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Fusarium species and mycotoxin profiles on commercial maize hybrids in Germany

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Abstract High year-to-year variability in the incidence of *Fusarium* spp. and mycotoxin contamination was observed in a two-year survey investigating the impact of maize ear rot in 84 field samples from Germany. *Fusarium verticillioides*, *F. graminearum*, and *F. proliferatum* were the predominant species infecting maize kernels in 2006, whereas in 2007 the most frequently isolated species were *F. graminearum*, *F. cerealis* and *F. subglutinans*. Fourteen *Fusarium*-related mycotoxins were detected as contaminants of maize kernels analyzed by a multi-mycotoxin determination method. In 2006, a growth season characterized by high temperature and low rainfall during anthesis and early grain filling, 75% of the maize samples were contaminated with deoxynivalenol, 34% with fumonisins and 27% with zearalenone. In 2007, characterized by moderate temperatures and frequent rainfall during the entire growth season, none of the 40 maize samples had quantifiable levels of fumonisins while deoxynivalenol and zearalenone were detected in 90% and 93% of the fields, respectively. In addition, 3-acetyldeoxynivalenol, 15-acetyldeoxnivalenol, moniliformin, beauvericin, nivalenol and enniatin B were detected as common contaminants produced in both growing seasons. The results demonstrate a significant mycotoxin contamination associated with maize ear rots in Germany and indicate, with regard to anticipated climate change, that fumonisins-producing species already present in German maize production may become more important.

Keywords Deoxynivalenol · Ear rot · *F. verticillioides* · *F. graminearum* · Fumonisin · Zearalenone

Abbreviations

Tef	translation elongation factor 1-alpha gene
DON	deoxynivalenol
FBs	fumonisins
ZEA	zearalenone

Introduction

Ear rot caused by *Fusarium* spp. is a major threat to maize production worldwide arising from the ability of most *Fusarium* species to produce mycotoxins that

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are potential health hazards for humans and animals consuming maize and maize-based products. Maize kernels infected by *F. verticillioides* (Saccardo) Nirenberg and *F. proliferatum* (Matsushima) Nirenberg are primarily contaminated with fumonisins (FBs) that may have cancer-promoting activities in humans. Epidemiological associations between maize consumption and oesophageal cancer (Marasas et al. 1981; Sun et al. 2007) have been reported, while neural tube defects in newborns have been associated with high consumption of FBs contaminated tortilla`s (Desjardins 2006). Leukoencephalomalacia in horses as well as pulmonary edema in swine are fatal diseases caused by FBs-contaminated feed (Thiel et al. 1991; Haschek et al. 2001). Trichothecenes (TCTCs) of types A and B are associated with a wide range of chronic and fatal toxicoses of humans and animals caused by inhibition of ribosomal protein-synthesis and immunosuppression (Desjardins 2006). Type A TCTCs, T-2 toxin (T-2), HT-2 toxin (HT-2), and mono- and di-acetoxyscirpenol are considered to be more toxic to human and animal consumers than type B TCTCs (Krska et al. 2001), but their occurrence is less frequent. Type B TCTCs deoxynivalenol (DON) and nivalenol (NIV) are frequent contaminants of maize ears, due to the common presence of *F. graminearum* Schwabe, *F. culmorum* (W.G. Smith) Saccardo and *F. cerealis* Burgess, Nelson & Toussoun (syn. *F. crookwellense*) (Logrieco et al. 2002). These *Fusarium* species are also capable of producing zearalenone (ZON) and its derivatives in cereal grains causing estrogenic syndromes in swine such as enlargement of mammary glands and organs of the genital tract, atrophy of ovaries, infertility and reduced piglet weight (Desjardins 2006). Several additional *Fusarium* mycotoxins, e.g. moniliformin (MON), beauvericin (BEA) and fusaproliferin have also been found in maize-based food and feed exhibiting different toxic activities (Logrieco et al. 2002; Desjardins 2006; Jestoi 2008).

Maize ear rots caused by *Fusarium* spp. have been differentiated into two distinct diseases called pink ear rot which is synonymous with *Fusarium* ear rot and red ear rot which is synonymous with *Gibberella* ear rot (Logrieco et al. 2002). Species within the *Liseola* section, in particular *F. verticillioides*, *F. proliferatum* and *F. subglutinans* (Wollenweber & Reinking) Nelson, Toussoun & Marasas, are the prevalent pathogens associated with pink ear rot in North America and many other temperate regions of the world (Munkvold and Desjardins 1997). The occurrence of *Fusarium* species depends primarily on environmental conditions; in Europe, *F. verticillioides* and *F. proliferatum* predominate in drier and warmer regions like Italy and Spain (Logrieco et al. 1995; Jurado et al. 2006). Under colder and more humid conditions, *F. subglutinans* is usually isolated more frequently than *F. proliferatum* and *F. verticillioides* (Lew et al. 1991; Logrieco et al. 2002). Red ear rot, primarily caused by *F. graminearum*, predominates in years and regions with frequent rainfall and moderate temperatures (Logrieco et al. 2002; Munkvold 2003). Other species often involved in red ear rot include *F. culmorum*, *F. subglutinans*, *F. cerealis* and *F. avenaceum* (Fries) Saccardo, all species more common in central and northern Europe. The type A TCTC producers *F. sporotrichioides* Sherbakoff, *F. poae* (Peck) Wollenweber and *F. equiseti* (Corda) Saccardo as well as *Fusarium oxysporum* Schlechtendahl emend. Snyder & Hansen, *F. venenatum* (Nirenberg) and *F. tricinctum* (Corda) Saccardo cause ear rot to a lesser extent (Logrieco et al. 2002).

