Damage to Corn by Fungi of the Genus *Fusarium* and the Presence of Fusariotoxins

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Abstract

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In 1998 and 1999 a total of 84 samples of corn, predominantly from localities in southern and central Moravia, were collected either directly from fields (entire ears at harvest maturity) or as grain from merchants. The objectives of the experiments were (a) to determine, on the basis of the results from mycological and toxicological analyses, the basic spectrum of fungal contaminants of corn in the Czech Republic with special reference to the genus *Fusarium*, and (b) to determine by enzyme immunoassay the presence of major toxic metabolites such as deoxynivalenol (DON), T-2 toxin (T-2), zearalenone (ZEA), and fumonisins (FUM) in grain samples. From naturally infected corn, representatives of seven fungal genera were isolated under *in vitro* conditions in both harvest years. Most frequent were species of the genus *Fusarium* (mean contamination of 44.6%). The next frequent genus was *Stemphylium* (29.3%). Eight species of *Fusarium* were found. In both years the most frequent species was *Fusarium graminearum* (1998 – 42.75%, 1999 – 41.8%), followed by *F. culmorum*. DON was found in 95.2% of the samples; its content ranged from 25 to 285 µg/kg. The content of T-2 varied more than that of DON, ranging from 12 to 875 µg/kg. Zearalenone content was more varied than that of the trichothecene-type compounds; 17% of the samples did not contain ZEA, the maximum content was 110 µg/kg. No FUM were found in 17% of the samples; in the others, FUM ranged from 12 to nearly 1000 µg/kg. Compared with the other three compounds, fumonisins showed generally the highest levels.

Keywords: *Fusarium* spp.; corn; mycotoxins; contamination; ELISA

Corn may be infected with a number of fungal pathogens during growth, harvest and storage. Species of the genera *Fusarium, Alternaria, Verticillium, Cladosporium* and *Epicoccum* affect it in the course of growth and harvest, while the dominant species in storage are species of the genera *Aspergillus* and *Penicillium* (KAMPHUIS *et al.* 1992). The list of economically and ecologically important diseases includes fungi of the genus *Fusarium.* They have been isolated from all parts of the plant, causing damage to emerging plants and inducing foot rot. Important diseases are also ear rot and grain rot (BOTTALICO *et al*. 1989).

The increased recent interest in members of the genus *Fusarium* stems mostly from their ability to produce toxic secondary metabolites. The contamination of food and feed causes great losses worldwide, chiefly by reducing the productivity of farm animals. The highest content of toxins occurs at the time of harvest and does not change much afterwards. Published data have revealed that 35 of the 61 *Fusarium* spp. isolated from plants had the potential to produce secondary toxic metabolites under laboratory conditions. So far, 137 secondary metabolic compounds have been described (DE NIJS *et al.* 1997).

The major groups of mycotoxins produced by *Fusarium* spp. are substances known as trichothecenes, zearalenone, fumonisins and fusaric acid.

Trichothecenes are a group of toxic compounds of sesquiterpenoid structure, which inhibit the synthesis of eukaryotic enzymes and cause mycotoxicoses of farm animals. They are also known to cause toxicoses such as

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alimentary toxic aleukia or akakabi disease in Japan (UENO 1983). Of this group T-2 toxin, HT-2 toxin, deoxynivalenol (syn. vomitoxin), nivalenol, diacetoxyscirpenol are most important. Zearalenone has a strong estrogenic effect connected with fertility disorders in farm animals (MIROCHA *et al*. 1977). In *Fusarium moniliforme* isolate (MRC 826), obtained from corn intended for human consumption in South Africa, the fumonisins B1 and B2 were isolated (GELDERBLOM *et al*. 1988; BEZUIDENHOST *et al*. 1988). Fumonisins are carcinogenic in laboratory rats and cause acute toxicoses of domestic animals (e.g. horse leucoencephalomalacia, pulmonary edema etc.). According to the International Agency of Cancer Research (IARC-WHO) they are classified as potential carcinogens for humans (class 2B). Fumonisins are structurally similar to sfingosin and can manifest their biological activity in blocking key enzymes of sfingolipid biosynthesis (NORRED 1993). An often neglected toxin produced by *Fusarium* spp. is fusaric acid. Its toxicity to warm-blooded animals is considerably lower than that of trichothecenes and fumonisins, but the latest experiments have indicated that this substance enhances the toxic effects of other fusariotoxins. This toxicological synergism can provide, to some extent, an explanation for the fact that feeding naturally contaminated feeds has, in many cases, markedly higher negative effects than feeding feeds artificially contaminated with the same toxin. Other toxins can also be synergistic.

