Fusarium Head Blight of Cereals in Denmark: Species Complex and Related Mycotoxins

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ABSTRACT

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Quantitative real-time polymerase chain reaction differentiating 10 *Fusarium* spp. and *Microdochium nivale* or *M. majus* was applied to a total of 396 grain samples of wheat, barley, triticale, oat, and rye sampled across Denmark from 2003 to 2007, along with selected samples of wheat and barley from 1957 to 2000, to determine incidence and abundance of individual *Fusarium* spp. The mycotoxins deoxynivalenol (DON), nivalenol, zearalenone, T-2, and HT-2 were quantified using liquid chromatography–double mass spectrometry. Major differences in the

Fusarium species complex among the five cereals as well as great yearly variation were seen. *Fusarium graminearum*, *F. culmorum*, and *F. avenaceum* were dominant in wheat, with DON as the dominant mycotoxin. *F. langsethiae*, *F. culmorum*, and *F. avenaceum* were dominant in barley and oat, leading to relatively high levels of the mycotoxins T-2 and HT-2. *F. graminearum*, *F. culmorum*, and *F. avenaceum* dominated in triticale and rye. The nontoxigenic *M. nivale/majus* were present in significant amounts in all cereal species. Wheat and barley samples from 1957 to 1996 exhibited no or very low amounts of *F. graminearum*, indicating a recent increase of this pathogen. Biomass and mycotoxin data exhibited good correlations between *Fusarium* spp. and their corresponding mycotoxins under field conditions.

Fusarium head blight (FHB) in small grain cereals is caused by a complex of toxigenic pathogens belonging to the genus *Fusarium* (*Gibberella*) and the nontoxigenic genus *Microdochium*. The result of FHB worldwide is lower grain yield and reduced quality due to mycotoxins. The most common *Fusarium* spp. in this complex found throughout Europe are *Fusarium graminearum* (*Gibberella zeae*), *F. avenaceum* (*G. avenacea*), and *F. culmorum* (5). However, *F. poae*, *F. tricinctum*, *F. sporotrichioides*, *F. equiseti*, and *F. langsethiae* are also frequently found (5,21,44). The distribution of the pathogens causing FHB is believed to be determined, in part, by climatic factors such as temperature and moisture (41). *F. graminearum* has traditionally been found to dominate in regions with warm and humid growth conditions; *F. avenaceum* and *F. culmorum* are both associated with cool, wet, and humid conditions; and *Microdochium nivale* and *M. majus* are associated with regions of relatively cool temperatures and frequent short rain showers (42).

In addition to climatic factors, agronomic factors are also very important for the occurrence of FHB. Soil tillage and previous crops have been shown to have a significant influence on FHB or mycotoxin contamination (7,10,27). The *Fusarium* spp. produce several mycotoxins which pose a health risk to humans and animals through food and feed prepared from contaminated crops (11). The mycotoxins produced by the *Fusarium* spp. include the

trichothecenes, zearalenone (ZEA), moniliformin, fumonisins, and the enniatins. Trichothecenes constitute the largest group of mycotoxins produced by *Fusarium* spp. in cereal grain; these can be divided into two groups: type A and type B (24). The type B trichothecenes are the most common trichothecenes and include deoxynivalenol (DON) and the closely related nivalenol (NIV). DON is produced by *F. graminearum* and *F. culmorum*, and NIV is produced by some isolates of these two species as well as by *F. poae* and *F. equiseti* (24). *F. graminearum* and *F. culmorum* are also producers of ZEA, which occurs commonly along with DON and similar derivatives (5). Type A trichothecenes includes T-2 mycotoxin, HT-2 mycotoxin, and diacetoxyscirpenol (DAS) and are considered to be more toxic to humans and animals than type B trichothecenes (22). The mycotoxins T-2 and HT-2 are produced primarily by *F. langsethiae* and *F. sporotrichioides*; also, a few isolates of *F. poae* have been reported as HT-2 and T-2 producers (35). DAS is produced by *F. poae* and *F. equiseti*, whereas *F. avenaceum* and *F. tricinctum* produce, among other metabolites, the mycotoxins moniliformin, beauvericin, and enniatins (35).

Due to differences in pathogenicity, toxigenicity, and fungicide sensitivity, it is important to obtain detailed knowledge of the occurrence of individual *Fusarium* spp., and a prerequisite for this is the correct identification and quantification of individual species. However, the classical morphological methods for identification of *Fusarium* spp. in grain samples are both laborious and require a high level of expertise in order to distinguish closely related species, and will only provide information on incidence of the pathogen infection in seed. In contrast to conventional tests, polymerase chain reaction (PCR)-based methods have the advantage of species specificity, sensitivity, and speed. Furthermore,

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* The e-Xtra logo stands for “electronic extra” and indicates that Figures 1, 2, and 3 appear in color online.

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quantitative (Q)PCR offers the opportunity for quantifying fungal biomass. Several QPCR assays have been developed for identification and quantification of individual *Fusarium* spp. (26), and QPCR methods have been developed which facilitate the detection and quantification of individual *Fusarium* spp. in cereals (25,37). Glynn and Edwards (12) compared QPCR assays to the traditional morphological approach and concluded that the amount of pathogen DNA provides a useful estimate of seed batch contamination for *Microdochium* but not for *Fusarium* pathogens, where large levels of variations were detected between the two methods. The variations can be explained by the fact that PCR-based methods detect all fungal DNA of interest without distinguishing live or dead cells and species competition, whereas morphological identification requires live organisms that are easily cultivable. Each kernel is colonized by a mixture of different fungal species, not all of them belonging to the FHB complex. There will be a competition between these different species in in vitro culture, and single species might outgrow the others (38). The morphological identification provides another difficulty in accurate incidence estimation because surface-sterilization of seed will destroy inoculum on the seed surface (29). Furthermore, PCR methods allow detection of degenerate and otherwise difficult cultures such as *F. langsethiae*, which is difficult to isolate from grain samples (44).

The perception as to which *Fusarium* spp. are responsible for FHB in Denmark has been based on survey studies performed in other European countries and from classical morphological studies of limited selected grain samples from Danish barley (1,33,39). The use of a QPCR method which is based on detection of the same gene, the translation elongation factor 1 α gene (TEF-1 α), in all *Fusarium* spp. makes it possible to obtain detailed information on the occurrence of *Fusarium* spp. in cereal grain. The present study represents the first comprehensive monitoring of its kind in Denmark and, indeed, is the first study where QPCR methodology targeting the same single gene in all the investigated species has been applied to a large number of samples covering a broad span of time originating from several cereal species anywhere. The samples of this study originate from a monitoring project established in 2003 by the Danish Knowledge Centre For Agriculture, Crop Production in order to study the extent of the problem of *Fusarium* mycotoxins in cereals and to study the effect of farming practices such as tilling and crop rotation on the mycotoxin production. The aim of the present study was to investigate the composition of the FHB species complex as well as to determine associations between the occurrence of *Fusarium* spp. and their mycotoxins in cereal samples in five seasons. In addition, a set of pooled grain samples representing the years 1957 to 2000 was investigated in order to obtain knowledge of the general composition of the FHB complex prior to 2003.

