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Toxigenic *Fusarium* species of *Liseola* section in pre-harvest maize ear rot, and associated mycotoxins in Slovakia

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Abstract

The occurrence of *Fusarium* species of *Liseola* section and related toxins was investigated for two years (1996 and 1998) on maize ear rot samples collected in the most important areas for maize growing in Slovakia. The species most frequently isolated was *F. verticillioides*, followed by *F. proliferatum* in 1996 and *F. subglutinans* in 1998. Most of the strains belonged to mating populations A, D, and E of the teleomorph *Gibberella fujikuroi. Fusarium graminearum* was also frequently recovered in both the years of investigations. Toxin analysis of maize ears showed that most of the samples (21 out of 22) were contaminated with at least one toxin. In particular, the concentration of fumonisin B_1, and fumonisin B_2 was up to 26.9 and 5.1 $\mu g\, g^{-1}$, respectively in 1996, and up to 12.1 and 6.3 $\mu g\, g^{-1}$, respectively in 1998. Beauvericin was detected only in one sample in 1996. Seven samples in 1996 were contaminated by fusaproliferin up to 8.2 $\mu g\, g^{-1}$, but just traces of the toxin were found in one sample in 1998. All 29 strains of *F. verticillioides*, two of three strains of *F. proliferatum* and none of eight *F. subglutinans* strains isolated from samples produced fumonisin B_1 in culture on whole maize kernels (0.1–5646 and 940–1200 $\mu g\, g^{-1}$, respectively). Two strains of *F. subglutinans* and two of *F. proliferatum* produced beauvericin (up to 65 and 70 $\mu g\, g^{-1}$, respectively). Ten strains of *F. verticillioides* produced beauvericin: 9 strains produced a low amount (up to 3 $\mu g\, g^{-1}$), while only one of them produced a high level of toxin (375 $\mu g\, g^{-1}$). Fusaproliferin was produced by two *F. proliferatum* strains (220 and 370 $\mu g\, g^{-1}$), by seven *F. subglutinans* (20–1335 $\mu g\, g^{-1}$) and by three *F. verticillioides* (10–35 $\mu g\, g^{-1}$). This is the first report on fusaproliferin production by *F. verticillioides*, although at low level.

Introduction

Among *Fusarium* species belonging to section *Liseola, F. verticillioides* (Sacc.) (syn. *F. moniliforme*), *F. subglutinans* (Wollenw. et Reinking) Nelson, Toussoun et Marasas and *F. proliferatum* (Matsushima) Nirenberg are well-known pathogens of maize, causing stalk and ear rot worldwide (Nelson et al., 1981; Leslie et al., 1990; Logrieco et al., 1993; Bottalico, 1998). These species colonize maize plants differently according to the stages of maturity of maize (Chulze et al., 1998), different geographical areas, and environmental conditions (Bottalico, 1998). *Fusarium subglutinans* has a lower optimum temperature for growth and predominates in more temperate areas than *F. verticillioides* (Nelson et al., 1981). Indeed, *F. subglutinans* was reported as the most prevalent ear rot agent in the main maize growing areas of central and northern Europe, such as Austria, Slovenia, and Poland (Lew et al., 1996; 1997; Levic et al., 1997; Milevoj, 1997). In contrast, *F. verticillioides* and *F. proliferatum*, although reported all over Europe, are more frequently isolated during hot, dry seasons principally in central and southern Europe, including

Italy (Logrieco et al., 1995; Ritieni et al., 1997), and Spain (Sanchis, Food Technology Department, Spain, personal communication).