The goals of the experiments were to determine on the basis of the results of mycological and toxicological analyses the basic spectrum of fungal contaminants of corn under conditions of the Czech Republic with special reference to the representatives of the genus *Fusarium*, and to make an attempt to detect the presence of basic toxic metabolites in grain samples.

MATERIAL AND METHODS

Samples of corn were collected predominantly from localities of southern and central Moravia in 1998 and 1999. They were taken directly from fields (entire corn ears at harvest maturity) or as grain supplied by merchants. In 1998, 42 samples were collected from 21 localities, and in 1999, 42 samples from 30 localities. The average weight of a sample of grain was 3000 g. On each sample a mycological analysis under *in vitro* conditions was done. One hundred grains/sample were placed on potato sucrose agar (PSA) in plates, the colonies of *Fusarium* spp. were then transferred onto fresh agar plates, and after monospore isolations the species were determined microscopically. Besides representatives of the genus *Fusarium*, other fungal contaminants of corn grain were also examined. The proportion of a particular fungal genus or species in the total number of colonies was expressed as percentage of contaminants (Tables 1 and 2).

An enzyme immunoassay for the determination of mycotoxin concentrations was applied to 42 samples. From each sample 1000 g grain was taken and ground. In accordance with the methodology for ELISA, 2–5 g of the representative sample was taken and after extraction an enzyme immunoassay was performed. The commercial kits Ridascreen were used for detection. The contents of the four basic toxic metabolites deoxynivalenol (DON), T-2 toxin (T-2), zearalenone (ZEA) and fumonisins (FUM) were determined. Each sample under study and each control sample containing a standard amount of a specific toxin were applied into two cells. Due to the capacity of the microtiter plate, 42 samples and 6 standards were analyzed. The ELISA kits used in the experiments are designed for comparative quantitative enzyme immunoassay of fusariotoxin residua not only in cereals, but also in animal feeds, blood serum, urine, beer etc.

RESULTS AND DISCUSSION

Contamination of corn grains with fungal pathogens

In both years, species of seven fungal genera were isolated from naturally infected corn grains. Species of the genus *Fusarium* occurred most frequently (mean contamination of 44.6%). The next frequent genus was *Stemphylium* (29.3%). Furthermore, representatives of the genera *Alternaria, Aspergillus, Mucor, Penicillium,* and *Rhizopus* were isolated. As there were no significant differences in the ratio of contaminating genera between the two years, only the spectrum of harvest year 1999 is shown in Table 1.

Occurrence of species of the genus *Fusarium*

The spectrum of isolates of this genus was comprised of eight species. In both harvest years the most frequent species was *Fusarium graminearum* (42.75% in 1998, 41.8% in 1999); it was followed by *F. culmorum* (35.8% in 1998, 32.3% in 1999). The occurrence of *F. avenaceum, F. moniliforme* and *F. proliferatum* was lower, while that of *F. poae, F. oxysporum* and *F. solani* was only light. Details of the species spectrum are given in Table 2.

Determination of fusariotoxins

The first group of chemically related fusariotoxins are the trichothecenes. They were used in the experiments to determine the concentrations of the two basic compounds deoxynivalenol and T-2 toxin. The first was found in 95.2% of the samples; DON concentrations ranged from 25 to 285 µg/kg, with most samples in the range of 100 to 200 µg/kg.