MATERIALS AND METHODS

Field samples. Fields samples of cereal grain were collected in 2003 to 2007 and consisted of harvested seed from fields evenly distributed throughout Denmark as part of a stratified monitoring program for mycotoxins in Danish cereal crops. A sample of 100 g was taken out of an original 1-kg grain sample harvested from farmers' fields. In total, 227 wheat samples from 2003 to 2007 (41 to 50 samples each year) were selected along with 61 barley and 53 triticale grain samples from 2005 to 2007 ($\approx$ 20 samples per year) and 22 oat and 17 rye grain samples from 2007. In general, the samples represent the broad variation in Danish farming practice with respect to cultivar, soil, fertilization, and fungicide treatment (G. C. Nielsen, unpublished data). However, there is an overrepresentation of wheat samples with wheat as previous crop as well as reduced tillage (23%) compared with a total area of 3% with these conditions in Denmark. After harvest, the grain samples were dried to a water content of 15 to 16%,

mycotoxin analyses were performed, and the samples were stored at -20°C before DNA extraction.

Historical samples. Milled grain samples of winter wheat and spring barley were obtained from the ASKOV long-term studies of animal manure and mineral fertilizers situated in Mid-Jutland. The ASKOV long-term studies began in 1894 (9) and the samples used for this survey were obtained during 1957 to 2000. Each sample was provided as a pooled sample consisting of pooled subsamples from 4 years based on four to five replicates pooled from each year. The samples originate from field plots fertilized according to common practice (9) and stored as milled grain in closed containers at room temperature.

Fungal isolates. The *Fusarium* isolates used for standard curves and positive controls were obtained from the IBT culture collection at the Technical University of Denmark: *F. graminearum* 1955, *F. culmorum* 9560, *F. avenaceum* 8500, *F. tricinctum* 8048, *F. poae* 8452, *F. langsethiae* 8150, *F. sporotrichioides* 1926, *F. equiseti* 8752, *F. verticilloides* 8002, *F. proliferatum* B8376B.

DNA extraction. Prior to DNA extraction, the fungal isolates were grown on potato dextrose agar (PDA) at 22°C under 12 h of light and 12 h of darkness. Mycelium was scraped off the agar using a spatula and ground in liquid N_2 with eight steel balls using a Geno/Grinder 2000 (OPS Diagnostics, Bridgewater, NJ). Ground mycelium (100 mg) was used for DNA extraction, using the cetyltrimethylammonium bromide method according to Nicolaisen et al. (25). The concentration of DNA from the fungal isolates used for standard curves was determined using NanoDrop 1000 (Thermo Fisher Scientific, MA). The same method was used for extraction of DNA from grain samples using 100 mg of powdered grain material.

Primers for real-time PCR. Primers designed by Nicolaisen et al. (25) were used for this study. These primers are based on the TEF-1 α in fungal and plant genomes. Primers for the following 10 different species were used: *F. graminearum*, *F. culmorum*/*F. cerealis*, *F. avenaceum*, *F. tricinctum*, *F. poae*, *F. langsethiae*, *F. sporotrichioides*, *F. equiseti*, *F. verticilloides*, and *F. proliferatum*. A specific primer pair identifying *M. nivale* and *M. majus* as one, and distinguishing this genus from *Fusarium*, was designed based on the TEF-1 α gene sequences of both species (13) using the Primer Express program (version 2.0; Applied Biosystems, Foster City, CA). The primers were Mniv283F: 5'-CCAAGCA GGTCGCTTTCGT-3' and Mniv333R: 5'-GCATGTTGTCGCG TTGA-3'. The specificity of the primers were tested on four isolates of *M. majus* (B-M, B-C, and REF from Scotland, kindly provided by V. Cockerell, Scottish Agricultural Science Agency; and CBS 106.90) and one isolate of *M. nivale* (CBS 162.7) together with reference isolates of *F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. tricinctum*, *F. poae*, *F. langsethiae*, *F. sporotrichioides*, *F. equiseti*, *F. verticilloides*, and *F. proliferatum* and cereal DNA as described by Nicolaisen et al. (25).

Quantification of *Fusarium* DNA. Real-time PCR was carried out in a total of 12.5 μl consisting of 6.25 μl of 2 $\times$ SYBR Green PCR Master Mix (Applied Biosystems), 250 nM each primer, bovine serum albumin at 0.5 $\mu\text{g}/\mu\text{l}$, and 2.5 μl of template DNA. PCR reactions were performed in duplicate on all samples. Genomic DNA from grain samples and pure cultures was diluted 1:10 before PCR. PCR was performed on a 7900HT Sequence Detection System (Applied Biosystems) using the following cycling protocol: 2 min at 50°C ; 95°C for 10 min; 40 cycles of 95°C for 15 s and 62°C for 1 min; followed by dissociation analysis at 60 to 95°C . For the plant assay, annealing and extension were performed at 60°C . The field samples were analyzed using the 11 specific assays. A standard curve was produced for the plant assay based on a field sample with known DNA concentration determined using NanoDrop 1000 (Thermo Scientific, Wilmington, DE). Individual standard curves were made of five-fold dilution series using pure fungal DNA and plant DNA.

Amplification efficiency of the plant assay was investigated on fivefold serial dilution (6,000 to 10 pg) of DNA extracted from samples of wheat, barley, triticale, rye, and oat in T0.1E (10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA). The amount of fungal DNA was calculated from the cycle threshold (Ct) values using the standard curve. Because SYBR Green binds to all double-stranded DNA, the results of each individual sample from each species-specific assay were evaluated by studying the dissociation curve and Ct value. The plant assay was used to determine the DNA yield and to ensure that no degradation had taken place during storage of the samples. The plant assay was further used to provide a normalized measurement for biomass in each sample, which was calculated as picograms of fungal DNA per micrograms of plant DNA according to Nicolaisen et al. (25). Amplification efficiency and cross reaction of species with nearly identical TEF-1 α genes were investigated by making fivefold serial dilutions of pure DNA of *F. langsethiae* (360 to 0.6 pg) in a constant background of pure DNA *F. sporotrichioides* (300 pg) as well as in T0.1E, respectively, using *F. langsethiae*-specific primers. The same investigations were made on fivefold serial dilutions of pure DNA of *F. graminearum* (150 to 0.24 pg) in a constant background of pure DNA of *F. culmorum* (500 pg) and in T0.1E using *F. graminearum*-specific primers. The efficiency [$E = 10^{(-1/\text{slope})} - 1$] was calculated from the slope of the linear relationship of the log₁₀ values of the DNA concentration and the cycle number (Ct).

Analysis of mycotoxins. Grain samples were analyzed for *Fusarium* mycotoxins DON, NIV, ZEA, HT-2, and T-2 by liquid chromatography–double mass spectrometry (LC-MS/MS) according to Nicolaisen et al. (25). The detection limits of the mycotoxins were 10 $\mu\text{g kg}^{-1}$ for DON, NIV, and HT-2; 2 $\mu\text{g kg}^{-1}$ for ZEA; and 5 $\mu\text{g kg}^{-1}$ for T2.

