	1	-	89
2008	3	25 to 97	50
Soybean			
2002	2	4 to 8	6
2003	1	-	5
2004	1	-	9
2005	7	6 to 11	9
2006	7	6 to 17	9
2007	9	4 to 8	7
2008	5	6 to 12	9
Pea			
2005	1	-	7
2007	1	-	8
2008	5	3 to 17	10

Nineteen *Fusarium* species were determined on grain of all four plant species analysed (Table 2). Eleven were determined on wheat grain, five on maize grain, 10 on soybean grain, and 10 on pea grain. The dominant species on wheat grain was *F. graminearum*, found in 27 % of isolates from wheat, followed by *F. poae* (22 %), *F. avenaceum* (16 %), and *F. verticillioides* (16 %). The most frequent species on maize grain was *F. verticillioides*, accounting for as much as 83 % of isolates from maize. On soybean grain *F. sporotrichioides* was the most common (34 %), followed by *F. verticillioides* (15 %), *F. equiseti* (13 %), and *F. semitectum* (11 %). *F. proliferatum* and *F. verticillioides* were the dominant species on pea grain, accounting for 29 % and 27 % of isolates from pea, respectively.

DISCUSSION

As *Fusarium* spp. contamination was determined in each grain sample analysed, it is possible that toxins produced by *Fusarium* spp. may have been present in all samples. Theoretically, *Fusarium* mycotoxins

can be produced on every plant and all plant parts infested by toxigenic *Fusarium* species. Over the last ten years, detection of mycotoxins and toxigenic *Fusarium* species has advanced significantly with the development of PCR-based techniques (13-16). However these techniques are still not widely used because they are expensive. This why standard mycological analysis of grain using blotters or agar media can still be useful in determining the extent of contamination with potentially toxigenic fungi such as *Penicillium* spp., *Aspergillus* spp., or *Fusarium* spp.

Our study suggests that the risk of contamination with *Fusarium* toxins is higher for maize and wheat than for soybean and pea. This is in accordance with several other studies conducted in Croatia, in which *Fusarium* mycotoxins were found in a high percentage of wheat or maize samples (17-20). Other Croatian studies confirmed high frequency of *Fusarium* species in maize grain (19, 21, 22). The main reason for this is probably because *F. verticillioides*, *F. proliferatum*, and *F. subglutinans* can grow systemically all along the plant, without causing symptoms on ears or kernels (5, 8). These symptoms may also not show if these fungi grow within the kernel and produce mycotoxins

Table 2 *Fusarium* species isolated from grain and toxigenic profiles of species determined

Plant species	Total number of isolates	Species isolated	Number of isolates	Mycotoxins potentially produced
Wheat	51	<i>F. graminearum</i>	14	DON, NIV, ZEN
		<i>F. poae</i>	11	NIV, BEA
		<i>F. avenaceum</i>	8	MON, BEA
		<i>F. verticillioides</i>	8	FUM
		<i>F. sporotrichioides</i>	3	T2, HT2
		<i>F. heterosporum</i>	2	-
		<i>F. crookwellense</i>	1	NIV, ZEN
		<i>F. tricinctum</i>	1	MON
		<i>F. semitectum</i>	1	MON, ZEN, BEA
		<i>F. oxysporum</i>	1	MON, BEA
Maize	41	<i>F. proliferatum</i>	1	FUM, BEA, MON
		<i>F. verticillioides</i>	34	FUM
		<i>F. graminearum</i>	4	DON, NIV, ZEN
		<i>F. sporotrichioides</i>	1	T2, HT2
		<i>F. solani</i>	1	-
Soybean	47	<i>F. sporotrichioides</i>	16	T2, HT2
		<i>F. verticillioides</i>	7	FUM
		<i>F. equiseti</i>	6	DAS, ZEN
		<i>F. semitectum</i>	5	MON, ZEN, BEA
		<i>F. pseudograminearum</i>	3	DON, ZEN
		<i>F. chlamyosporum</i>	2	MON
		<i>F. sambucinum</i>	2	DON, ZEN, MON
		<i>F. poae</i>	1	NIV, BEA
		<i>F. compactum</i>	1	-
Pea	48	<i>F. solani</i>	1	MON, DAS
		<i>F. proliferatum</i>	14	FUM, BEA, MON
		<i>F. verticillioides</i>	13	FUM
		<i>F. sporotrichioides</i>	4	T2, HT2
		<i>F. scirpi</i>	4	DAS, ZEN
		<i>F. semitectum</i>	3	MON, ZEN, BEA
		<i>F. oxysporum</i>	3	MON, BEA
		<i>F. poae</i>	2	NIV, BEA
		<i>F. avenaceum</i>	2	MON, BEA
		<i>F. equiseti</i>	2	DAS, ZEN
		<i>F. culmorum</i>	1	DON, NIV, ZEN