Table 1. Fungal contamination of corn grains in 1999

Samples	Percentage of contaminants																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Alternaria spp.	20	25	15	20	20	50	50	40	35	30	30	30	30	25	20	20	15	0	0	0	5	5
Aspergillus spp.	0	0	10	0	0	0	0	0	0	0	0	5	10	5	0	0	0	0	0	0	10	5
Fusarium spp.	65	75	10	20	60	0	0	10	25	30	35	15	20	20	20	20	15	50	55	50	10	10
Penicillium spp.	15	0	0	0	5	0	0	0	0	0	0	0	5	10	0	0	0	0	0	0	10	5
Stemphylium spp	0	0	75	60	15	50	50	50	30	40	40	30	25	30	60	60	65	50	45	50	0	0
Mucor spp.	0	0	0	0	0	0	0	0	0	0	0	20	10	10	0	0	5	0	0	0	50	60
Rhizopus spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	15	10

	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	A
Alternaria spp.	5	0	5	0	0	0	0	0	0	0	0	0	10	20	20	0	0	0	0	0	13.0
Aspergillus spp.	5	0	5	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.4
Fusarium spp.	10	15	15	20	100	80	60	60	100	50	100	80	80	40	25	100	70	60	100	90	44.6
Penicillium spp.	5	20	25	15	0	0	0	0	0	0	0	20	10	10	10	0	20	15	0	0	5.0
Stemphylium spp.	0	50	50	45	0	20	40	40	0	50	0	0	0	30	45	0	10	15	0	10	29.3
Mucor spp.	60	15	0	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5.8
Rhizopus spp.	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.9

A = average (%)

T-2 was detected in 92.9% of the samples. The concentrations of T-2 fluctuated more than those of DON and were in the range of 12 to 875 µg/kg. Many samples were, however, below the level of 100 µg/kg. Only in three cases was the concentration close to or higher than 400 µg/kg.

The concentration of zearalenone varied more than that of the trichothecene-type compounds. Some samples had very low concentrations (near zero), the maximum concentration did not exceed 110 µg/kg. This toxin was not found in 17% of the samples.

Fumonisins were not detected in seven samples out of 42 (17%). In the other samples the concentration ranged from 12 to nearly 1000 µg/kg. Compared with the other three toxins, this substance had the highest average level. A graphical presentation of the results of enzyme immunoassay of all four compounds is given in Fig. 1.

The overall results comparing the total concentrations of all four fusariotoxins in individual samples suggested that these four toxins were present in most samples, only in some samples was one of the compounds absent. Although individual toxic compounds have a rather different mechanism of action on target organisms, an attempt was made to summarize the concentrations of fusariotoxins in individual samples. It is evident from Fig. 2 that in several samples the total concentration exceeded the level of 1000 µg/kg, and that the largest percentage of samples fell into the category with a maximum level of 600 µg/kg.

