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Predominance and association of pathogenic fungi causing Fusarium ear blight in wheat in four European countries

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Abstract

Two years of field sampling aimed to establish the predominance and association among the fungal pathogens causing Fusarium ear blight (FEB) in four European countries (Hungary, Ireland, Italy and the UK). A PCR-based method was used to detect four *Fusarium* species and two varieties of *Microdochium nivale* present in the samples. The prevalence of FEB pathogens differed significantly between countries. Overall, all pathogens were commonly detected in Ireland and to a lesser extent in the UK. In contrast, only two species, *F. graminearum* and *F. poae*, were regularly detected in Italy and Hungary. *Fusarium culmorum* was rarely detected except in Ireland. Log-linear models were used to determine whether there is the independence of the six FEB pathogens at each sampling site. Significant two-pathogen interactions were frequently observed, particularly in harvest samples; all these significant two-pathogen interactions were of the synergistic type, except between *F. poae* and *F. culmorum*, and were generally consistent over the 2 years and four countries. *Fusarium graminearum* and *F. poae* were least frequently involved in two pathogen interactions but were involved in most of the nine significant three-pathogen interactions. However, only the interaction between *F. graminearum, F. avenaceum* and *F. poae* was significant in both years. Potential implications of the present results in FEB management are discussed.

Introduction

Fusarium ear blight of wheat (FEB) is recognised as a significant but sporadic threat to wheat production world-wide (Parry et al., 1995; Goswami and Kistler, 2004). The main causal agents of FEB are *Fusarium culmorum, F. graminearum, Microdochium nivale* var. *nivale* and var. *majus* (formerly *F. nivale*), *F. avenaceum* and *F. poae*. In addition to causing significant yield losses, FEB is of greater significance under certain conditions because of associated mycotoxin accumulation which can occur in infected grain. The toxigenic capabilities of the pathogens causing FEB differ. *Fusarium graminearum, F. culmorum, F. avenaceum* and *F. poae* can produce a range of mycotoxins (Bottalico and Perrone, 2002), whereas apparently *M. nivale* does not produce mycotoxins. Contaminated grain is unsuitable for animal and human consumption because of adverse effects of such

toxins on health (IARC, 1993; Li et a1., 1999; Bennett and Klich, 2003).

The distribution and predominance of the FEB pathogens is, to a large extent, determined by climatic factors, particularly temperature and moisture (Doohan et al., 2003). Crop husbandry practices also have an important influence on the predominance of FEB pathogens. Overwintered plant debris favours infection by *F. graminearum* via ascospores. In hotter regions of Europe, *F. graminearum* predominates, whereas in cooler maritime areas, *M. nivale* is favoured (Parry et al., 1995). However, combinations of these pathogens can often occur on wheat ears and the impact of the environment on such pathogen complexes is not well understood. The sporadic nature of FEB is largely attributed to the observation that wheat plants are most susceptible to the disease during anthesis. FEB is most severe when warm and wet conditions prevail during anthesis in cereals (Parry et al., 1995; Xu, 2003). However, there are insufficient quantitative data to forecast FEB development and production of associated mycotoxins using information on environmental conditions. In countries where there are histories of severe epidemics of FEB, a scheduled fungicide application is generally adopted to manage this disease. However, this approach to FEB management is undesirable both in terms of cost and environmental concern.

It is of paramount importance to know the exact FEB pathogens causing FEB symptoms at a particular site for several reasons. First, it is known that the effects of environmental conditions on FEB development may differ considerably between FEB pathogens (Parr et al., 1995; Rossi et al., 2001, 2002). Second, FEB pathogens may differ in their responses to different fungicides used to control FEB (Jennings et al., 2000). Finally, FEB pathogens differ in toxin-producing abilities. Therefore, it is essential to understand the exact nature of the FEB complex for reliable disease predictions and management, and for reducing the food risks associated with mycotoxins. Recent advances in molecular detection of FEB pathogens enable researchers to detect and quantify individual FEB pathogen faster and more reliably (Nicholson et al., 2003).