Statistical analysis. Correlation analyses were performed for levels of mycotoxins (DON, NIV, ZEA, HT2, and T2) and biomass of *F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. tricinatum*, *F. poae*, *F. sporotrichioides*, *F. langsethiae*, and *M. nivale/majus* (i.e., 13 variables in total). Samples with mycotoxin or fungal biomass levels below the detection limits were set to 5 and 2, respectively, in order to facilitate log transformation of data. Replacing 0 for mycotoxin determination with 5 reflects the approximate detection limit for the five individual mycotoxins analyzed. Biomass amounts of 0 were replaced with the arbitrary value 2, which will not give a large deviation when log transformed. All data were subsequently log transformed to stabilize variances, and exploratory principal component analysis (PCA) on the correlation matrix of the variables was performed using the statistical program Paleontological Statistics (PAST) (14). The PCAs decompose the correlated variables into underlying uncorrelated latent structures (principal components) capturing the highest degree of the total variation across all variables in as low a dimension as possible. PCA is a bilinear model that produces a scores matrix (n,k) and a loading matrix (p,k), also sometimes referred to as the left and right eigenvectors, respectively; “n” represents the number of samples, “p” the number of variables, and “k” the number of principal components. The score matrix shows how the different samples are distributed with respect to the main variation described in the first k components. The loading matrix shows why (i.e., which variables span the variation in the different components). PCA can be used as an explorative tool to reveal associations among variables, and allows interpretations that would not otherwise be apparent (19). Furthermore, PCA splits data into a systemic part, captured by the first and most significant principal components, and an unsystematic part, captured in a residual matrix. Consequently, the underlying significance of the principal components is less uncertain than for the original variables where compared directly. The results of the PCA were interpreted graphically by drawing biplots, where scatter plot of the score matrix as point and the loading matrix as

arrows for the first two components are superimposed for a condensed representation of the correlation between the original variables (*Fusarium* spp. and mycotoxins) and the distribution of the samples.

RESULTS

Reproducibility and validation of assays. Quantification of DNA of the five different cereal species showed a linear relationship between log₁₀ values of the amount of genomic DNA and Ct values, with R^2 values of 0.989 to 0.993 and E values of 91 to 100%, where barley and oat generally had the lowest E values and rye and wheat the highest.

The specificity of the species-specific assays has previously been verified in Nicolaisen et al. (25) on DNA from pure fungal isolates. In the present study, the influence of closely related species with almost identical TEF-1 α genes was investigated by making serial dilutions of *F. langsethiae* DNA in a constant background of *F. sporotrichioides* DNA ($E = 110\%$, $R^2 = 0.989$) and serial dilutions of *F. langsethiae* in T0.1E ($E = 91\%$, $R^2 = 0.991$) using *F. langsethiae*-specific primers. The same test was performed for serial dilutions of *F. graminearum* DNA in a constant background of *F. culmorum* DNA ($E = 105\%$, $R^2 = 0.987$) and *F. graminearum* in T0.1E ($E = 93\%$, $R^2 = 0.992$) using *F. graminearum*-specific primers. In both tests, the efficiency of the assays increased when a high constant background of competitive DNA was present, which may lead to overestimation of either *F. langsethiae* or *F. graminearum* in very high backgrounds of closely related species. The amounts of *F. sporotrichioides* and *F. culmorum* DNA used in the experiments were higher than detected in any field samples.

Incidences of *Fusarium* spp. in Danish cereals. Species-specific QPCR assays were used to quantify incidence and biomass (DNA) of *Fusarium* spp. and *Microdochium* spp. in 379 grain samples from 2003 to 2007. The frequency in which eight *Fusarium* spp. and *M. nivale/majus* were found in wheat, barley, triticale, oat, and rye is described in Table 1. The most frequent species in wheat through the period of study (2003 to 2007) were *F. avenaceum*, *F. graminearum*, and *F. culmorum*, which were all found in >80% of the samples. Lower incidences of *F. poae*, *F. langsethiae*, and *F. tricinatum* were recorded; the frequency varied between 60 and 70% of the samples. *F. sporotrichioides* occurred very rarely, in <11% of the samples, and *F. equiseti* was found only in a single wheat sample. In barley, the most frequent species in the period 2005 to 2007 were *F. poae*, *F. culmorum*, *F. avenaceum*, *F. langsethiae*, and *F. graminearum*, which were found, on average, in >85% of the samples. *F. tricinatum* was found in 67% of the samples, *F. sporotrichioides* in 15%, and *F. equiseti* in 2%. In triticale, the most frequent species in the period 2005 to 2007 were *F. poae* and *F. culmorum*, which were found in >74% of the samples. *F. avenaceum* and *F. graminearum* were found in $\approx 50\%$ of the triticale samples, whereas *F. langsethiae* and *F. tricinatum* were found in <20% of the samples and *F. sporotrichioides* and *F. equiseti* were not found at all. In oat, *F. avenaceum* was the most frequent species in 2007, with an incidence of 100%, followed by *F. poae* and *F. langsethiae*, both exhibiting an incidence of 86%, and *F. graminearum* and *F. culmorum*, with 82%. *F. tricinatum* and *F. sporotrichioides* were less frequent, with 55 and 27%, respectively. In rye, the most frequent species in 2007 were *F. avenaceum* and *F. culmorum*, both with an incidence of 94%, followed by *F. poae* and *F. graminearum*, with an incidence of 88 and 71%, respectively. *F. langsethiae* was found in 18% of the rye samples and *F. sporotrichioides* and *F. tricinatum* were found at an even lower frequency, with 6% incidence for both. No *F. equiseti* was found in rye. *F. proliferatum* and *F. verticillioideus* were not found in any of the cereal samples. The nontoxigenic *M. nivale/majus* was, on average, found in >90% of the samples across years and cereal species.

***Fusarium* and *M. nivale/majus* biomass and mycotoxin.** The biomass of *Fusarium* spp. and *M. nivale/majus* (DNA) and the amount of *Fusarium* mycotoxins found in Danish cereal grain samples from 2003 through 2007 are shown in Tables 2 to 5. For each species, there is a large variation between years but also large variability between the samples within each year, and this is reflected in the 95% confidence interval. The nontoxigenic *M. nivale/majus* generally occurred at a higher biomass than most of the *Fusarium* spp. in all years across the cereal species, with the exception of *F. graminearum* in wheat. Compared with the other cereal species, the amounts of *M. nivale/majus* in wheat were generally lower.

It is clear that a relatively larger biomass was present for *F. graminearum* than for any other *Fusarium* spp. throughout the study (Table 2). Relatively lower amounts of *F. avenaceum*, *F. langsethiae*, and *F. culmorum* and even lower amounts of *F. poae* and *F. tricinctum* were observed; finally, only traces of *F. sporotrichioides* were found. *F. graminearum* exhibited ≤ 10 -fold differences in biomass between the years 2003 and 2004, where high amounts of this species were found, compared with 2006, where low levels were recorded. Relatively high levels of *F. avenaceum* were seen in 2003 and 2004. The type B trichothecenes (DON and NIV) were the most prevalent mycotoxins during the years. Especially in 2003 and 2004, the amounts of DON were

TABLE 1. Incidences of *Fusarium* spp. and *Microdochium nivale/majus* in wheat (2003 to 2007), barley (2005 to 2007), triticale (2005 to 2007), oat (2007), and rye (2007) and across years for wheat, barley, and triticale, given as percentages^a