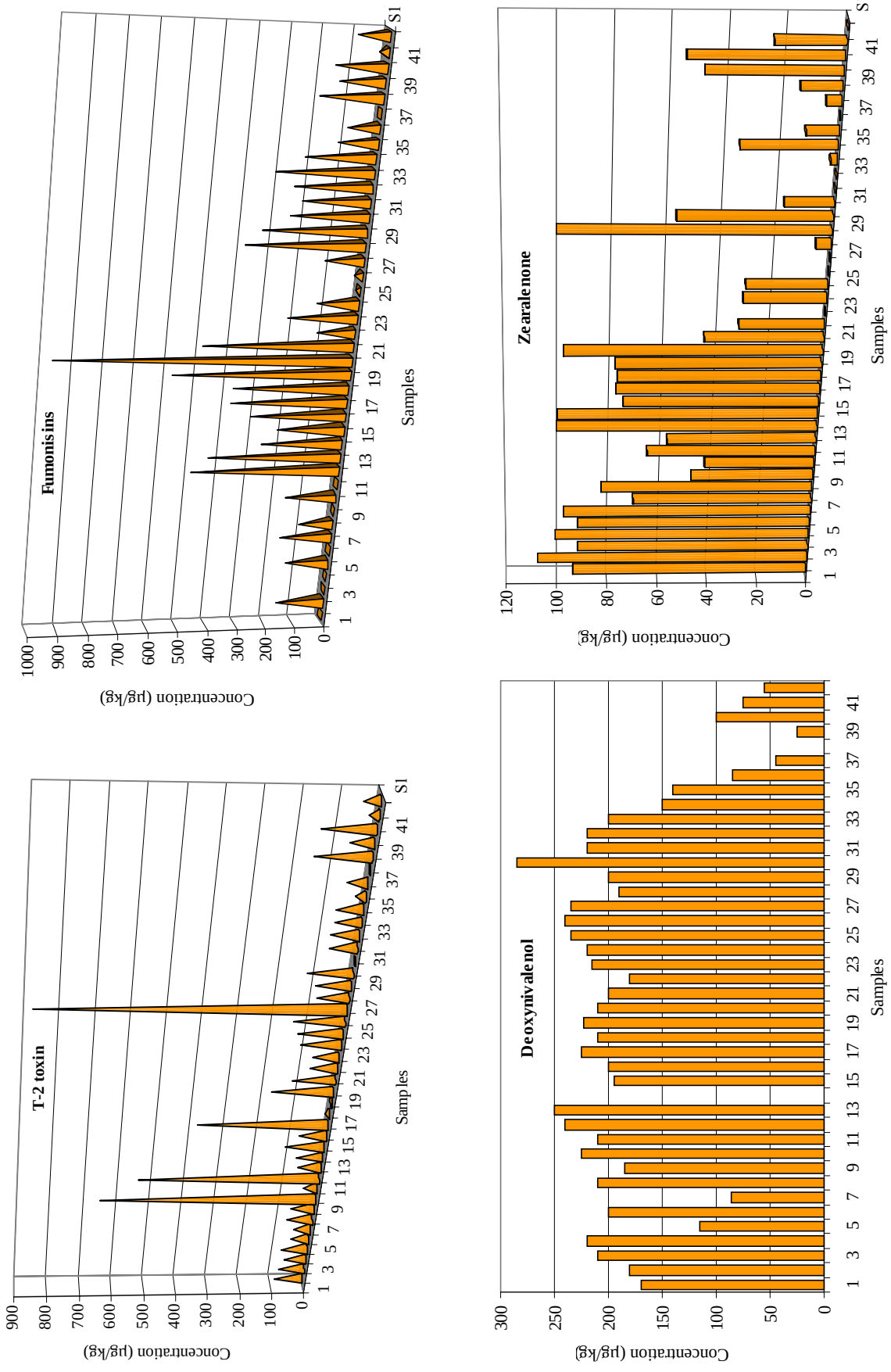

Fig. 1. Concentration of mycotoxins in corn grains

Table 2. Spectrum of *Fusarium* spp. on corn grains in 1999

	Percentage of contaminants																					
Fusarium	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
avenaceum	0	15	0	0	10	10	5	0	5	0	0	5	0	0	10	10	0	5	5	0	5	0
culmorum	30	25	75	40	35	35	45	45	45	40	35	35	50	30	10	5	20	10	10	15	40	30
gramin-earum	65	50	25	50	30	35	50	45	25	25	35	55	30	30	40	40	35	40	35	35	15	30
moniliforme	5	10	0	10	15	10	0	10	5	5	5	10	10	10	20	5	15	10	15	20	10	5
oxysporun	0	0	0	0	10	10	0	0	10	5	15	0	10	5	0	0	5	5	20	5	5	10
poae	0	0	0	0	0	0	0	0	0	5	0	0	0	10	5	10	15	15	0	0	10	15
proliferatum	0	0	0	0	0	0	0	0	10	20	10	0	0	15	15	20	10	10	15	10	10	15
solani	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	5	5	5
Others	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	10	0	0
		23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	A
avenaceum		5	0	5	0	10	10	5	0	0	0	10	10	10	5	5	0	0	0	0	0	3.9
culmorum		25	50	45	30	25	30	25	30	256	10	20	40	35	45	30	50	45	40	35	20	32.0
graminearum		20	50	50	30	50	50	65	50	70	50	70	40	45	40	50	50	50	40	35	35	42.0
moniliforme		10	0	0	10	15	10	5	10	5	10	0	5	10	10	10	0	5	5	10	10	8.2
oxysporun		10	0	0	20	0	0	0	10	0	20	0	5	0	0	0	0	0	15	10	5	4.9
poae		20	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	15	3.1
proliferatum		10	0	0	0	0	0	0	0	0	10	0	0	0	0	5	0	0	0	10	15	4.9
solani		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.6
Others		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.3

A = average (%)

The results from the mycological analyses that elucidated the species spectrum and quantified the occurrence of *Fusarium* spp. in corn samples, were compared with the total concentrations of toxins (Fig. 3). Through it is a comparison of relative values, the graph reveals a certain relationship between the two parameters. Most samples with a higher occurrence of *Fusarium* spp. also had a higher total concentration of fusariotoxins. Yet toxins were also found in samples in which no *Fusarium* species were mycologically detected (e.g. samples 6 and 7). This can result from some experimental mistake, but it is more likely a result of a phytopathological process during the vegetation period.