The overall objective of this study was to develop a quantitative assessment model to predict the risk of FEB and the associated production of mycotoxins. In this paper, we report results from one particular aspect of this study, i.e., understanding the predominance of FEB pathogens and potential interactions among them in Europe based on a pan-European field sampling of FEB occurrence. Field sampling was conducted in four European countries (UK, Hungary, Italy and Ireland); these four countries represent three contrasting climates (Mediterranean, Atlantic and Continental-arid) where winter wheat is produced in Europe. Individual pathogen of the FEB disease complex was detected and quantified using the newly developed molecular techniques (Nicholson et al., 2003).

Materials and methods

Field sampling

A number of sites were allocated across four European countries in 2001 and 2002: UK (20 sites), Ireland (12 in 2001 and 13 in 2002), Italy (19) and Hungary (15). These sites were chosen to represent important areas of wheat production in a range of climatic conditions in Europe where FEB is likely to be a serious problem on winter wheat. The location of sampling sites in 2001 was limited in the UK and Ireland because of the foot and mouth epidemic in farm animals. Fungicides effective against FEB were not applied at the sites in UK, Hungary or Italy, whereas about 50% of the sites in Ireland received fungicides after ear emergence.

At each site, a sample of 200 ears was collected using the random W-shape sampling strategy at growth stage (GS)69 (end of anthesis) and GS77 (milky ripe) (Zadoks et al., 1974). The ears were visually assessed for ear blight by recording the number of spikelets infected on each ear. These ears were then ranked for disease intensity from least (i.e., labelled as ear 1) to most diseased (i.e., labelled as ear 200). Three bulk samples were then produced: bulk sample 1 for ears 1–30 (least diseased), bulk sample 2 for ears 86–115 and bulk sample 3 for ears 171–200 (most diseased). The dry weight of the bulked ears was then determined before extracting DNA from the three bulks (groups) for diagnostic PCR testing. Depending on the incidence of ears with symptoms, sometimes only one or two bulk samples were generated. For

the third sample, at harvest, 400 ears instead of 200 were randomly collected. Disease intensity was not assessed since accurate assessment was not possible at harvest. These ears were hand-harvested and threshed. The total weight and the moisture content of the harvested grains were then determined before they were being milled for DNA extraction.

Diagnostic PCR test for FEB pathogens

Fusarium DNA was extracted from milled flour using a modification of the CTAB (hexadecyl-trimethyl-ammonium bromide) buffer method described by Nicholson et al. (1996). Approx, 4 g of milled, freeze-dried samples were extracted in 20 ml of CTAB buffer (CTAB 22 mM, sarkosyl 34 mM, sorbitol 137 mM, EDTA 22 mM, polyvinylpolypyrolidone (PVPP) 1% NaCl 1.2 mM) for 60 min at 65 °C. One-third volume of 5 M potassium acetate was added along with 1 ml chloroform:isoamyl alcohol (24:1), mixed and held at −20 °C for 20 min. The mixture was then centrifuged at $1900 \times g$ for 15 min and the aqueous phase was added to two volumes of 100% ethanol. The DNA was precipitated at $850 \times g$ and the pellet was washed twice with 70% (v/v) ethanol before being dissolved in TE buffer (10 mM Tris–HCl pH 8.0, 0.1 mM EDTA). DNA was quantified according to the method described by Hopwood et al. (1997). Aliquots of each DNA sample were added to a solution containing 100 ppm SYBR Green (Flowgen, Lichfield, UK) and assayed using a plate reader set to measure emission at 538 nm after excitation at 485 nm. The DNA concentration was ascertained by comparison with a serial dilution (0–1.8 ng μl^{-1}) of λ DNA cut by restriction enzyme *Hind*III on each plate.