In Germany, with a production area of two million ha in 2008, maize is one of the most important agricultural products and a major component of animal feed. Maize is also the most important substrate for biogas production and consequently, the current expansion of maize acreage is associated with shorter crop rotations and a higher risk of toxic contamination. Despite the well-known threat of maize ear rot to human and animal health as well as legislated regulation of maximum acceptable levels of FBs, DON and ZON (EU Commission 2007), limited information is available regarding incidence of *Fusarium* spp. and mycotoxin contamination on maize in Germany. Therefore, a two-year survey was initiated, in order to determine the severity of maize ear rot, identify the spectrum of *Fusarium* species involved, and quantify the mycotoxin contamination. Maize kernels were sampled at harvest from 44 fields in 2006 and from 40 fields in 2007, two growing seasons with contrasting environmental conditions during anthesis and early grain filling of maize.

Materials and methods

Maize samples

Forty-four and 40 maize fields from the major maize growing areas in Germany were randomly sampled at

harvest in 2006 and 2007 (Fig. 1). All samples (1 to 2 kg) intended for animal consumption were harvested mechanically and stored for up to 3 days at -20°C until analysis. Three hundred kernels per sample were cultured to determine the frequency of *Fusarium* infection as well as to identify the species present. For mycotoxin analysis, 50 g kernels per sample were ground using an ultra-centrifugal mill (Retsch, Haan, Germany).

Mycological analysis

Kernels of each sample were surface sterilized for two minutes in 1.3% aqueous sodium hypochlorite, rinsed twice with sterile distilled water and transferred onto Czapek-Dox-Iprodion-Dichloran (CZID) agar (Abildgren et al. 1987). After incubation at 23°C for 6 days, the percentage of infected kernels was recorded. Cultures with different growth characteristics (pigmentation, shape and colour of mycelium, growth rate, etc.) were transferred to potato dextrose agar (PDA) plates to obtain monohyphal isolates required for identification.

Identification of *Fusarium* isolates obtained in 2006 was primarily carried out microbiologically. PDA was used for the observation of colony characteristics and carnation leaf agar was employed for the examination of conidial morphology (Leslie and Summerell 2006).

Identity of selected isolates from the microbiological identification was confirmed by sequencing the partial translation elongation factor 1-alpha gene (*tef*) as well as by species-specific PCR. Pure cultures were grown in half-strength potato dextrose broth (3 g/100 ml) by continuous shaking at 75 rpm in the dark at 25°C for 7 days. Mycelium was harvested by filtration, lyophilized and ground in an ultra-centrifugal mill (Retsch, Haan, Germany) to a fine powder (<0.1 mm). DNA was extracted from 20 mg of the powder using the Wizard® Magnetic DNA Purification System for Food (Promega, Mannheim, Germany).

Sequences of *tef* genes were generated and the isolates were identified by blasting the sequences in the FUSARIUM-ID v 1.0 database (http://fusarium.cbio.psu.edu). Sequences of *tef* were obtained by performing PCRs using primers EF1T (ATGGG TAAGGAGGACAAGAC) and EF2T (GGAAGTAC CAGTGATCATGTT) (O'Donnell et al. 1998). Amplification reactions were done in volumes of 50 µl containing 20 ng of template DNA, 4 µl dNTPs