Information on the geographical distribution of these species is of importance in predicting exposure of maize to toxic secondary metabolites produced by these fungi and in the assessment of the relative toxicological risk. Each of these species has a specific toxin profile and can produce potent mycotoxins, including fumonisins (Nelson et al., 1993), fusaric acid (Bacon et al., 1996), and moniliformin (Marasas et al., 1984), and the more recently reported beauvericin (BEA) and fusaproliferin (FUP) (Moretti et al., 1996). Among these mycotoxins, fumonisin B_1 (FB_1), a toxin produced by both *F. verticillioides* and *F. proliferatum* (Nelson et al., 1993), is a well-known contaminant of maize (Munkvold and Desjardins, 1997) and has been related to oesophageal cancer in South Africa (Rheeder et al., 1992) and is responsible for several animal diseases, for example, leukoencephalomalacia in horses (Kellerman et al., 1990) and pulmonary oedema in swine (Harrison et al., 1990). Fusaproliferin is a toxic metabolite isolated from cultures of *F. proliferatum* and *F. subglutinans* (Moretti et al., 1996). It is a sesquiterpene compound that has toxic activity against brine shrimp (*Artemia salina* L.), insect cells, and human B lymphocytes, and has a teratogenic effect on chicken embryos (Logrieco et al., 1996; Ritieni et al., 1997). Beauvericin is a cyclodepsipeptide compound, known to have insecticidal properties (Grove and Pople, 1980) and to be produced by *F. proliferatum* and *F. subglutinans* (Moretti et al., 1996). It is also highly toxic to human cell lines (Macchia et al., 1995) and to be a very potent channel-forming molecule inducing pores in biological membranes (Lemmens et al., 2000). Evidence suggests that mycotoxins may have synergistic effects *in vivo* (Marasas et al., 1984). Therefore, the detection of more than one toxin with different biological activity occurring in infected maize samples is useful to better evaluate the risk due to human and animal consumption of contaminated maize.

Gibberella fujikuroi (Sawada) Ito in Ito and K. Kimura is the teleomorph of *F. verticillioides, F. proliferatum*, and *F. subglutinans* (Leslie, 1995). *Fusarium subglutinans* from maize usually corresponds to mating population E of *G. fujikuroi*, whereas *F. proliferatum* and *F. vericillioides* from maize correspond to mating populations D and A, respectively (Leslie, 1995). Each mating population has its own toxicological profile, that could reflect important differences in pathogenicity, natural history, and ecology of mating populations (Leslie, 1995; Moretti et al., 1996).

The aims of this study were: (a) to investigate the most commonly occurring *Fusarium* species causing ear rot in the main maize areas in Slovakia over two years of analysis; (b) to detect the possible contamination of *Fusarium* toxins on maize kernels; (c) to assess the mating population of the strains isolated from maize kernels and belonging to the *G. fujikuroi* complex, and to analyse the toxicological profile of each strain.

Materials and methods

Maize samples

Samples (around 50 visibly mouldly maize ears per sample) were collected at pre-harvest from different maize fields in the 11 most important maize production areas of Slovakia (see Table 1) during the 1996 and 1998 crop seasons. The samples were stored at 4 °C.

Mycological analyses

One hundred visibly infected kernels from each sample (20 kernels per ear from five randomly chosen ears) were placed in Petri dishes (five kernels per plate, with each broken into two pieces) containing a modified pentachloronitrobenzene medium selective for *Fusarium* (Nelson et al., 1983). Single-spores of putative *Fusarium* colonies were transferred to carnation leaf agar for identification, then mycelium and conidia from each strain were frozen in sterile 18% glycerol–water and stored at −75 °C in the Istituto Tossine e Micotossine da Parassiti Vegetali (ITEM) culture collection and given an accession number. One or more strains from each sample were characterized for mating population and *in vitro* production of BEA, FB_1, and FUP.

Fertility tests

Tester strains for mating population tests were received from J.F. Leslie, Kansas State University. Strains in the present study were crossed on carrot agar as male parents with tester strains of mating population A through F (Klittich and Leslie, 1988). Mated cultures were considered fertile if perithecia formed within 6 weeks. All strains were crossed twice with both testers from each mating population.

In vitro *toxin production*

Single-conidium strains of fungal cultures were grown on 100 g of autoclaved yellow maize kernels adjusted to about 45% moisture in 500 ml Erlenmeyer flasks and inoculated with 2 ml of an aqueous suspension containing approximately 10^7 conidia ml^{-1}. Cultures were incubated at 25 °C for 4 weeks. The harvested culture material was dried in a forced draft oven at 60 °C for 48 h, finely ground, and stored at 4 °C until use. Controls were treated the same way, except that they were not inoculated.