Following determination of the DNA concentration, the samples were adjusted to a common concentration, e.g. 20 ng μl^{-1}, for use in PCR. The species-specific PCR primer pairs used to detect each pathogen were those described in Nicholson et al. (2004). PCR reactions were carried out in volumes of 50 μl PCR buffer containing 200 ng DNA sample, 100 μM each of dATP, dGTP, dCTP and dTTP, 5 pmol of each specific forward and reverse primer, and 0.8 units of *Taq* polymerase (Boehringer Mannheim). PCR buffer comprised 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 100 g ml^{-1} gelatine, 0.05% vv Tween 20 and 0.05% (vv) Nonidet P-40. Reactions were overlaid with mineral oil prior to PCR. Samples were applied to a preheated PCR block and denatured at 95 °C for 2 min prior to cycling. DNA was amplified using 'touchdown' PCR (Don et al., 1991) to ensure specificity of product amplification. The annealing temperature was 66 °C for the first five cycles and 64 °C for the next five cycles, followed by 25 cycles at 62 °C. The temperature cycle consisted of denaturation (95 °C) for 30 s, annealing (as described above) for 20 s and extension (72 °C) for 45 s with maximal ramping rates between temperatures. A final extension step of 5 min was incorporated followed by cooling to 10 °C until recovery of samples.

Statistical methods

In this study, we first used logistic regression analysis (Cox and Snell, 1989) to determine whether the incidence of each FEB pathogen over all the sampling sites differed significantly between four countries and between years. FEB incidence and severity (as number of spikelets infected per ear), and their relationship within each sampling site have already been separately dealt with (Xu et al., 2004). In logistic regression analysis, the presence of each FEB pathogen at each site was assumed to be distributed binomially. A FEB pathogen is deemed to be present at a site if it was detected in any of the bulk samples (usually three) for each sampling time.

Next we used log-linear models to test the independence of the six FEB pathogens in the FEB complex within each sampling site assuming a Poisson sampling. Because of the sampling method used, we can only determine whether the occurrence of one pathogen was related to the other at the same site. In the current context, Poisson sampling means that a fixed amount of effort was used to sample the wheat ears across sites, which were then categorised into cells in the table depending on the presence/absence of the six pathogens at each site. Specifically, it was assumed that neither the total number of individual pathogen nor the total occurrence of all pathogens was known before the sampling took place.

In the log-linear model, each count in the individual cell was treated as a Poisson response variable, which was then regressed on independent

variables/factors. In the present study, there were eight factors, i.e., FEB pathogens (*F. graminearum*, *F. avenaceum*, *F. poae*, *F. culmorum*, *M. nivale* var. *nivale* and *M. nivale* var. *majus*), country and year. For the six pathogens, each factor had two levels: present and absent. Because of the limited number of sites used, it was not practical to test pathogen interactions of high order (>3). There are many possibilities for testing two- or three-pathogens interaction depending on the terms already included in the model. In the present study, we used a simple and straightforward approach to test whether there were significant interactions between the pathogens under consideration. For example, to test whether there were significant interactions between two pathogens, represented by F1 and F2, we fitted the following model to the 2×2 contingency table:

$$\ln(y) = u + \mathrm{F1} + \mathrm{F2}$$

where y and u are the counts in the 2×2 table and the overall mean, respectively, and F1 and F2 are the two factors representing the respective two pathogens. The model has a residual deviance with 1 degree of freedom, due to the interaction between the two pathogens (since if we include the interaction F1.F2 in the above model, deviance would be zero as a full model is fitted). Therefore, the significance of the interaction can then be determined as this deviance statistic has a chi-square distribution with one degree of freedom. Similarly, we can use this approach to test significance of three-pathogens interaction using the residual deviance (with 1 degree of freedom) from the following model:

$$\ln(y) = u + \mathrm{F1} + \mathrm{F2} + \mathrm{F3} + \mathrm{F1.F2} + \mathrm{F2.F3} + \mathrm{F1.F3}$$

Country and year can be similarly included in the above model. The only difference is that when testing the interaction with country, the residual deviance has three degrees of freedom.