Species	Wheat					Barley			Triticale			Oat	Rye	Wheat	Barley	Triticale
	2003	2004	2005	2006	2007	2005	2006	2007	2005	2006	2007	2007	2007	All	All	All
<i>Fg</i>	100	100	83	67	86	75	85	100	78	53	44	82	71	87	87	58
<i>Fc</i>	83	91	72	60	96	85	100	90	67	71	83	82	94	86	92	74
<i>Fa</i>	100	100	96	71	98	90	80	100	28	35	67	100	94	93	90	43
<i>Ft</i>	63	41	51	53	88	55	70	81	0	6	11	55	6	60	67	6
<i>Fp</i>	88	75	60	60	70	100	100	100	72	82	94	86	88	70	100	83
<i>Fs</i>	17	32	4	2	2	10	20	14	0	0	0	27	6	11	15	0
<i>Fe</i>	0	0	0	0	2	0	0	5	0	0	0	0	0	>1	2	0
<i>Fl</i>	78	27	64	78	52	95	100	81	6	35	17	86	18	59	92	19
<i>M</i>	98	100	94	60	100	100	100	100	100	94	100	100	100	90	100	98

^a Abbreviations: *F. graminearum* (*Fg*), *F. culmorum* (*Fc*), *F. avenaceum* (*Fa*), *F. tricinctum* (*Ft*), *F. poae* (*Fp*), *F. sporotrichioides* (*Fs*), *F. langsethiae* (*Fl*), *M. nivale/majus* (*M*). Wheat: 2003, $n = 41$; 2004, $n = 44$; 2005, $n = 47$; 2006, $n = 45$; 2007, $n = 50$; total $n = 227$. Barley: 2005, $n = 20$; 2006, $n = 20$; 2007, $n = 21$; total $n = 61$. Triticale: 2005, $n = 18$; 2006, $n = 17$; 2007, $n = 18$; total $n = 53$. Oat: $n = 22$. Rye: $n = 17$. All = all years.

TABLE 2. Amounts of *Fusarium* spp., *Microdochium nivale/majus*, and the mycotoxins deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEA), HT-2, and T-2 in wheat grain samples collected in Danish fields in 2003 to 2007 described by mean values and 95% confidence interval^a

Year	n	Amount of <i>Fusarium</i> DNA (pg of fungal DNA/ μ g of plant DNA)								<i>Fusarium</i> mycotoxins (μ g kg ⁻¹)				
		<i>Fg</i>	<i>Fc</i>	<i>Fa</i>	<i>Ft</i>	<i>Fp</i>	<i>Fs</i>	<i>Fl</i>	<i>M</i>	DON	NIV	ZEA	HT-2	T-2
2003	41	1,480 $\pm$ 356	342 $\pm$ 106	638 $\pm$ 184	178 $\pm$ 96	143 $\pm$ 39	10 $\pm$ 8	570 $\pm$ 201	579 $\pm$ 173	562 $\pm$ 188	28 $\pm$ 8	1 $\pm$ 1	1 $\pm$ 2	0
2004	44	8,359 $\pm$ 7,433	305 $\pm$ 98	815 $\pm$ 337	81 $\pm$ 70	81 $\pm$ 34	16 $\pm$ 8	92 $\pm$ 92	750 $\pm$ 252	916 $\pm$ 723	19 $\pm$ 7	99 $\pm$ 79	3 $\pm$ 3	1 $\pm$ 1
2005	47	1,765 $\pm$ 1,530	257 $\pm$ 83	272 $\pm$ 79	93 $\pm$ 65	71 $\pm$ 33	1 $\pm$ 1	412 $\pm$ 194	298 $\pm$ 92	475 $\pm$ 299	17 $\pm$ 6	0	0	0
2006	45	1,067 $\pm$ 850	67 $\pm$ 34	82 $\pm$ 43	43 $\pm$ 23	128 $\pm$ 51	0	213 $\pm$ 61	45 $\pm$ 18	153 $\pm$ 61	8 $\pm$ 5	25 $\pm$ 22	3 $\pm$ 2	1 $\pm$ 1
2007	50	1,334 $\pm$ 1,238	224 $\pm$ 80	234 $\pm$ 87	93 $\pm$ 57	141 $\pm$ 75	9 $\pm$ 17	99 $\pm$ 51	959 $\pm$ 183	327 $\pm$ 228	16 $\pm$ 8	18 $\pm$ 20	2 $\pm$ 2	0.3 $\pm$ 0.6

^a Abbreviations: *F. graminearum* (*Fg*), *F. culmorum* (*Fc*), *F. avenaceum* (*Fa*), *F. tricinctum* (*Ft*), *F. poae* (*Fp*), *F. sporotrichioides* (*Fs*), *F. langsethiae* (*Fl*), *M. nivale/majus* (*M*); n = number of tested samples; 95% = 95% of the samples are found in this interval. Detection limits: DON, NIV, and HT-2 > 10 μ g kg⁻¹; ZEA > 2 μ g kg⁻¹; T-2 > 5 μ g kg⁻¹.

TABLE 3. Amounts of *Fusarium* spp., *Microdochium nivale/majus*, and the mycotoxins deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEA), HT-2, and T-2 in barley grain samples collected in Danish fields in 2003 to 2005 described by mean values and 95% confidence interval^a

Year	n	Amount of <i>Fusarium</i> DNA (pg fungal DNA/ μ g of plant DNA)								<i>Fusarium</i> mycotoxins (μ g kg ⁻¹)				
		<i>Fg</i>	<i>Fc</i>	<i>Fa</i>	<i>Ft</i>	<i>Fp</i>	<i>Fs</i>	<i>Fl</i>	<i>M</i>	DON	NIV	ZEA	HT-2	T-2
2005	20	114 $\pm$ 140	114 $\pm$ 44	192 $\pm$ 124	319 $\pm$ 187	626 $\pm$ 312	8 $\pm$ 12	1,197 $\pm$ 543	1,373 $\pm$ 562	32 $\pm$ 17	37 $\pm$ 22	0	0	0
2006	20	57 $\pm$ 51	1,645 $\pm$ 2,677	923 $\pm$ 1,548	565 $\pm$ 365	987 $\pm$ 779	36 $\pm$ 39	4,197 $\pm$ 2,230	542 $\pm$ 388	19 $\pm$ 13	21 $\pm$ 13	0	20 $\pm$ 9	5 $\pm$ 3
2007	21	218 $\pm$ 185	427 $\pm$ 337	1,243 $\pm$ 595	389 $\pm$ 241	937 $\pm$ 411	6 $\pm$ 8	670 $\pm$ 397	12,851 $\pm$ 4,454	91 $\pm$ 45	65 $\pm$ 34	2 $\pm$ 3	25 $\pm$ 15	5 $\pm$ 5

^a Abbreviations: *F. graminearum* (*Fg*), *F. culmorum* (*Fc*), *F. avenaceum* (*Fa*), *F. tricinctum* (*Ft*), *F. poae* (*Fp*), *F. sporotrichioides* (*Fs*), *F. langsethiae* (*Fl*), *M. nivale/majus* (*M*); n = number of samples tested; 95% = 95% of the samples are found in this interval. Detection limits: DON, NIV, and HT-2 > 10 μ g kg⁻¹; ZEA > 2 μ g kg⁻¹; T-2 > 5 μ g kg⁻¹.