The species spectrum of *Fusarium* spp. isolated from infected corn plants is usually relatively wide, depending predominantly on the locality and the climatic conditions. In the United States, five species have been isolated (in order of frequency): *Fusarium graminearum, F. moniliforme, F. subglutinans, F. proliferatum* and *F. oxysporum.* When these were tested for their toxicogenic properties on rats, in most cases they caused bleeding and mortality (ABBAS *et al*. 1988). The most frequent species isolated in Italy were *Fusarium moniliforme, F. culmorum* and *F. proliferatum* (BOTTALICO *et al*. 1989). An interesting result, contradictory to some extent to the above, was published in 1993 (LOGRIECO *et al*. 1993). The authors did a mycological analysis of 14 corn samples from localities near Warsaw and in all of them the dominant species was *Fusarium subglutinans*, belonging systematically like *F. moniliforme* to the section *Liseola*. As the authors reported, the complete absence of *F. moniliforme* in naturally infected corn ears was quite unexpected. The explanation can be found in previous studies that indicated a certain geographic distribution of *F. moniliforme,* or its ecological niche may differ from that of *F. subglutinans.* Another study reported that the two most frequent pathogenic species occurring on corn worldwide were *F. moniliforme* and *F. proliferatum* (BULLERMAN & WEI-YUN 1994). In another study, these two species together with *F. subgluti-*