Toxin analyses

For BEA, FUP, and fumonisins extraction the procedure described by Munkvold et al. (1998) was followed. Standards of FB_1 and FB_2 and BEA were purchased from Sigma Chemical Co., St. Louis, MO; fusaproliferin was isolated in the laboratory of the Department of Scienza degli Alimenti from maize kernels inoculated by a *F. proliferatum* strain (Ritieni et al., 1995). Analysis of FB_1 and FB_2 were performed by following procedures of Doko and Visconti (1994), while for strains isolated from 1996 samples, FB_1 was analysed following the procedure previously described by Munkvold et al. (1998). The amount of BEA and FUP were determined by high-performance liquid chromatography (Munkvold et al., 1998). The detection limit was 0.1 $\mu g\,g^{-1}$ for BEA, and 0.025 $\mu g\,g^{-1}$ for FUP. All analyses were run in triplicate and the mean values are reported. Calculated standard deviation was always lower than 5%.

Results

Occurrence of Fusarium *species*

The species most frequently isolated from maize kernels were the anamorphs of *G. fujikuroi* and *F. graminearum* Schwabe (Tables 1 and 2) in both years of the investigations. In particular, 12–100% of the ears were contaminated with *F. verticillioides* in 1996 and 4–95% were contaminated in 1998. In addition, *F. proliferatum* occurred in 10–94% in all samples in 1996, but in just four samples in 1998 (from 8% to 88%), while *F. subglutinans* was very seldom identified in 1996 (6–11%), although it was found in 10 samples in 1998, at a level of 7–42% (Tables 1 and 2). Three other species were occasionally isolated from the maize samples: *F. avenaceum* was isolated from one sample in 1996 (12%), *F. compactum* from one sample in 1998 (4%), and *F. equiseti* from three samples in both years (from 3% to 56%).

Toxin contamination of maize samples

Data on toxin contamination are presented in Table 1 for 1996 and Table 2 for 1998. The maize samples were contaminated with FB_1, and FB_2 (up to 26.9 and 5.1 $\mu g\,g^{-1}$, respectively) in 1996, while in 1998 the contamination by these toxins was at a lower concentration (up to 12.1 and 6.3 $\mu g\,g^{-1}$, respectively). Beauvericin was detected just in one sample in 1996 but it was not detected in any sample in 1998. Seven samples in 1996 were contaminated by FUP (up to 8.2 $\mu g\,g^{-1}$), but just traces of the toxin were found in one sample in 1998. Among the samples from both years, only sample 8/96 was contaminated with all four toxins for which tests were carried out.

Toxin production and mating population of Fusarium *strains*

The data on the production by cultures of BEA, FB_1, FB_2, and FUP *in vitro* and the results of fertility tests are reported in Table 3. Twenty-eight strains from 1996 were investigated and 12 from 1998. Most strains proved to be fertile (32 out of 40): 25 out of 29 *F. verticillioides* strains belonged to mating population A, 2 out of 3 *F. proliferatum* strains belonged to mating population D and 6 of 8 strains of *F. subglutinans* belonged to mating population E. Among the 29 strains of *F. verticillioides*, FB_1 was produced at a significant level by 26 strains (from 230 to 5645 $\mu g\,g^{-1}$), while 3 strains produced it at a very low level (up to 2 $\mu g\,g^{-1}$); 2 of 3 *F. proliferatum* strains produced FB_1 (up to 1200 $\mu g\,g^{-1}$), and none of the 8 strains of *F. subglutinans* analysed produced the toxin. Beauvericin was produced by 2 of 3 strains of *F. proliferatum* and by 2 of 8 strains of *F. subglutinans* (up to 65 $\mu g\,g^{-1}$). Finally, BEA was produced among 10 strains of *F. verticillioides* at a low level (traces to 1 $\mu g\,g^{-1}$) and 1 strain, ITEM 2636, at a high level (375 $\mu g\,g^{-1}$). Fusaproliferin was produced by 3 strains of *F. verticillioides* (10–35 $\mu g\,g^{-1}$), 2 of *F. proliferatum* (220–370 $\mu g\,g^{-1}$) and 7 strains of *F. subglutinans* (20–335 $\mu g\,g^{-1}$).