TABLE 4. Amounts of *Fusarium* spp., *Microdochium nivale/majus*, and the mycotoxins deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEA), HT-2, and T-2 (μ g kg⁻¹) in triticale grain samples collected in Danish fields in 2003 to 2005 described by mean values and 95% confidence interval^a

Year	n	Amount of <i>Fusarium</i> DNA (pg fungal DNA/ μ g of plant DNA)								<i>Fusarium</i> mycotoxins (μ g kg ⁻¹)				
		<i>Fg</i>	<i>Fc</i>	<i>Fa</i>	<i>Ft</i>	<i>Fp</i>	<i>Fs</i>	<i>Fl</i>	<i>M</i>	DON	NIV	ZEA	HT-2	T-2
2005	18	3,369 $\pm$ 3,319	530 $\pm$ 387	113 $\pm$ 123	0	134 $\pm$ 71	0	46 $\pm$ 89	2,287 $\pm$ 1,279	433 $\pm$ 231	19 $\pm$ 10	0	0	0
2006	17	101 $\pm$ 110	172 $\pm$ 101	92 $\pm$ 155	9 $\pm$ 19	394 $\pm$ 431	0	49 $\pm$ 47	113 $\pm$ 92	20 $\pm$ 17	20 $\pm$ 18	0	0	0
2007	18	62 $\pm$ 61	2,138 $\pm$ 1,977	569 $\pm$ 453	55 $\pm$ 180	209 $\pm$ 82	0	94 $\pm$ 143	4,675 $\pm$ 2,352	282 $\pm$ 231	22 $\pm$ 10	4 $\pm$ 6	0	0

^a Abbreviations: *F. graminearum* (*Fg*), *F. culmorum* (*Fc*), *F. avenaceum* (*Fa*), *F. tricinctum* (*Ft*), *F. poae* (*Fp*), *F. sporotrichioides* (*Fs*), *F. langsethiae* (*Fl*), *M. nivale/majus* (*M*); n = number of tested samples; 95% = 95% of the samples are found in this interval. Detection limits: DON, NIV, and HT-2 > 10 μ g kg⁻¹; ZEA > 2 μ g kg⁻¹; T-2 > 5 μ g kg⁻¹.

higher compared with the amounts found in other years. In 2005, no ZEA, HT-2, or T-2 was detected and, apart from 2004, the amount of ZEA was found to be low in all years. The levels of type A trichothecenes HT-2 and T-2 were generally low.

For barley (Table 3), *F. langsethiae* was generally the predominant *Fusarium* sp. in terms of biomass across the years. However, the biomass of *M. nivale/majus* was higher. This was followed by *F. culmorum*, *F. avenaceum*, *F. tricinctum*, and *F. poae*, which again exhibited higher amounts of DNA than *F. graminearum*, whereas *F. sporotrichioides* was less abundant. There were large variations in biomasses between years (e.g., *F. langsethiae* showed a difference of sixfold between years, whereas it was fairly stable for others, such as *F. tricinctum*, which differed less than twofold across the years). The *Fusarium* mycotoxins present were found to reflect the presence of both type A- and type B-producing species, with generally low amounts of both types and very low amounts of ZEA.

F. graminearum and *F. culmorum* were the species with the highest biomass in triticale across years (Table 4). These were followed by *F. avenaceum* and *F. poae*, which both had biomass at relatively higher levels than *F. langsethiae* and *F. tricinctum*. *F. sporotrichioides* was not found in any of the samples. There was large variation in biomass of the individual species between years (e.g., *F. graminearum* and *F. culmorum*). Thus, in 2005, the biomass of *F. graminearum* was 6-fold higher than that of *F.*

culmorum but, in 2006, the biomass of both was relatively low and, in 2007, the biomass of *F. culmorum* was 34-fold higher than for *F. graminearum*. The *Fusarium* mycotoxins present were found to reflect the presence of mainly type B-producing species. In 2005 and 2007, the biomass of either *F. graminearum* or *F. culmorum* was relatively high, which was consistent with the high levels of DON observed. Low levels of NIV were found in all years, ZEA was only found at very low levels in 2007, and no HT-2 and T-2 was detected.

F. langsethiae and *F. avenaceum* were the dominant species in oat samples (Table 5). These species were followed by *F. graminearum*, *F. poae*, *F. tricinctum*, and *F. culmorum*, all of which were present at higher levels than *F. sporotrichioides*. In rye, the species showing the highest amount of biomass were *F. avenaceum* and *F. culmorum*. These were followed by *F. graminearum* and *F. poae*, which had relatively larger biomass than *F. langsethiae*, *F. tricinctum*, and *F. sporotrichioides*. The *Fusarium* mycotoxins found in oat reflected the presence of both type A- and type B-producing species whereas mainly type B-producing species were found primarily in rye. Low levels of ZEA were found in both oat and rye.

The occurrence of *Fusarium* spp. and *M. nivale/majus* in wheat and barley is described in Tables 6 and 7, respectively, for 1957 to 2000. *F. culmorum* and *F. poae* were found in all years in both wheat and barley. Low levels of *F. graminearum* were found in

TABLE 5. Amounts of *Fusarium* spp., *Microdochium nivale/majus*, and the mycotoxins deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEA), HT-2, and T-2 in oat and rye grain samples collected in Danish fields in 2007 described by mean values and 95% confidence interval^a

Cereal	n	Amount of <i>Fusarium</i> DNA (pg fungal DNA/μg of plant DNA)								<i>Fusarium</i> mycotoxins (μg kg ⁻¹)				
		Fg	Fc	Fa	Ft	Fp	Fs	Fl	M	DON	NIV	ZEA	HT-2	T-2
Oat	22	1,409 ± 2,548	646 ± 270	2,467 ± 926	692 ± 335	903 ± 500	57 ± 83	2,595 ± 1,028	8,955 ± 3,741	44 ± 25	45 ± 30	1 ± 1	57 ± 26	19 ± 21
Rye	17	258 ± 202	819 ± 307	1,787 ± 827	17 ± 33	184 ± 112	5 ± 10	31 ± 39	6,806 ± 5,845	56 ± 25	3 ± 3	2 ± 2	2 ± 3	0

^a Abbreviations: *F. graminearum* (Fg), *F. culmorum* (Fc), *F. avenaceum* (Fa), *F. tricinctum* (Ft), *F. poae* (Fp), *F. sporotrichioides* (Fs), *F. langsethiae* (Fl), *M. nivale/majus* (M); n = number of tested samples; 95% = 95% of the samples are found in this interval. Detection limits: DON, NIV, and HT-2 >10 μg kg⁻¹; ZEA >2 μg kg⁻¹; T-2 >5 μg kg⁻¹.

TABLE 6. Quantification of *Fusarium* and *Microdochium nivale/majus* biomass and the mycotoxins deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEA), HT-2, and T-2 in eight pooled historical wheat samples from the ASKOV location in 1957 to 2000 described by mean values^a

Year	Amount of <i>Fusarium</i> DNA (pg fungal DNA/μg of plant DNA)								<i>Fusarium</i> mycotoxins (μg kg ⁻¹)				
	Fg	Fc	Fa	Ft	Fp	Fl	M		DON	NIV	ZEA	HT-2	T-2
1957–1960	0	122	0	0	43	0	198		66	12	nd	nd	nd
1965–1968	119	0	100	0	0	0	345		69	17	nd	nd	nd
1977–1980	0	121	456	0	12	66	200		39	nd	13	nd	nd
1981–1984	0	171	18	0	76	0	378		nd	nd	nd	nd	nd
1985–1989	0	199	300	0	80	0	620		62	26	nd	nd	nd
1989–1992	0	33	16	0	100	0	151		nd	11	nd	nd	nd
1993–1996	0	313	8	0	649	28	172		27	32	nd	nd	nd
1997–2000	76	8,866	1,131	0	492	225	839		859	206	14	nd	nd

^a Abbreviations: *F. graminearum* (Fg), *F. culmorum* (Fc), *F. avenaceum* (Fa), *F. tricinctum* (Ft), *F. poae* (Fp), *F. sporotrichioides* (Fs), *F. langsethiae* (Fl), *M. nivale/majus* (M); nd = not determined. Detection limits: DON, NIV, and HT-2 >10 μg kg⁻¹; ZEA >2 μg kg⁻¹; T-2 >5 μg kg⁻¹.