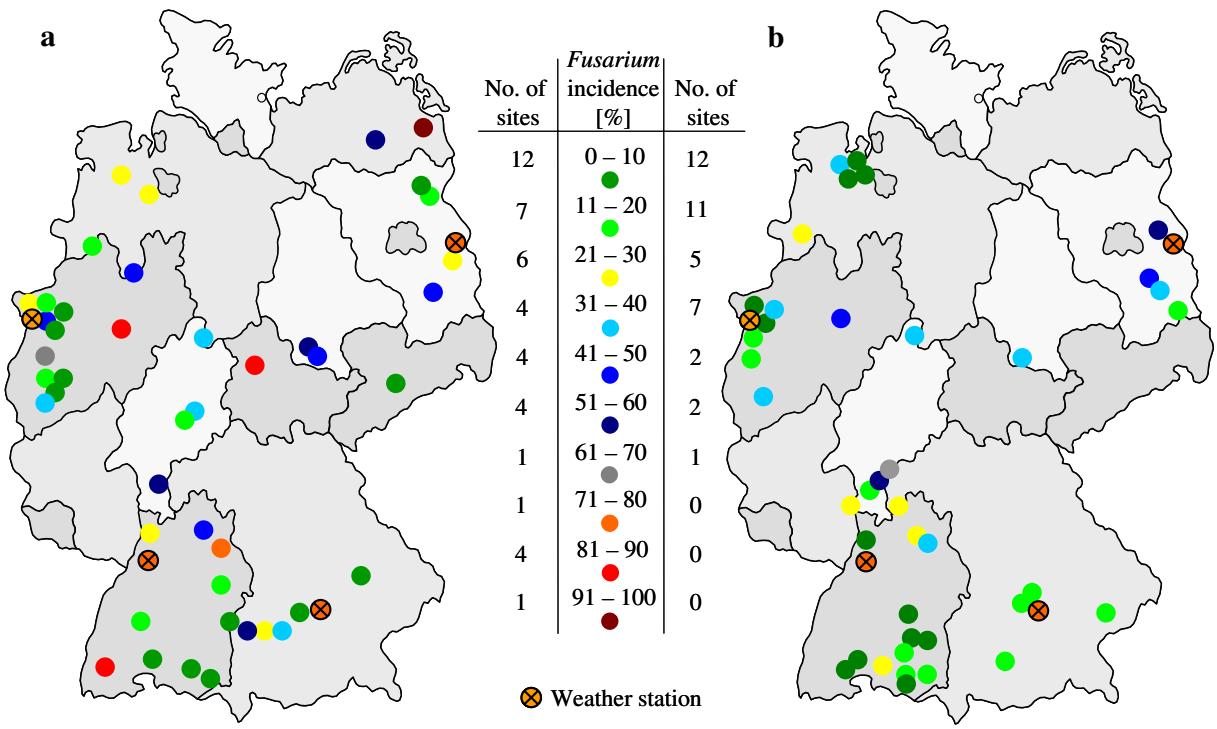

Fig. 1 Incidence of maize ear rot caused by *Fusarium* spp. in maize fields in Germany in 2006 (**a**) and 2007 (**b**)

(600 μM), 1.5 μl of each primer (6 μM), 5 μl PCR buffer and 0.3 μl Taq DNA Polymerase (Roche Diagnostics, Mannheim). The amplification protocol consisted of one cycle of 2 min at 95°C, 35 cycles of 30 s at 95°C (denaturation), 30 s at 54°C (annealing), 1 min at 72°C (extension) and one cycle of 10 min at 72°C. Sequencing reactions were performed in volumes of 10 μl including 4 μl of amplified DNA, 2 μl buffer, 2 μl of DYEnamic ET terminator cycle sequencing kit (GE Healthcare, Freiburg) and 0.7 μl of one primer (EF1T or EF2T). Thermocycler program for sequencing was 24 cycles of 20 s at 94°C, followed by 15 s at 50°C and 1 min at 60°C. Sequencing reactions were run on ABI 3700 (Applied Biosystems) equipment.

Conventional PCR was performed by hybridizing isolated DNA with species-specific primers (Table 1) with the following thermocycler profile: a single cycle of 2 min at 94°C, 40 cycles of 1 min at 94°C, 30 s at 60°C and 1 min at 72°C and one cycle of 10 min at 72°C. For each reaction, 20 ng of template DNA were mixed with 28 μl PCR mastermix containing 2 μl species-specific primer (6 μM), 6 μl PCR buffer, 4 μl dNTPs (600 μM) and 0.2 μl Taq DNA polymerase (Promega, Mannheim, Germany).

Multiplex PCRs were applied for the simultaneous detection of 10 *Fusarium* species isolated from the 2007 samples (Fig. 2). Pure cultures of *Fusarium* isolates were grown on PDA for 10 days. Harvested mycelium was lyophilized and ground and DNA was extracted from aliquots of 20 mg fine powder using the Wizard® Magnetic DNA Purification System for Food. Species-specific primers described in Table 1 were divided into two reaction mixtures (Table 2). Each reaction mixture contained 6 μl 5x PCR-Buffer, 3.5 μl dNTPs (600 μM), 0.2 μl Taq DNA Polymerase (Promega, Mannheim, Germany), 20 ng template DNA and the respective primer pairs. *Fusarium*