Table 1. Occurrence of *Fusarium* species and toxin contamination ($\mu g\, g^{-1}$) in maize kernel samples collected in 1996 in Slovakia

Sample	Origin	*Fusarium* species	(%)	FB_1*	FB_2*	BEA*	FUP*
1	Trnovec nad Váhom	*F. graminearum*	(95)	0.04	0.05	n.d	0.35
		F. proliferatum	(10)				
		F. verticillioides	(65)				
2	Kollárovo	*F. graminearum*	(64)	0.02	0.01	n.d.	n.d.
		F. verticillioides	(54)				
		F. subglutinans	(11)				
3	Vlčkovce	*F. proliferatum*	(86)	0.25	0.04	n.d.	n.d.
		F. verticillioides	(45)				
		F. graminearum	(4)				
4	Zemplínska Šírava	*F. proliferatum*	(94)	20	0.01	n.d.	2.2
		F. graminearum	(43)				
5	Topolníky	*F. proliferatum*	(92)	0.03	0.01	n.d.	n.d.
		F. verticillioides	(12)				
		F. graminearum	(8)				
6	Demandice	*F. verticillioides*	(100)	26.9	5.1	n.d.	n.d.
		F. proliferatum	(15)				
7	Čalovo	*F. verticillioides*	(65)	0.02	0.02	n.d	0.7
		F. graminearum	(18)				
8	Vojany	*F. proliferatum*	(87)	0.01	0.01	3.0	8.2
		F. verticillioides	(40)				
		F. graminearum	(12)				
9	Ožďany	*F. verticillioides*	(80)	0.01	0.01	n.d.	7.3
		F. proliferatum	(70)				
10	Vel'ké Kapušany	*F. proliferatum*	(75)	n.d.	0.005	n.d.	7.7
		F. equiseti	(56)				
		F. verticillioides	(13)				
		F. subglutinans	(11)				
11	Ivanka pri Dunaji	*F. verticillioides*	(72)	0.01	0.01	n.d.	0.2
		F. graminearum	(21)				
		F. avenaceum	(12)				

*: FB_1 = fumonisin B_1; FB_2 = fumonisin B_2; BEA = beauvericin; FUP = fusaproliferin; n.d. = not detected.

Discussion

Slovakia is a region that has not been well investigated for the incidence of *Fusarium* ear rot on maize. The data reported here show that *F. graminearum* and species belonging to section *Liseola* occurred on maize kernels in both years. Occurrence and prevalence of *Fusarium* species in maize ears from different regions and years mainly depended on the temperature and rainfall (Bottalico, 1998). The data reported here agree with reports from central and northern Europe that *F. graminearum* is one of the main species isolated from maize ear rot (Chelkowski, 1989; Bocarov-Stancic et al., 1997). The data on *F. verticillioides* confirmed that it was one of the main species from maize in central Europe, as reported in Croatia (Jurjevic et al., 1997) and Yugoslavia (Levic et al., 1997). *Fusarium subglutinans*, reported as the most important species on maize in Austria (Lew et al., 1997), Poland (Logrieco et al., 1993), and together with *F. verticillioides* in Yugoslavia (Levic et al., 1997) occurred mainly during the second year of investigation in the samples from Slovakia, while *F. proliferatum* was more frequently isolated in the first year. The occurrence of these two species could be due to different climatic conditions occurring during the two years of investigation.

The significant infection by *Fusarium* species of all maize samples explained the contamination of all the samples with at least one of the toxins analysed. The co-occurrence of *Fusarium* toxins was reported from several areas (Logrieco et al., 1993; Ritieni et al., 1997; Munkvold et al., 1998). However, this is the first report of the occurrence of *Fusarium* species and maize contamination by fumonisins, BEA, and FUP in Slovakia, and is a further contribution to the knowledge of the distribution of toxigenic fungi and related

Table 2. Occurrence of *Fusarium* species and toxin contamination ($\mu g\,g^{-1}$) in maize kernel samples collected in 1998 in Slovakia