TABLE 7. Quantification of *Fusarium* and *Microdochium nivale/majus* biomass and the mycotoxins deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEA), HT-2, and T-2 in eight pooled historical barley samples from the ASKOV location in 1957 to 2000 described by mean values^a

Year	Amount of <i>Fusarium</i> DNA (pg fungal DNA/μg of plant DNA)								<i>Fusarium</i> mycotoxins (μg kg ⁻¹)				
	Fg	Fc	Fa	Ft	Fp	Fl	M		DON	NIV	ZEA	HT-2	T-2
1957–1960	0	0	225	0	48	0	110		13	15	nd	nd	nd
1965–1968	8	101	243	0	109	0	506		11	19	nd	nd	nd
1977–1980	0	190	1,086	0	276	199	983		49	16	nd	nd	nd
1981–1984	0	91	53	0	27	0	790		nd	10	nd	nd	nd
1985–1989	0	52	255	0	83	72	2,111		nd	21	nd	nd	nd
1989–1992	0	55	162	102	77	218	4,049		nd	18	nd	12	nd
1993–1996	0	90	78	161	195	0	1,144		nd	19	nd	nd	nd
1997–2000	0	168	924	110	100	209	18,793		17	49	nd	12	nd

^a Abbreviations: *F. graminearum* (Fg), *F. culmorum* (Fc), *F. avenaceum* (Fa), *F. tricinctum* (Ft), *F. poae* (Fp), *F. sporotrichioides* (Fs), *F. langsethiae* (Fl), *M. nivale/majus* (M); n = number of samples tested; nd = not determined. Detection limits: DON, NIV, and HT-2 >10 μg kg⁻¹; ZEA >2 μg kg⁻¹; T-2 >5 μg kg⁻¹.

both wheat and barley in 1965 to 1968 and then again in 1997 to 2000 in wheat. *F. tricinatum* was found exclusively on barley in 1989 to 2000. No *F. sporotrichioides*, *F. equiseti*, *F. proliferatum*, or *F. verticilloides* were found in these samples whereas *M. nivale* and *M. majus* were found in all years in both wheat and, especially, barley. The occurrence of mycotoxins in both barley and wheat showed a dominance of type B trichothecenes and very low amounts of the type A trichothecenes. NIV was found more abundantly than DON in barley, whereas DON was found more abundantly than NIV in wheat. No T-2 was found in either wheat or barley and HT-2 was only found in barley after 1989. ZEA was only found in a few samples of wheat in 1977 to 1980 and 1997 to 2000.

Correlations between year, the species of the FHB complex, and mycotoxins by PCA analysis. Wheat samples ($n = 219$) from 2003 to 2007 were plotted as a biplot in Figure 1. This shows both the distribution of the samples in the two most descriptive dimensions of data and the variables spanning these two directions. On the x-axis, component 1 describes 29% of the variability and, on the y-axis, component 2 describes an additional 14% of the original variability. From the PCA, it is evident that levels of the mycotoxins DON, NIV, and ZEA are correlated with the biomass of *F. graminearum* and *F. culmorum* but also with *F. avenaceum* and *M. nivale/majus* in the direction of component 1. The biomass of *F. tricinatum*, *F. poae*, and *F. langsethiae* spans component 2 and is correlated with the mycotoxin HT-2, whereas the biomass of *F. sporotrichioides* and the mycotoxin T-2 is not well described by the two first components. The samples are marked with the year of sampling, and there appears to be a pattern of separation between the 5 years, indicated by the concentration ellipsis which encompass 50% of data for a given year. The correlations in the direction of component 1 and 2 are mainly due to the year; 2003 and 2004 were years with

much fusarium infection, whereas the years 2005 and 2007 were less conducive to infection and, therefore, influenced the correlation less. Finally, 2006 appears not to be conducive to infection, and this is also apparent in Table 2. Exploration of patterns with respect to *F. sporotrichioides* and T-2 was done by examination of higher, less-descriptive, components, which indicate that the presence of *F. sporotrichioides* is correlated with the presence of both *F. poae* and HT-2 toxins (data not shown), whereas T-2 finds no description in higher components due to extremely low levels of this mycotoxin in wheat (Table 2).

Barley samples ($n = 59$) from the 2005 to 2007 were plotted as a biplot in Figure 2 (component 1 = 24% and 2 = 18%). From the PCA, it is evident that the mycotoxins DON and NIV correlate with the biomass of *F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. poae*, and *M. nivale/majus* in the direction of component 1. Levels of the mycotoxins HT-2 and T-2 correlate with the biomass of *F. langsethiae* and *F. tricinatum* and span component 2. Neither the mycotoxin ZEA nor pathogen *F. sporotrichioides* are described well by the two first components. The samples are, as above, marked with the year, and there is a pattern of separation between the 3 years. The correlations in the direction of component 1 are ascribed to the year 2007 whereas 2006 mainly influenced the correlations in the direction of component 2 and 2005 appeared to have been a less conducive year to *Fusarium* infections in barley, which is also observed in Table 3. Exploration of patterns with respect to ZEA and *F. sporotrichioides* can be done by examination of higher, less descriptive, components, which indicate that the presence of both ZEA and *F. sporotrichioides* are correlated with the presence of both T-2 and HT-2 (data not shown).

Triticale samples ($n = 55$) from 2005 to 2007 are plotted as a biplot in Figure 3 (component 1 = 26% and 2 = 17%). From the PCA, it is evident that the mycotoxins DON and NIV correlate

Fig. 1. Wheat biplot of the principal component analysis of levels of mycotoxins deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEA), HT-2, and T-2 and biomass of *Fusarium graminearum*, *F. culmorum*, *F. avenaceum*, *F. tricinatum*, *F. poae*, *F. sporotrichioides*, *F. langsethiae*, and *Microdochium nivale/majus*. Each ellipse encompasses 50% of the data for a given year. Year: 2003 (+), 2004 (□), 2005 (◇), 2006 (○), and 2007 (△).

with the biomass of *F. graminearum*, *F. culmorum*, *F. avenaceum*, and *M. nivale/majus* in the direction of component 1. The biomass of *F. langsethiae* and *F. tricinctum* and ZEA span component 2. The biomass of *F. poae* and *F. sporotrichioides* and the mycotoxins HT-2 and T-2 are not well described by the two first components. The samples are, as for barley, marked with the year, and there appears to be a pattern of separation between the 3 years. The correlations in the direction of component 1 and 2 are attributable to the years 2005 and 2007 whereas 2006 appears to have been a less conducive year to *Fusarium* infections in this crop. Exploration of patterns with respect to *F. poae* and *F. sporotrichioides* and the mycotoxins HT-2 and T-2 was performed by examination of higher, less descriptive, components, which indicated that the presence of *F. poae* correlated with the presence of *F. avenaceum*, whereas *F. sporotrichioides*, HT-2, and T-2 cannot be described in higher components (data not shown) due to low levels of these mycotoxins in triticale (Table 4).