Table 1 Species-specific primers for the identification of *Fusarium* species

Target species	Primer name	Sequence	Product size (bp)	Reference[a]
F. avenaceum	JIAf/r	GCTAATTCTTAACTTACTACTAGGGGCC CTGTAATAGGTTATTTACATGGGCG	220–300	A
F. cerealis	CRO-AF/R	CTCAGTGTCCACCGCGTTGCGTAG CTCAGTGTCCCAATCAAATAGTCC	842	B
F. culmorum	Fc01F/R	ATGGTGAACTCGTCGTGGC CCCTTCTTACGCCAATCTCG	570	C
F. equiseti	FEF1/R1	CATACCTATACGTTGCCTCG TTACCAGTAACGAGGTGTATG	400	D
F. graminearum	Fg16F/R	CTCCGGATATGTTGCGTCAA GGTAGGTATCCGACATGGCAA	400–500	C
F. oxysporum	CLOX1/2	CAGCAAAGCATCAGACCACTATAACTC CTTGTCAGTAACTGGACGTTGGTACT	534	E
F. poae	Fp82F/R	CAAGCAAACAGGCTCTTCACC TGTTCCACCTCAGTGACAGGTT	220	F
F. proliferatum	PRO1/2	CTTTCCGCCAAGTTTCTTC TGTCAGTAACTCGACGTTGTTG	585	G
F. sporotrichioides	AF330109CF/R	AAAAGCCCAAATTGCTGATG TGGCATGTTCATTGTCACCT	332	H
F. subglutinans	SUB1/2	CTGTCGCTAACCTCTTTATCCA CAGTATGGACGTTGGTATTATATCTAA	631	G
F. venenatum	VEN-BF/R	GGCGGATAAAGGATAGTGGTAGAAG GGCGGATAAGCAAATAAGATGCTT	276	B
F. verticillioides	VERT-1/2	GTCAGAATCCATGCCAGAACG CACCCGCAGCAATCCATCAG	800	I

[a] Reference: A - Turner et al. (1998); B - Yoder and Christianson (1998); C - Nicholson et al. (1998); D - Mishra et al. (2003); E - Mulé et al. (2004a); F - Parry and Nicholson (1996); G - Mulé et al. (2004b); H - Demeke et al. (2005); I - Patino et al. (2004)

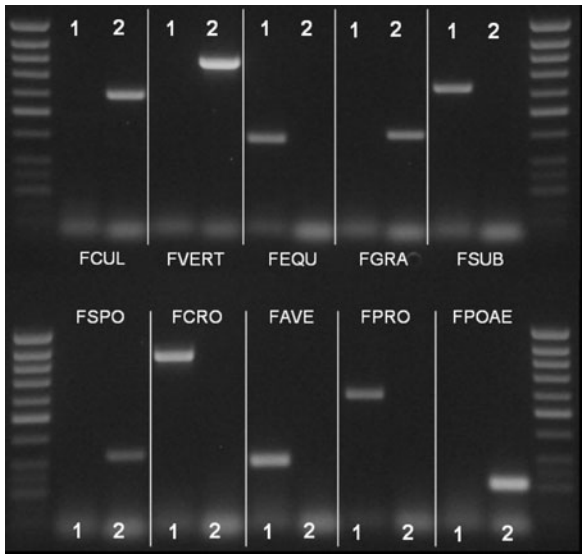

Fig. 2 Multiplex PCR for the simultaneous detection of ten *Fusarium* species: 1 = reaction mixture 1; 2 = reaction mixture 2; FAVE, *F. avenaceum*; FCUL, *F. culmorum*; FCRO, *F. cerealis*; FEQU, *F. equiseti*; FGRA, *F. graminearum*; FPOAE, *F. poae*; FPRO, *F. proliferatum*; FSPO, *F. sporotrichioides*; FSUB, *F. subglutinans*; FVERT, *F. verticillioides*

isolates, for which species-specific primers have not been published, like *F. tricinctum* and *F. venenatum*, could not be identified by multiplex PCRs and were therefore typed morphologically.

Mycotoxin analysis

Thirty-two mycotoxins were quantified by liquid chromatography-mass spectrometry as described by Herebian et al. (2009). Briefly, after homogenization of ground maize and addition of internal standards, mycotoxins were extracted acetonitrile/water/acetic acid (79:20:1, v/v/v) by vortexing at full speed for 15 s and extracting for 90 min on a rotary shaker. Extracts were centrifuged for 3 min at 1,000 rpm and supernatants were analyzed directly.

HPLC analysis of the extracts was performed using a C18 column. Gradient elution was performed using methanol/water/acetic acid (10:89:1, v/v/v) and methanol/water/acetic acid (97:2:1, v/v/v). Selected reaction monitoring was performed using a TSQ quantum ultra AM mass spectrometer (ThermoFinnigan, Bremen, Germany) equipped with an ESI ion source operating in both, positive and negative mode. Nitrogen was employed as both the drying and nebulizer gas. The transitions of the two most intense and specific fragment ions of the precursor ion were monitored in the multiple reaction monitoring mode for quantification and confirmation of target analytes. External calibration was performed in the range 0.001–3.5 μg/ml and standard solutions were stored in the dark at –20°C. Recoveries calculated from blank samples spiked with a known amount of standards taken through the whole process ranged from 73 to 152%. Relative standard deviation for five replicates was < 10.8%, indicating high reproducibility of the method. Excellent linearity (r^2>0.998) was obtained for all external calibration curves over the range from the limit of detection to at least 800 ng/g. Limits of quantification (LOQ) for the *Fusarium* toxins analyzed are given in Table 4.