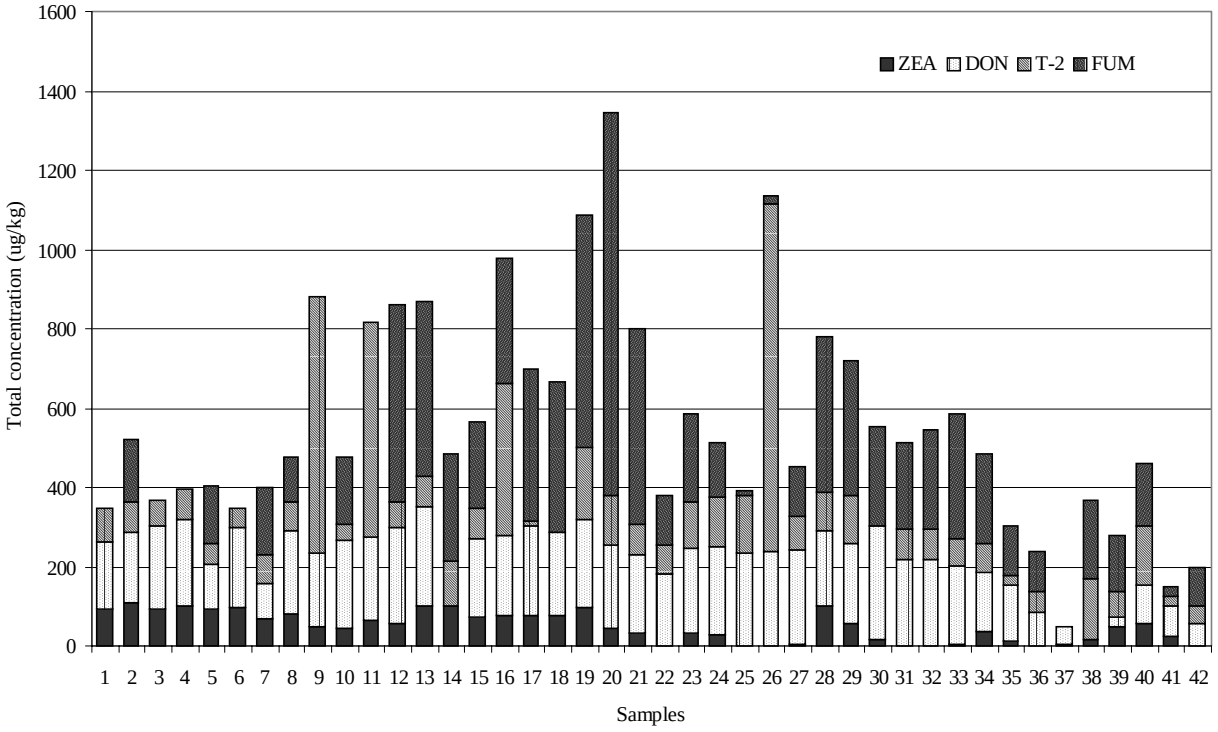

Fig. 2. Total amount of fusariotoxins in corn grains

nans were again described as best known on corn (COTTEN & MUWKVOLD 1998). In New Zealand the dominant species are *F. graminearum, F. culmorum, F. crookwelense, F. semitectum* and *F. poae*. Of interest is the low occurrence of *F. moniliforme* (DI MENNA *et al.* 1997). The species spectrum detected in corn samples in the present study is not much different, the dominant species are *F. graminearum* and *F. culmorum.*

The occurrence of mycotoxins produced by *Fusarium* spp. on corn has been reported from a number of world regions. Information about the worldwide contamination of cereals, including corn, were published by Japanese authors (TANAKA *et al*. 1988). A survey of 19 countries revealed the importance of mycotoxins of *Fusarium* spp. and their worldwide distribution. Of 500 samples, 244 were positive for nivalenol, 223 for deoxynivalenol and 219 for zearalenone. Detailed data on the contamination of cereal foods from samples obtained in 1997–1998 in the market in Germany were published in 1998 by USLEBER & MARTLBAUER. The highest level of contamination was in corn-based products. For example, the maximum concentration of deoxynivalenol was 910 µg/kg and that of fumonisins was 2600 µg/kg. In the United States corn-based products were also analyzed for the presence of fumonisins and the maximum concentration was up to 7450 ng/g (CASTELO *et al*. 1998). The studies carried out in New Zealand showed that corn grains were contaminated with detectable levels of NIV and DON (LAUREN 1991). In Korea corn grains were analyzed for the presence of trichothecenes DON and NIV, and zearalenone by using GLC and HPLC. Sample contamination ranged from 10.9% (NIV) to 65.2% (DON) (KIM *et al.* 1993). The natural occurrence of mycotoxins of *Fusarium* spp. was recorded in China in regions designated as high-risk areas for esophageal cancer. The authors concentrated on three trichothecenes and zearalenone (LUO *et al*. 1990). From southeastern Asia come findings concerning the production of fumonisin (MILLER *et al*. 1993).

Monitoring of the occurrence of fusariotoxins is being initiated in the Czech Republic. Data about the situation in the Czech Republic reported that after harvest in 1996, 54% of wheat and barley samples were contaminated, predominantly with DON and zearalenone at an average concentration of 605 µg/kg. In the same year, 76 samples of corn-based raw materials were also analyzed for the presence of fumonisins. The range of concentrations was from 9 to 984 µg/kg (PAULOVÁ 1998). As legislation determining the maximum allowable levels of fusariotoxins in food raw materials and feeds is being prepared, it is very difficult to interpret similar results. The first comprehensive document stating the limits of mycotoxin contents is EU standard 1525/98, effective 1 January 1999. According to FAO documents, only in some countries were the levels of contamination determined that may induce clinical symptoms of mycotoxicoses. In Austria the values for deoxynivalenol in wheat and rice are 500 µg/kg and for zearalenone in wheat and rice 60 µg per kg. The limits for zearalenone in foods in France are 200 µg/kg, the limits for fumonisins in Switzerland are 1000 µg/kg. The limits in North America for deoxyni-