Sample	Origin	*Fusarium* species	(%)	FB_1*	FB_2*	BEA*	FP*
1	Trnovec nad Váhom	*F. verticillioides*	(85)	1.85	0.46	n.d	n.d.
		F. subglutinans	(42)				
		F. proliferatum	(7)				
		F. graminearum	(18)				
2	Kollárovo	*F. proliferatum*	(88)	4.55	1.02	n.d.	traces
		F. verticillioides	(47)				
		F. graminearum	(12)				
		F. subglutinans	(7)				
3	Vlčkovce	*F. verticillioides*	(95)	12.1	6.35	n.d.	n.d.
		F. subglutinans	(18)				
4	Včeláre	*F. graminearum*	(98)	0.01	0.04	n.d.	n.d.
		F. subglutinans	(18)				
		F. verticillioides	(4)				
		F. equiseti	(3)				
5	Topol'níky	*F. graminearum*	(74)	0.01	0.03	n.d.	n.d.
		F. subglutinas	(18)				
		F. verticillioides	(11)				
6	Demandice	*F. verticillioides*	(68)	1.25	0.25	n.d.	n.d.
		F. graminearum	(34)				
		F. proliferatum	(8)				
7	Čalovo	*F. graminearum*	(72)	0.01	0.02	n.d	n.d.
		F. subglutinans	(17)				
		F. proliferatum	(14)				
8	Vojany	*F. graminearum*	(82)	0.01	0.05	n.d.	n.d
		F. verticillioides	(18)				
		F. equiseti	(21)				
		F. subglutinans	(9)				
		F. compactum	(4)				
9	Ožďany	*F. verticillioides*	(85)	9.2	3.0	n.d.	n.d.
		F. subglutinans	(12)				
		F. graminearum	(9)				
10	Hradište	*F. graminearum*	(78)	0.1	0.25	n.d.	n.d.
		F. verticillioides	(4)				
11	Ivanka pri Dunaji	*F. graminearum*	(98)	0.01	0.01	n.d.	n.d.
		F. verticillioides	(59)				
		F. subglutinans	(14)				

*: FB_1 = fumonisin B_1; FB_2 = fumonisin B_2; BEA = beauvericin; FUP = fusaproliferin; n.d. = not detected.

toxins in Europe. Some of the samples were contaminated with the toxins at significant levels (Tables 1 and 2) and in particular, samples 8/1996 was contaminated with all four toxins. Fumonisin B_1 was reported to induce apoptosis in several types of cultured human cells (Tolleson et al., 1996) and also showed clear evidence for its carcinogenicity in male rats and female mice (US NTP, 1999). The occurrence of several samples with levels of FB_1 contamination higher than $4\,\mu g\,g^{-1}$ (Tables 1 and 2), which is the recommended limit for human foods by US FDA (FDA home page: *http://vm.cfsan.Fda.gov*), is cause for concern in relation to the possible consumption of such contaminated maize. Moreover, BEA has also been shown to be highly toxic to human cell lines, inducing apoptosis (Macchia et al., 1995), and FUP was reported to cause apoptosis in a mammalian cell line (Di Paola et al., 1998). Therefore, the assessment in the same sample of all these toxins is necessary not only because of the serious risk for the health of humans and animals but also because of their possible synergistic effects.

Most of the strains tested were fertile and belonged to mating populations A, D, and E of *G. fujikuroi*. This agrees with previous reports that considered these mating populations as the most frequent on maize (Leslie, 1995). With respect to toxin production, each

Table 3. Toxin production ($\mu g\,g^{-1}$) by *Fusarium* strains of *Liseola* section isolated from maize kernels in Slovakia and results of mating population (MP) identification