BEA=beauvericin; DAS=diacetoxyscirpenol; DON=deoxynivalenol; FUM=fumonisin; HT2=HT-2 toxin; MON=moniliformin; NIV=nivalenol; T2=T-2 toxin; ZEN=zearalenone

in an apparently healthy grain. Our 2007 and 2008 samples were asymptomatic, but high percentage of *Fusarium* spp. was still found on grain. The 2005 maize samples were mostly collected from plants affected with *Fusarium* ear rot (8).

Contamination of wheat samples in our study was high in 2002, 2003, and 2008, and relatively low in 2007. Contamination of wheat grain with *Fusarium*

spp. highly depends on environmental conditions from flowering to the harvest (3). Environmental conditions affect colonisation of wheat heads with different *Fusarium* spp. Regardless, *Fusarium* species are expected to colonise wheat grain every year and in every location where wheat is grown. Other studies from Croatia confirm that *Fusarium* spp. are among the most common fungi on wheat grain (23, 24).

In contrast to cereals, *Fusarium* spp. contamination of soybean and pea grain has received less attention. Judging from *Fusarium* spp. infestation, the risk of contamination with *Fusarium* toxins might be lower for soybean and pea than for maize and wheat. Nevertheless, several studies from different countries have confirmed the presence *Fusarium* mycotoxins in soybean and pea. Schollenberger et al. reported T-2 tetraol, HT-2 toxin, deoxynivalenol (DON), and zearalenone (ZEN) in soya meal. Interestingly, none of the pea samples contained *Fusarium* toxins (25). Park et al. (26) confirmed the toxicity of *Fusarium* isolates from soybean grain in experiments with rats, while Martinelli et al. (27) confirmed the ability of *F. graminearum* to produce DON and nivalenol (NIV) in soybean pots and seeds during vegetation. Clear et al. (28) detected DON and HT-2 toxin in soybean seeds, while Saber et al. (29) detected T-2 toxin in pea. Ćosić et al. (30) reported much lower *Fusarium* spp. contamination of soybean seed than in our study, but the presence of these fungi confirm that *Fusarium* toxins could be found on soybean in Croatia.

Nineteen *Fusarium* species found on wheat, maize, soybean, and pea grain show that this genus infests plant parts in a rich variety of species (31). Population structure of *Fusarium* spp. on grain can indicate which types of mycotoxins could be expected in a sample, based on the toxigenic profile of each species. *Fusarium* species found on grain in this study according to Leslie and Summerell (1) can produce DON, NIV, ZEN, fumonisins (FUM), beauvericin (BEA), diacetoxyscirpenol (DAS), HT-2 toxin (HT2), T-2 toxin (T2), moniliformin (MON), and a range of other less investigated toxins (1). Exposure to these toxins can seriously affect human or animal health (5-7, 17, 32-34). ZEN is an immunotoxic, haematotoxic, hepatotoxic, and genotoxic oestrogenic toxin (32) which can cause infertility, hyperoestrogenism or poor performance in domesticated animals (7, 32), and has been implicated in hyperoestrogenic syndromes in humans (32). MON is a cytotoxic and haematotoxic toxin which can cause poor performance in livestock or mortality in animals (7), and has been suspected as a cause of human heart disorder known as Keshan disease (7). BEA is cytotoxic and can cause apoptosis of murine and human cell lines (7). FUM is a cancer-promoting hepatotoxic and nephrotoxic toxin (5, 6), and can cause fatal animal diseases like leukoencephalomalacia in horses and pulmonary oedema in swine (5). Trichothecenes like DON, NIV, DAS, HT2, or T2 are haematotoxic,

cytotoxic, immunotoxic and embryotoxic (7, 33), and can cause reduced growth, postnatal mortality, foetal skeleton abnormalities, leucopenia, intrauterine growth retardation, pulmonary adenomas, or epithelial hyperplasia in animals (33, 34).