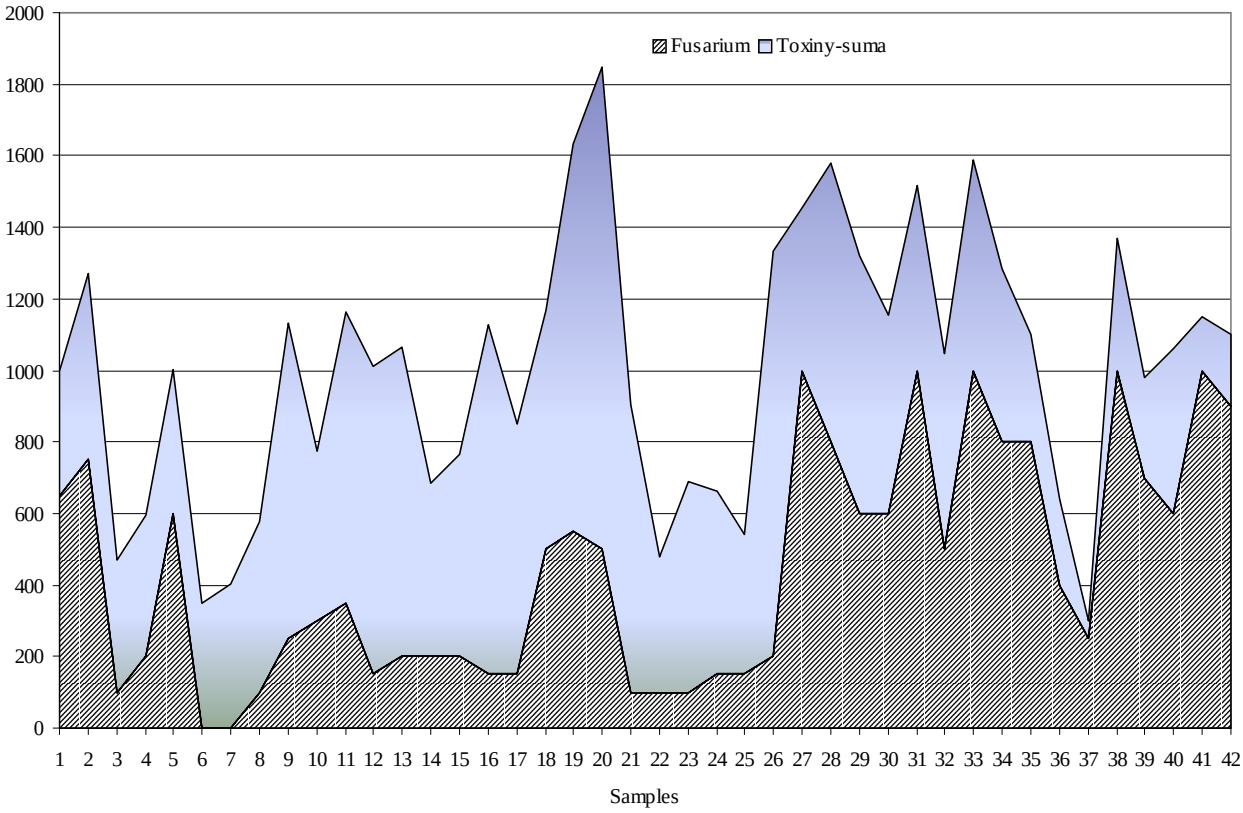

Fig. 3. Relationships between occurrence of *Fusarium* spp. and total amount of fusariotoxins

valenol in wheat are between 1000 µg/kg (United States) and 2000 µg/kg (Canada) (USLEBER & MARTLBAUER 1998). In general, the limits for most fusariotoxins in foods will be 1 mg/kg, depending, of course, also on acute toxicity. For illustration, the LD_{50} orally in mice is 70 mg per kg for deoxynivalenol, 20 mg/kg for zearalenone, but only 10 mg/kg for T-2 toxin.

Mycotoxins are detected by different techniques. Generally, the most accurate method, which is considered a standard procedure, is liquid chromatography. For routine screening, enzyme immunoassays (ELISA) or thin-layer chromatography are employed. Serological tests ELISA for the detection of fusaric mycotoxins in cereal grains were recently used in the United States for fumonisins (CASTELO *et al.* 1998) and for DON, ZEA and FUM in Germany (USLEBER & MARTLBAUER 1998). PESTKA *et al.* (1995) presented a broad survey (70 literature citations) on immunological techniques for the detection of mycotoxins in cereals and cereal-based food products. All these methods are sufficiently accurate and sensitive, and the comparison of results obtained by HPLC and ELISA techniques revealed high correlations (CASTELO *et al.* 1998).