Table 2 Use of 10 species-specific primer pairs in two reaction mixtures for the identification of *Fusarium* species

Reaction mixture	Target species	Primer pair	Product size (bp)	Concentration [nM]
1	*F. crookwellense*	Cro-AF/Cro-AR [a]	842	330
	F. subglutinans	SUB1/SUB2 [b]	631	400
	F. proliferatum	PRO1/PRO2 [b]	585	350
	F. equiseti	FEF1/FER1 [c]	400	400
	F. avenaceum	JIAf/JIAr [d]	220–300	350
2	*F. verticillioides*	VERT1/VERT2 [e]	800	300
	F. culmorum	Fc01F/Fc01R [f]	570	300
	F. graminearum	Fg16F/Fg16R [f]	400–500	350
	F. sporotrichioides	AF330109CF/AF330109CR [g]	332	400
	F. poae	Fp82F/Fp82R [h]	220	300

[a] Yoder and Christianson (1998); [b] Mulé et al. (2004b); [c] Mishra et al. (2003); [d] Turner et al. (1998); [e] Patino et al. (2004); [f] Nicholson et al. (1998); [g] Demeke et al. (2005); [h] Parry and Nicholson (1996)

Meteorological data

Meteorological data were obtained from weather stations operated by the German Meteorological Service (Fig. 3). In both years, weather information was retrieved daily for the period from May to October.

Statistical analysis

Statistical analysis was performed applying SPSS version 17.0 (SPSS Inc., Chicago, USA). Annual differences in average mycotoxin concentration and mean number of infected kernels per site were statistically analyzed using the non-parametric Mann-Whitney-U-test. Tests were performed at a probability level of $p=0.05$. Spearman`s correlation coefficients were generated to determine the relationship between mycotoxin levels and the incidence of *Fusarium* spp.. For samples with mycotoxin concentrations below the detection limit, statistical analyses were performed using half value of the detection limit.

Results

Incidence of maize ear rot caused by *Fusarium* species

In 2006 and in 2007, *Fusarium* species causing ear rot of maize were detected in all samples investigated. The frequency of kernels colonized by *Fusarium* spp. in 2006 ranged from 0.7% to 99.7%. In 12 fields, the frequency was less than 10% while in five samples the incidence exceeded 80% (Fig. 1). The average frequency of infected kernels was 34.2%. In 2007, the highest frequency of *Fusarium*-infected maize kernels recorded was 64%. In 23 fields, the frequency of kernel infection was below 20%, while the average frequency of maize kernels affected by *Fusarium* spp. was 21.7%.

Spectrum of *Fusarium* species

Thirteen *Fusarium* species were identified as pathogens of maize ear rot in 2006 (Table 3). The predominant species was *F. verticillioides*, isolated from 82% of maize fields. In addition, *F. graminearum*, *F. proliferatum* and *F. equiseti* were commonly associated with maize ear rot. *Fusarium cerealis* and *F. culmorum* were present at 48% of the sites. *Fusarium avenaceum* and *F. sporotrichioides* were detected in more than 30% of samples investigated. *Fusarium oxysporum* and *F. poae* occurred in several fields, whereas *F. subglutinans*, *F. venenatum* and *F. tricinctum* were isolated infrequently from maize in 2006.

With the exception of *F. oxysporum*, the same spectrum of *Fusarium* species was isolated in 2007 (Table 3). *Fusarium graminearum* was by far the most frequent species detected in all samples. The mean number of kernels infected by *F. graminearum* was significantly higher in 2007 than in 2006. *Fusarium cerealis* was the second most frequent

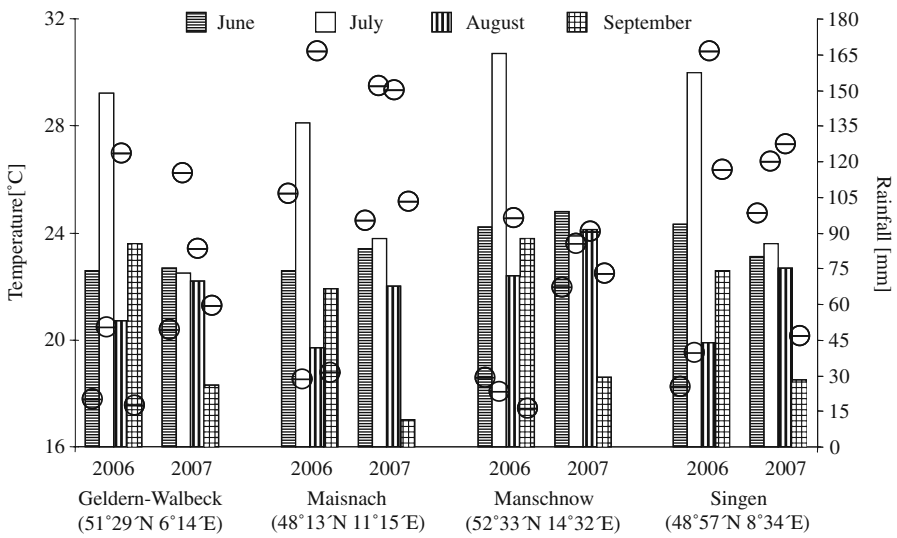

Fig. 3 Mean maximum temperatures (columns) and rainfall (circles) on four locations in Germany in 2006 and 2007