The dominance of *F. verticillioides* in maize grain in our study is in accordance with other studies conducted in Croatia (21, 22, 24). Its toxigenic profile implies that the most common mycotoxins in maize should be fumonisins or other toxins produced by this species. Research in Croatia has confirmed this assumption; Domijan et al. (19) found FB₁ in all tested samples, while Jurjević et al. (20) also found beauvericin.

In our study, *F. graminearum* was the most common species in wheat grain. These results are in accordance with Ćosić et al. (24) and Jurković et al. (35), confirming that *F. graminearum* is the dominant species on wheat in Croatia. The most important mycotoxins produced by *F. graminearum* are DON and ZEN. Several Croatian studies (17, 18, 36) have confirmed these mycotoxins as very frequent on wheat in Croatia.

F. poae, *F. avenaceum*, and *F. verticillioides* were relatively common on wheat in our study. *F. avenaceum* and *F. verticillioides* were also common in the study of Ćosić et al. (24). Their toxigenic profile of *F. avenaceum* and *F. verticillioides* implies that MON and FUM could be rather common in Croatian wheat. While MON is detected in wheat grain in many countries (9), fumonisins in wheat have been poorly investigated. Bottalico and Perrone (9) suggested that FB₁ could occur in wheat and other small-grain cereals.

A relatively high percentage of *F. sporotrichioides* in two soybean samples and of *F. proliferatum* and *F. verticillioides* in three pea samples point to a risk of T-2 toxin or fumonisin in these samples. The adverse effects of these two mycotoxins are well known in animals (5, 7, 32-34), and their presence in soybean or pea intended for human consumption could human health at risk. This calls for further research and surveillance of *Fusarium* mycotoxins in crops other than cereals in Croatia.

To the best of our knowledge, some of the *Fusarium* species have been reported by this study for the first time in Croatia. Although *Fusarium* species were often reported by other authors using different older names, no records have been found in Croatia of *F. heterosporum*, *F. crookwellense*, *F. pseudograminearum*, *F. sambucinum*, and *F.*

compactum or of their synonyms. Save for *F. heterosporum*, all of these species are potentially toxigenic (1, 2).

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Sažetak**KONTAMINACIJA ZRNA PŠENICE, KUKURUZA, SOJE I GRAŠKA VRSTAMA *FUSARIUMA* U HRVATSKOJ**

U periodu od 2002. do 2008. g. analizirana je prisutnost vrsta *Fusariuma* na 208 uzoraka zrna pšenice, kukuruza, soje i graška. Kontaminacija vrstama *Fusariuma*, izražena kao postotak sjemenki s kolonijama *Fusarium* spp., kretala se od 5 % do 69 % na pšenici, od 25 % do 100 % na kukuruzu, od 4 % do 17 % na soji te od 3 % do 17 % na grašku. Prosječna kontaminacija vrstama *Fusariuma* u različitim godinama varirala je od 10 % do 46 % na pšenici, od 50 % do 91 % na kukuruzu, od 5 % do 9 % na soji te od 7 % do 10 % na grašku. Vrste *Fusariuma* koje se javljaju na zrnu izolirane su i determinirane s odabranih uzoraka pšenice, kukuruza, soje i graška. Skupljeno je 187 izolata, a utvrđeno je 19 vrsta: *F. graminearum*, *F. poae*, *F. avenaceum*, *F. verticillioides*, *F. sporotrichioides*, *F. heterosporum*, *F. crookwellense*, *F. tricinctum*, *F. semitectum*, *F. oxysporum*, *F. proliferatum*, *F. solani*, *F. equiseti*, *F. pseudograminearum*, *F. chlamydosporum*, *F. sambucinum*, *F. compactum*, *F. scirpi* i *F. culmorum*. Dominantne vrste bile su *F. graminearum* na pšenici (27 % izolata), *F. verticillioides* na kukuruzu (83 % izolata), *F. sporotrichioides* na soji (34 % izolata) te *F. proliferatum* na grašku (29 % izolata). U Hrvatskoj su prvi put utvrđene vrste *F. heterosporum*, *F. crookwellense*, *F. pseudograminearum*, *F. sambucinum* i *F. compactum*.

KLJUČNE RIJEČI: determinacija vrsta, gljive, hrana, krma, mikotoksini

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