DISCUSSION

This study is the first of its kind to give this detailed information, in terms of frequency and biomass for 11 *Fusarium* spp. causing FHB in five different small grain cereals over 1 to 5 years. The QPCR assays not only provide a measure for the fungal biomass but also reveal the true predominance of the individual species in the FHB complex. This information was then linked by PCA analysis to the occurrence of the relevant *Fusarium*-related mycotoxins. Although higher amounts of mycotoxins were seen in some years, they never exceeded the limits of *Fusarium* mycotoxins in unprocessed grain permitted by the European Union (EU). Major differences were observed between the FHB complex in the five different cereal species as well as substantial year-to-year variation, and analysis of historical barley and wheat samples indicated a shift in the FHB complex toward more *F. graminearum*. Correlations between the biomass of indi-

vidual *Fusarium* spp. and the corresponding mycotoxins illustrated that, by monitoring the individual *Fusarium* spp., it is possible to predict the potential for mycotoxin contamination.

There is generally limited knowledge of the *Fusarium* spp. composition on the major cereals (wheat, barley, oat, triticale, and rye), let alone in Denmark. Prior knowledge of the frequency of *Fusarium* spp. in Danish cereals was based on three morphological studies of *Fusarium* spp. on barley. A study on barley from harvested plots in 1967 across Denmark found *Fusarium* spp. on $\approx 5\%$ of the seed, with a dominance of *F. avenaceum*, *F. poae*, *F. graminearum*, *M. nivale/majus*, and *F. culmorum* (39). Another study of *Fusarium* spp. isolated from Danish malting barley found *F. tricinctum* and *F. avenaceum* as the dominant species followed by *F. poae* (1). A minor study of Danish cereals by Thrane (33) showed that *F. avenaceum* and *F. culmorum* were the most frequent species. It has been assumed, based on the study by Thrane (33) and the species composition in other northern European countries within the same climatic zone, that *F. avenaceum* and *F. culmorum* were the dominant *Fusarium* spp. in Danish cereals (5). The incidence data in the present study confirm the assumptions made by Bottalico and Perone (5) and further add that *M. nivale* and *M. majus* were the most frequent species across the years and crops. Furthermore, the conclusions of the three older morphological studies (1,33,39) were also reflected in the 16 historical samples of wheat and barley analyzed in this study. Here, *F. culmorum*, *F. avenaceum*, and *M. nivale/majus* were found in samples from the period 1957 to 2000 but *F. graminearum* was absent in most years and only present in low amounts in wheat samples from the period 1965 to 1968 and again in samples from the period 1997 to 2000.

Because both frequency and biomass were measured, a more detailed picture of the FHB complex in wheat, barley, triticale, oat, and rye was obtained. *F. graminearum* was found to be the most abundant species in wheat, in terms of biomass, and this was consistent for all years. *F. langsethiae*, *F. avenaceum*, and *F. poae*

Fig. 2. Barley biplot of the principal component analysis of levels of mycotoxins deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEA), HT-2, and T-2 and biomass of *Fusarium graminearum*, *F. culmorum*, *F. avenaceum*, *F. tricinctum*, *F. poae*, *F. sporotrichioides*, *F. langsethiae*, and *Microdochium nivale/majus*. Each ellipse encompasses 50% of the data for a given year. Year: 2005 ($\diamond$), 2006 ($\circ$), and 2007 ($\triangle$).

were the predominant *Fusarium* spp. over the years in barley and oat. In contrast, in triticale, the predominant species was *F. culmorum* and, in rye, *F. avenaceum*. In the present study, *M. nivale/majus* was generally the most abundant in terms of biomass over years and cereal species, especially in 2007, where very high amounts of biomass were detected in all cereal species. *M. nivale* and *M. majus* are well-known pathogens of cereals included in the species complex of foot rot and FHB (5). At temperatures <18°C, conidia of *M. nivale* and *M. majus* are more competitive than species belonging to the *Fusarium* genus (7). In cooler areas of Europe, such as Denmark, this species might have an advantage over the other species in the FHB complex. These two species, which previously belonged to the *Fusarium* genus, have often caused confusion in predictions of DON content based on visual symptoms because their symptoms resemble those of *F. graminearum* and *F. culmorum*. Waalwijk et al. (37) showed that the nontoxigenic *M. majus* was almost completely absent from wheat ears where high levels of toxigenic *Fusarium* spp. were found. In the present study, there was a constant occurrence of *M. nivale/majus* with type B trichothecene-producing *Fusarium* spp.; further studies must be made to reveal whether it is *M. nivale* or *M. majus* that is the dominant species.

Although only a limited number of historical samples were available for this study, the results indicate that there has been a recent change in the FHB complex composition toward more *F. graminearum* in Denmark around year 2000, which has also been reported for several European countries (16,17,27,32,37,42). The change has been proposed to be linked to an increase in minimum tillage and maize cropping (3,10,23,31). Wheat samples originating from crop rotations with maize as the previous crop were not included in this study, because <1% of the wheat area in Denmark has maize as the previous crop. However, a separate analysis of 21 samples from fields having maize as the previous crop showed very high and dominating levels of *F. graminearum* and very high amounts of DON compared with wheat samples

from other cropping systems (data not shown). This clearly indicates that wheat grown in rotation with maize has a high risk for being contaminated with mycotoxins produced by *F. graminearum*, especially, which has also been shown by several studies (8,10,27). Maize was introduced as a crop in Denmark in the beginning of the 1980s; maize area increased to 50,000 ha in 2000 and, since then, it has further increased to 150,000 ha in 2008 (representing 5.6% of the agricultural area). This is most likely to be responsible for promoting the shift toward *F. graminearum*. A general increase in the winter cereal area in Denmark as well as an increasing trend toward cropping using reduced tillage and stubble management could also have contributed to the changes, as also proposed by Champeil et al. (8). Another feasible explanation for the changes toward more *F. graminearum* may involve a gradual adaptation of the fungus to the cooler climate and, at the same time, changes in climate toward warmer and more humid summers could also have an effect in the increase of *F. graminearum* (41). Superior competitiveness of *F. graminearum* in wheat over other *Fusarium* spp. may provide yet another explanation for its increase in cooler regions of Europe (43).

In the present survey, 2003 and 2004 favored the development of *F. graminearum* and DON production. Rainfall and consequent high humidity are important for infection (18,28). Although no specific analysis was carried out on local weather data from the individual fields and the occurrences of FHB, high precipitation during flowering was recorded in both of these seasons (www.dmi.dk). *F. graminearum* is described to have temperature optimum for both infection and growth of ≈28 to 29°C (30). This is considerably higher than the Danish average temperature in June, which has varied between 13.5 and 16.0°C in the time period 2003 to 2007 (www.dmi.dk). In June and July 2007, the precipitation was twice the seasonal average; even so, it was dry during flowering, which limited the initial infection. In the 2007 season, *M. nivale/majus* and *F. avenaceum* dominated on the

Fig. 3. Triticale biplot of the principal component analysis of levels of mycotoxins deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEA), HT-2, and T-2 and biomass of *Fusarium graminearum*, *F. culmorum*, *F. avenaceum*, *F. tricinctum*, *F. poae*, *F. sporotrichioides*, *F. langsethiae*, and *Microdochium nivale/majus*. Each ellipse encompasses 50% of the data for a given year. Year: 2005 (◇), 2006 (○), and 2007 (△).

grain, and clear leaf symptoms of *M. nivale/majus* were also observed in that year, indicating that these weather conditions were very favorable for *M. nivale/majus*. This is in accordance with previous studies, which showed that *F. avenaceum* and *M. nivale/majus* are the greatest producers of conidia under moist and cool conditions (30). The historical samples of barley showed an increase in the amount of *M. nivale/majus* in barley during the time span 1957 to 2000, as opposed to wheat in the same time period; however, the possible explanation for this change is not clear.