Table 3 Incidence of *Fusarium* spp. isolated from maize kernels produced in Germany in 2006 and 2007

Fusarium species	Sites [%]		Mean infected kernels/site [n]		Total infected kernels [%]	
	2006	2007	2006	2007	2006	2007
F. avenaceum	37	33	2.8[a*]	1.3[a]	0.9	0.4
F. cerealis	48	53	5.3[a]	6.5[a]	1.8	2.2
F. culmorum	48	20	5.6[a]	2.4[b]	1.9	0.8
F. equiseti	57	13	5.9[a]	0.5[b]	2.0	0.2
F. graminearum	71	100	20.8[a]	38.8[b]	6.9	12.9
F. oxysporum	25	0	1.7[a]	0[b]	0.6	0.0
F. poae	18	13	0.5[a]	0.4[a]	0.2	0.1
F. proliferatum	64	8	8.3[a]	0.8[b]	2.8	0.3
F. sporotrichioides	32	3	1.9[a]	0.1[b]	0.6	0.04
F. subglutinans	11	40	1.3[a]	5.4[b]	0.4	1.8
F. tricinctum	2	18	0[a]	0.5[b]	0.01	0.2
F. venenatum	7	10	0.1[a]	0.1[a]	0.05	0.04
F. verticillioides	82	5	31.4[a]	0.6[b]	10.5	0.2

* Means in each row followed by the same letter are not significantly different

species, encountered in more than 50% of the maize fields. Regarding the impact of *F. cerealis*, no significant differences were observed comparing 2006 and 2007. *Fusarium verticillioides*, *F. proliferatum* as well as *F. equiseti* were less frequently detected in 2007. The mean number of kernels infected by *F. verticillioides, F. proliferatum* and *F. equiseti* was significantly lower compared to 2006. *Fusarium subglutinans* (40%) and *F. tricinctum* were significantly more prevalent in 2007. *Fusarium avenaceum* was often identified, while the frequency of occurrence of *F. venenatum* and *F. poae* was slightly reduced in 2007. The average number of kernels infected by *F. sporotrichioides*, *F. culmorum* and *F. oxysporum* was significantly lower in 2007, compared to 2006.

Among all sites investigated in 2006, *F. verticillioides* was the predominant species isolated from 10.5% of the 13200 kernels evaluated (Table 3), followed by *F. graminearum* and *F. proliferatum*. Infections by *F. equiseti*, *F. culmorum* and *F. cerealis*, respectively, were observed in about 2% of the kernels. *Fusarium avenaceum*, *F. sporotrichioides*, *F. oxysporum* or *F. subglutinans* were isolated from less than one percent of kernels. The percentage of kernel infections by *F. poae*, *F. venenatum* or *F. tricinctum* was 0.2% or less.

Out of the 12,000 kernels analyzed in 2007, 12.9% were infected by *F. graminearum*, and about 2% by *F. cerealis* and *F. subglutinans*, respectively. Infections by *F. culmorum*, *F. avenaceum* and *F. proliferatum* were observed in less than 1% of kernels. *Fusarium verticillioides*, *F. equiseti*, *F. tricinctum*, *F. poae*, *F. sporotrichioides* or *F. venenatum* were detected in 0.2% of kernels or less.

Mycotoxin contamination caused by *Fusarium* spp

In 2006, 75% of the maize samples had DON levels above the LOQ (Table 4). Mean and maximum levels of DON were 1.78 μg/g and 19.57 μg/g, respectively. The acetylated derivatives 3-AcDON and 15-AcDON were present in 70% and 50% of the samples, respectively, both with mean concentrations above 0.20 μg/g. The cyclic hexadepsipeptides BEA and ENNB, and MON were frequently detectable in the maize crops. However, mean and maximum contamination of BEA, ENNB and MON were distinctly lower compared to DON. In 2006, thirty-four percent of the maize samples had detectable amounts of FB_1, while FB_2 was detected in 23% of the samples. Maximum concentrations of FB_1 and FB_2 were 20.68 μg/g and 6.71 μg/g, respectively; the total maximum concentration of FB_1 + FB_2 observed in one sample was 25.09 μg/g. ZON was detected in less than 30% of the fields investigated with an average contamination of 0.07 μg/g. Contamination with NIV were even more infrequently detectable, in 23% of the crops, while the type A TCTCs T-2, HT-2 and monoacetoxyscirpenol (MAS) were detected in 18% of the samples or less.