In spite of that there are risk factors, which are to be minimized in mycotoxin analyses. The first is sample collection. With respect to the importance of this experimental stage, the sampling procedure was specified in 1994 in EU directive 98/53/EC. This directive determines the procedure of collecting feed samples for the detection of mycotoxin contents. The second factor is sample purification and extraction. If purification is not complete, mycotoxins may remain masked by other substances and this could give false negative results. In connection with the analysis of mycotoxins it is necessary to point out that mycotoxin content need not be correlated with the occurrence of native agents of these substances or with the content of their spores. The visible occurrence of a mycelial coating (white or pink in *Fusarium* spp.) may indicate a potential problem, but this must be argumented by some analytical detection of toxic substances.

In association with the consumption of contaminated feeds the potential occurrence of mycotoxin residua in food sources of animal origin is often discussed. The latest knowledge is that fumonisin residua are possible in milk, they are also probable at small amounts in pig kidneys and livers, while in pork and chicken meat and eggs they are improbable.

In the Czech Republic the problem of mycotoxicoses is limited predominantly to fusariotoxins, which are important contaminants of food and feed raw materials. In

growing seasons with excessive rainfall and relatively cold weather there is a great potential of increased contamination with these substances. On the basis of the results obtained so far, the hygienically and economically most important mycotoxins are DON and zearalenone. Especially DON could be considered a sort of indicator of total contamination with mycotoxins. The first Czech legal standard determining the level of mycotoxins is Food Act 110/97 of the Collection of Laws and regulation 298/97 stating the limits for aflatoxins, patulin, ochratoxin A and deoxynivalenol (e.g. the limit for flour is 1 mg/kg).

The facts presented emphasize the importance of studies of these pathogens and the diseases caused by them in the Czech Republic. Knowledge of the species spectrum of *Fusarium* spp. is essential not only for the studies of toxicological properties but also for the selection of sources of resistance in breeding programs. This type of protection will play an important role in the future and the testing of the level of resistance in initial breeding lines will become a necessity.

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Souhrn

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V letech 1998 a 1999 bylo shromážděno celkem 84 vzorků kukuřice především z lokalit jižní a střední Moravy. Vzorky byly odebírány jednak přímo z porostů (celé palice ve sklizňové zralosti), jednak bylo získáno zrno z výkupních závodů. Cílem experimentů bylo na základě výsledků mykologických a toxikologických analýz určit základní spektrum houbových kontaminantů kukuřice v podmínkách ČR se zvláštním zřetelem na zástupce rodu *Fusarium* a pokusit se ve vzorcích zrna imunoenzymatickou cestou detekovat přítomnost základních toxických metabolitů: deoxynivalenol (DON), T-2 toxin (T-2), zearalenon (ZEA) a fumonisiny (FUM). Z přirozeně infikovaných kukuřičných zrn byli v obou sledovaných sklizňových letech v *in vitro* podmínkách izolováni zástupci celkem 7 houbových rodů. S nejvyšší intenzitou se vyskytovaly druhy rodu *Fusarium* (průměrná kontaminace 44,6%), druhým nejčastěji se vyskytujícím byl rod *Stemphylium* (29,3 %). Spektrum izolovaných druhů rodu *Fusarium* bylo tvořeno 8 druhy. V obou sledovaných sklizňových ročnících byl druhem s nejvyšší frekvencí *Fusarium graminearum* (1998 – 42,75 %, resp. 1999 – 41,8 %), druhým nejčastěji se vyskytujícím druhem bylo *F. culmorum*. Na obsah deoxynivalenolu bylo positivně analyzováno 95,2 % vzorků. Koncentrace DON se pohybovaly v rozpětí od 25 do 285 µg/kg. Koncentrace T-2 byly zaznamenány v širším rozpětí než u předešlého toxinu a pohybovaly se od 12 do 875 µg/kg. Koncentrace zearalenonu byly variabilnější než v případě sloučenin trichothecénového typu. V některých vzorcích byly zaznamenány nulové koncentrace (17 % vzorků), maximální koncentrace nepřesáhla 110 µg/kg. Nulová koncentrace fumonisinů byla u 7 vzorků z celkového počtu 42 (tj. 17 %), u ostatních vzorků se detekované rozpětí pohybovalo od 12 do téměř 1000 µg/kg. Ve srovnání s ostatními třemi zkoumanými sloučeninami bylo u této látky dosaženo v průměru nejvyšších hodnot.

Klíčová slova: *Fusarium* spp.; kukuřice; mykotoxiny; kontaminace; ELISA

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