ITEM	Sample	Species	MP	FB_1*	BEA*	FUP*
2617	1–96	*F. verticillioides*	MATA-2	1350	n.d.	n.d.
2618	1–96	*F. verticillioides*	MATA-2	450	n.d.	10
2619	1–96	*F. verticillioides*	MATA-1	1125	n.d.	n.d.
2621	2–96	*F. proliferatum*	n.f.	n.d.	55	220
2622	2–96	*F. verticillioides*	MATA-2	650	n.d.	n.d.
2624	2–96	*F. subglutinans*	MATE-1	n.d.	n.d.	800
2625	3–96	*F. verticillioides*	MATA-2	400	n.d.	n.d.
2628	3–96	*F. verticilliodes*	MATA-1	600	n.d.	n.d.
2629	3–96	*F. verticillioides*	MATA-2	870	n.d.	n.d.
2631	4–96	*F. proliferatum*	MATD-2	1200	n.d.	n.d.
2633	4–96	*F. subglutinans*	MATE-2	n.d.	n.d.	340
2635	4–96	*F. proliferatum*	MATD-2	940	70	370
2636	5–96	*F. verticillioides*	MATA-2	650	375	15
2637	5–96	*F. verticillioides*	MATA-2	860	n.d.	n.d.
2638	5–96	*F. verticillioides*	MATA-2	525	n.d.	n.d.
2640	6–96	*F. verticillioides*	MATA-1	620	n.d.	n.d.
2642	6–96	*F. verticillioides*	n.f.	280	n.d.	n.d.
2643	6–96	*F. verticillioides*	n.f.	230	n.d.	n.d.
2644	8–96	*F. verticillioides*	n.f.	270	n.d.	n.d.
2645	8–96	*F. subglutinans*	MATE-1	n.d.	n.d.	1010
2646	8–96	*F. subglutinans*	MATE-1	n.d.	n.d.	n.d.
2647	8–96	*F. subglutinans*	MATE-1	n.d.	n.d.	880
2648	9–96	*F. subglutinans*	n.f.	n.d.	65	620
2649	9–96	*F. verticillioides*	MATA-1	430	n.d.	35
2650	9–96	*F. verticillioides*	MATA-2	1220	n.d.	n.d.
2652	10–96	*F. verticillioides*	n.f.	250	n.d.	n.d.
2653	10–96	*F. verticillioides*	n.f.	200	n.d.	n.d.
2657	10–96	*F. subglutinans*	MATE-1	n.d.	n.d.	1335
3412	8–98	*F. verticillioides*	MATA-2	0.2	1	n.d.
3413	8–98	*F. verticilioides*	MATA-2	0.1	n.d.	n.d.
3414	11–98	*F. subglutinans*	n.f.	n.d.	30	20
3415	11–98	*F. verticillioides*	MATA-1	0.1	traces	n.d.
3416	8–98	*F. verticiolliodes*	MATA-2	1625	1	n.d.
3417	8–98	*F. verticillioides*	MATA-2	470	3	n.d.
3418	8–98	*F. verticillioides*	MATA-2	2100	1	n.d.
3419	8–98	*F. verticillioides*	MATA-2	3280	1	n.d.
3420	8–98	*F. verticillioides*	MATA-2	3160	2	n.d.
3421	8–98	*F. verticillioides*	MATA-2	1150	1	n.d.
3422	8–98	*F. verticillioides*	MATA-2	5645	1	n.d.
3423	11–98	*F. verticillioides*	MATA-1	2820	1	n.d.

*: FB_1 = fumonisin B_1; FB_2 = fumonisin B_2; BEA = beauvericin; FUP = fusaproliferin; n.d. = not detected; n.f. = not fertile.

mating population is considered to possess its own toxicological profile (Leslie et al., 1992; Moretti et al., 1996). Among the isolates tested in this study, FB_1 was produced only by strains of mating populations A and D, while data relating to BEA and FUP were different to our previous reports, since an isolate of mating population A (ITEM-2636) produced both these toxins (Moretti et al., 1996). Further studies using molecular tools could help to understand the biology of ITEM-2636. Since BEA was produced at low levels by few of the tested strains, we believe that the exposure of maize to this toxin in Slovakia could be low. However, further data are needed to support this observation. Since three strains of mating population A produced FUP, we can conclude that this is the first report of FUP production by members of mating population A

and consequently of the *F. verticillioides* anamorph. Some of the strains were not fertile, but they produced typical mycotoxins of the anamorphs to which they belong. However, the high level of fertility found among the strains reported, confirmed previous investigations (Leslie, 1995). This is important because of the high possibility of genetic recombination in the field that could increase the genetic pool available for pathogenetic and toxigenic populations of different species of *Liseola* section on maize in Slovakia.

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