Table 4 Mycotoxin contamination of maize samples from Germany, in 2006 and 2007

Mycotoxin	Positive samples [%]		Concentration [ng/g]			
			Mean (SEM•)		Maximum	
	2006	2007	2006	2007	2006	2007
Beauvericin (10)*	52	33	390[a†] (183)	240[a] (151)	6402	5100
Deoxynivalenol (40)	75	90	1780[a] (492)	2240[a] (440)	19570	16250
3-AcDON (15)	70	80	230[a] (41)	70[a] (10)	890	280
15-AcDON (20)	50	98	290[a] (83)	680[b] (121)	2740	3390
Enniatin B (4)	41	30	70[a] (35)	160[a] (87)	1210	2960
Fumonisin B_1 (100)	34	0	1910[a] (738)	50[b] (0)	20690	50
Fumonisin B_2 (50)	23	0	460[a] (205)	25[b] (0)	6710	25
Moniliformin (10)	43	45	280[a] (99)	110[a] (51)	3330	1850
Nivalenol (20)	23	48	160[a] (101)	210[b] (69)	4410	2120
Monoacetoxyscirpenol (18)	18	23	142[a] (39)	20[a] (3)	1080	120
T-2 toxin (4)	14	10	4[a] (1)	10[a] (8)	30	340
HT-2 toxin (4)	11	15	7[a] (2)	20[a] (12)	80	500
α-Zearalenole (4)	0	10	2[a] (0)	5[a] (3)	2	120
Zearalenone (3)	27	93	70[a] (27)	480[b] (363)	860	14580

* Numbers in the parantheses indicate the limit of quantification (ng/g). • Standard error of the mean. † Means in each row followed by the same letter are not significantly different.

In 2007, 90% of the samples were positive for DON (Table 4). The average concentration of DON was higher compared to 2006 as well to the other metabolites detected in 2007. The number of samples positive for 3-AcDON also increased, however, mean and maximum concentrations of 3-AcDON were lower in 2007. In contrast, in addition to the higher number of 15-AcDON-contaminated samples, average concentration of 15-AcDON was significantly higher in 2007. With 93% contaminated maize samples, impact as well as the mean concentration of ZON increased significantly in 2007, compared to 2006. Its derivative zearalenole (α-ZOL) was detected at four sites. NIV contamination with a maximum level of 2.12 μg/g was detected in almost 50% of the fields in 2007. Compared to 2006, mean NIV concentration was significantly higher in 2007. With 45% positive samples, MON contamination were observed in similar frequencies as in 2007; however, mean and maximum concentrations were lower. BEA and ENNB were detected in 33% and 30% of the samples, reaching average levels of 0.24 μg/g and 0.16 μg/g, respectively. Twenty-three percent of the samples tested positive for MAS, whereas contamination by T-2 and HT-2 were infrequently detected. In 2007, concentrations of FB_1 and FB_2 were below the LOQ for all maize samples, in conclusion significantly lower than in 2006.

Discussion

Quantification of a broad range of mycotoxins and identification of the associated *Fusarium* species in a large-scale survey in two growing seasons with contrasting environmental conditions provides comprehensive information on the impact of maize ear rots and the associated mycotoxin contamination of maize grown for feed production in Germany.

Mainly associated with the common occurrence of *F. graminearum*, DON and the acetylated derivatives 3-AcDON and 15-AcDON were frequently detected in concentrations alarming for animal consumption in both growth periods. In 15 samples in 2006 and in 18 samples in 2007 DON concentrations exceeded 1.75 μg/g, the maximum level for unprocessed maize in the EU (EU Commission 2007). Median levels for DON in 2006 and 2007 demonstrate that high DON concentrations can be expected regularly in maize production in Germany. Kernel infections by *F. culmorum* may also contribute to contamination with DON and its derivatives, but in both years, the number of *F. culmorum*-infected kernels was markedly lower than infections by *F. graminearum*. The higher frequency of contamination with type B-trichothecenes, in particular 15-AcDON and NIV reflected the higher incidence of *F. graminearum* and *F. cerealis* in 2007, a growing

season obviously promoting infection and colonization of maize by both species. The number of kernels infected by *F. graminearum* correlated with concentrations of DON, 3-AcDON and 15-AcDON (data not shown). In addition, significant relationships were also observed between DON- and 15-AcDON-concentrations, and *F. cerealis*, a species generally described to produce rather NIV than DON. A significant relationship between *F. cerealis* and NIV contamination was also observed in the current survey. However, the correlation between DON and *F. cerealis* is probably due to joint sample infections by *Fusarium* species with different mycotoxin profiles.

Temperatures of about 28°C are optimal for *F. graminearum* infection (Reid et al. 1999) and Sutton (1982) reported that high levels of moisture around the silking growth stage, associated with moderate temperatures and high rainfall during early kernel maturation favoured *Gibberella* ear rot. Hence, moderate temperatures and frequent precipitation recorded from early growth stages until end of grain filling throughout Germany in 2007 might explain the high frequencies of *F. graminearum* and *F. cerealis* and concomitantly higher ZON accumulation (Fig. 3). ZON, however, correlated only with the incidence of *F. cerealis*. The number of maize samples positive for ZON in 2007 was markedly higher than in 2006. Incidence of ZON has been reported to be correlated with summer precipitation in Canada (Sutton 1982). In our study, infrequent ZON detection and low rainfall from June until the end of anthesis in August 2006 contrasting with 93% ZON positive samples and high precipitation during anthesis in 2007 support the relationship between rainfall and ZON accumulation.