F. langsethiae and *F. poae* were abundant, especially in barley and oat. *F. langsethiae* was found to be present at high levels in barley, not only in recent years but also in the samples from 1977 to 1980, demonstrating that this species was present before it was first described (36). An increasing occurrence of *F. poae* has been recorded in recent years (2,43). *F. poae* is generally described as less pathogenic (6,40) as well as a secondary invader, colonizing the weakened ears sometimes already infected by other, more aggressive *Fusarium* spp. such as *F. graminearum* (2). Even though *F. poae* has been described to be associated with warm and dry weather (42), it has been found commonly in Nordic regions, where it is clearly associated with content of NIV (44).

A multivariate statistical analysis (PCA) was employed to investigate correlations between the individual 10 *Fusarium* spp. and *M. nivale/majus* as well as the associated mycotoxins. The PCA analysis generally showed positive correlations between the biomass of individual *Fusarium* spp. and their corresponding mycotoxins, verifying the ability of the different species to produce specific mycotoxins under field conditions. In wheat, two defined groups are seen describing the *Fusarium* spp., producing either type A or type B trichothecenes. For type B trichothecenes, levels of DON and ZEA were found to correlate with *F. graminearum* and *F. culmorum* but also to other species such as *F. avenaceum* and *M. nivale/majus*. Although the latter do not produce DON and NIV, this correlation might indicate that these species are favored by the same environmental conditions. It is possible that, due to competition between species, these species are stimulating DON production in *F. graminearum* and *F. culmorum*. In the group of type A trichothecenes, HT-2 was found to correlate with *F. langsethiae*, *F. tricinctum*, and *F. poae*. *F. langsethiae* is a known producer of HT-2 but the PCA analysis indicates that *F. poae* might also be responsible for some HT-2 because a few isolates of *F. poae* have been shown to produce T-2 and HT-2 (35). *F. tricinctum* is considered to be nontrichothecene producer (34) but may be stimulated by the same growth conditions as *F. langsethiae* and *F. poae*. The type B trichothecene NIV is located on the plot between the two groups described above, indicating a correlation with both *F. graminearum* and *F. culmorum* in the first group as well as *F. poae* in the second group. It was seen that levels of the two trichothecene groups did not correlate, and the correlations were greatly affected by the different years.

In barley, as in wheat, two groups separating type A and type B trichothecenes were seen; however, ZEA was not detected and, therefore, not described, and *F. poae* correlated with the type B trichothecene group. The reasons for the low levels of ZEA appear unclear. A clear separation of the years was seen: 2007 showed a preference for type B, 2006 defined type A, and 2005 did not contribute to the correlations. In triticale, the type A trichothecenes were not detected but DON correlated with *F. graminearum* and *F. culmorum* correlated with NIV. Again, *M. nivale/majus* levels were found to correlate with *F. graminearum* and *F. langsethiae* was seen to correlate with *F. tricinctum*, indicating that these species are stimulated by the same environmental conditions or agricultural practices.

The existence of a complex of *Fusarium* spp. within single field samples has previously been shown (2,4,15). In the present study, up to seven different *Fusarium* spp., along with *M. nivale/*

majus, were often found in single grain samples, confirming the existence of a diverse *Fusarium* species complex. Audenaert et al. (2) recorded up to nine *Fusarium* spp. in addition to *M. nivale* in one sample and found clear associations between *F. poae* and *F. avenaceum* as well as between *F. graminearum* and *M. nivale/majus*. The former finding was not confirmed in our data, whereas the latter finding is in accordance with the Danish data from wheat, barley, and triticale, where the biomass of *M. nivale/majus* seems to be correlated with the biomass of *F. graminearum*.

Interesting observations concerning the ratio between DON and NIV in wheat and barley are apparent in Tables 2 and 3. In wheat, the DON/NIV ratio is $\approx 20:1$ whereas, in barley, the DON/NIV ratio is close to 1:1. These differences might be related either to the ratio between the NIV producers *F. graminearum* or *F. culmorum* and *F. poae* or the fact that NIV chemotypes of *F. graminearum* and *F. culmorum* are associated with barley. However, Tables 2 and 3 clearly show differences in the ratio between the DON-producing species *F. graminearum* and *F. culmorum* and NIV-producing *F. poae*. In wheat, the *F. graminearum* or *F. culmorum*/*F. poae* ratio varies from 10:1 to 100:1, whereas, in barley, the ratio varies from 2:1 to 1:3. Bottalico and Perone (5) reported that NIV found in cereals in Sweden and northern Europe most likely is produced by *F. poae*.

In the present study, it was possible to study the presence of 11 individual fungal species in 396 grain samples, a task which would have been extremely laborious and difficult to carry out using traditional culturing methods, assuming that it would have been possible to extract viable spores from these samples. The same DNA-extraction method was used for all cereal species and the amplification efficiencies were comparable in the different cereal backgrounds. The fungal biomass was calculated as the amount of fungal DNA normalized with the amount of plant DNA for each sample in order to compensate for variations in DNA yield and presence of PCR inhibitors. However, due to differences in genome sizes, comparison of biomass data from different cereal species should be avoided. Validation experiments using mixtures of DNA from closely related species showed that PCR amplification of low amounts of target DNA from one species may be affected by the presence of high amount of DNA from a closely related species, and this may lead to some overestimation of the species present in low amounts. However, the QPCR methods will allow detection and quantification of species present in very low amounts, in contrast to conventional methods, where the presence of especially slow-growing or dead species will be underestimated.

FHB is not considered a serious threat to Danish grain production because crop rotations and tillage methods are most commonly nonconductive to severe attack by FHB. Nevertheless, this study has shown that the *Fusarium* spp. capable of producing mycotoxins are common on Danish grain, and can give rise to widespread attack in seasons where climate stimulates infection and growth of *Fusarium* spp. The mycotoxin monitoring project by Knowledge Centre for Agriculture, Crop Production, shows that from common agricultural practices in Denmark no samples collected during 2005 to 2010 exceed the permitted limits for DON given by the EU (G. C. Nielsen, unpublished data). In 2003 and 2004, where the grown cultivars were more susceptible and weather more favorable for *Fusarium*, several wheat samples exceeded the permitted limits for DON. The wheat cultivars grown in Denmark generally exhibit low to medium resistance to FHB (20) and that, in general, farmers do not apply fungicides to control FHB, because the risk is considered low and trials have shown low yield responses for control. Because the fungal species involved in FHB differ in pathogenicity, mycotoxin production, and response to cropping practice and fungicides, the correct identification and quantification is considered important for further development of risk assessments and control strategies.

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