The year-to-year variability in contamination of maize with FB_1 and FB_2 is likely to be caused by contrasting weather conditions during the growth periods resulting in differences in the incidence of *F. verticillioides* and *F. proliferatum*. In 2006, weather in many regions was characterized by high temperature and low rainfall in July and September (Fig. 3), favouring infection and colonization of maize ears by FBs-producing species (Vigier et al. 1997; Marasas et al. 2000; Munkvold 2003). In contrast, in 2007 moderate temperatures were well below the optimum temperature of 30°C reported for *F. verticillioides* (Marin et al. 1999; Reid et al. 1999). High FBs levels and frequent kernel infections by *F. verticillioides* and *F. proliferatum* in 2006 can be associated with maize crops exposed to drought stress. Previous studies on FBs in maize confined high levels of FBs to southern Europe, but in 2006, levels of FB_1 and FB_2 in Germany were comparable with FBs concentrations from other regions in the world (Kedera et al. 1999; Placinta et al. 1999; Adejumo et al. 2007). This study is the first report of considerable FBs concentrations in maize occurring above 52°N latitude and suggests that high FBs concentrations are likely to occur more frequently in Germany and other temperate regions with anticipated climate change.

In 2006, contamination with MON, BEA and ENNB were expected to be associated with the infestation of kernels by *F. proliferatum* as well as *F. avenaceum*, well-known producers of these mycotoxins (Logrieco et al. 2003). A correlation analyses for 2006, however, indicated significant relationships between *F. oxysporum* and concentrations of MON, BEA and ENNB (data not shown). Those correlations resulted most likely from cross-infections by other MON-, BEA- and ENNB-producing *Fusarium* species since *F. oxysporum* is generally considered to produce no mycotoxins (Desjardins 2006). In 2007, the MON- and BEA-producing species *F. subglutinans* and *F. avenaceum* were determined as frequent colonizers of maize kernels. *Fusarium subglutinans* is reported as a common pathogen causing maize ear rot in years characterized by cooler weather and more frequent rainfall. Under these conditions, the proliferation of *F. subglutinans* is favoured at the expense of *F. proliferatum* (Lew et al. 1991; Vigier et al. 1997; Logrieco et al. 2002). According to the profiles of mycotoxins and *Fusarium* species observed in both years, MON and BEA may be frequent contaminants in maize irrespective of the prevailing environmental conditions. Both derivatives have been reported as frequent contaminants of maize worldwide, with maximum concentrations often significantly higher than those detected in this survey (Jestoi 2008). Chelkowski et al. (2007) detected ENNB in 15 out of 27 maize samples in Poland, with a maximum of 46 μg/g. However, to date less data are available regarding the natural occurrence of ENNB in maize.

Corresponding to the infrequent occurrence of *F. sporotrichioides* and *F. poae* in 2006 and 2007, T-2, HT-2 and MAS were detected in lower frequencies. Year-to-year variability in the incidence of *F. equiseti,* which is a secondary invader and less pathogenic on maize (Logrieco et al. 2002; Leslie and Summerell

2006), may also be explained indirectly by environmental conditions, such as dry conditions that aggravate kernel damage caused by birds (Sutton et al. 1980). Mechanical kernel damage could also explain the infections by *F. oxysporum* observed in 2006.

Mean ear rot incidence of 34.2% in 2006 and 21.7% in 2007, the detection of *Fusarium* species in all fields investigated, and local infection frequencies up to 99.7% demonstrate the high impact of maize ear rot in Germany. With regard to the current expansion of maize acreage in particular, including the cultivation of maize for biogas production, it is likely that damage caused by *Fusarium* spp. and their mycotoxins will continue to worsen. As many of the *Fusarium* species are capable of colonizing multiple hosts, this will not only affect maize but also other crops in the rotations, in particular small grains. More narrow crop rotations and the expected spread of both the European corn borer (*Ostrinia nubilalis* Hbn.) (Schmitz et al. 2002) and the Western corn rootworm (*Diabrotica virgifera* LeConte) in Germany (Hummel et al. 2008) will further complicate the ability to comply with maximum tolerable mycotoxin concentrations in maize and other cereal commodities. Both insects have been implicated in the dispersal of *Fusarium* spp. and are known to increase the incidence of ear rots and mycotoxin contamination in maize (Munkvold et al. 1997; Munkvold and Desjardins 1997; Munkvold 2003).

The current results emphasize the importance of continued research on the influence of environmental conditions on the spectrum of *Fusarium* species and their secondary metabolites as well as on management strategies to prevent mycotoxin contamination, such as breeding for ear rot resistance in maize, management of plant debris (crop rotation, tillage) and/or chemical control.

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