We first tested the significance of interactions within each year, then over both years, and finally tested whether the interaction varied significantly with year. In assessing interactions of two pathogens, we also used Fisher's exact test, which, as the name indicates, provides an exact probability for the observed data, but is restricted in its application to two-pathogen interactions. All the statistical analyses were carried out using Genstat™ version 6.1 (Payne, 2002)

Results

Table 1 shows the overall incidence of each FEB pathogen detected by the PCR technique in four countries over the 2 years. The prevalence of FEB pathogens differed considerably between countries as well as between years. Overall, incidence of all pathogens except *F. poae* was significantly greater at harvest in 2002 than in 2001 ($P < 0.01$). For example, *F. graminearum* was detected in 22 out of 66 sites in 2001, compared to 33 out of 67 sites in 2002. Apart from a few cases, the incidence of

Table 1. Overall incidence (proportion of sampling sites) of pathogenic fungi capable of causing Fusarium ear blight in four European countries. Presence of a FEB pathogen was determined by a diagnostic PCR technique

Country	No. of sites	Year	*F. graminearum*			*F. avenaceum*			*F. poae*			*F. culmorum*			*M. nivale* var. *nivale*			*M. nivale* var. *majus*		
			T1[a]	T2	T3	T1	T2	T3	T1	T2	T3	Tl	T2	T3	T1	T2	T3	T1	T2	T3
Hungary	15	2001	0.07	0.00	0.27	0.00	0.13	0.47	0.47	0.80	0.80	0.07	0.07	0.07	0.00	0.00	0.00	0.00	0.00	0.00
	15	2002	0.00	0.13	0.40	0.00	0.13	0.20	0.00	0.07	0.67	0.07	0.07	0.07	0.00	0.07	0.07	0.00	0.07	0.07
Ireland	12	2001	0.00	0.25	0.42	0.00	0.33	0.58	0.00	0.42	0.25	0.00	0.50	0.67	0.08	0.00	0.00	0.00	0.25	0.42
	13	2002	0.00	0.62	0.69	0.08	0.69	1.00	0.00	0.00	0.39	0.08	0.00	0.69	0.00	0.31	0.46	0.15	0.85	1.00
Italy	19	2001	0.05	0.00	0.21	0.05	0.00	0.05	0.21	0.11	0.63	0.05	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.32
	19	2002	0.00	0.26	0.53	0.16	0.58	0.00	0.21	0.37	0.58	0.05	0.11	0.00	0.00	0.00	0.00	0.05	0.26	0.05
UK	20	2001	0.00	0.05	0.45	0.00	0.00	0.15	0.30	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.15	0.00	0.00	0.40
	20	2002	0.00	0.35	0.40	0.00	0.25	0.20	0.80	0.90	0.90	0.00	0.15	0.35	0.15	0.75	0.85	0.25	0.85	0.80
Mean incidence			0.02	0.20	0.41	0.04	0.25	0.29	0.28	0.49	0.68	0.04	0.10	0.20	0.03	0.15	0.22	0.06	0.28	0.38

[a]Sampling time; Tl – anthesis (GS69); T2 – milky ripe stage (GS77); and T3 – harvest.

FEB pathogens detected increased with sampling time within each season. For instance, incidence of *F. graminearum* increased from 2 to 55 out of 133 sites over the 2 years from anthesis to harvest, whereas *F. poae* increased from 37 to 91 out of 133 sites in the same period.

Of the six FEB pathogens, *F. poae* and *F. culmorum* were the most and least frequently detected, respectively. At harvest, their overall incidences were respectively 68% and 20%. Within each country, the incidence of the six FEB pathogens varied considerably. In Ireland, all six pathogens were frequently detected, apart from *M. nivale* var. *nivale* in 2001. Except in Ireland, *F. poae* was the pathogen most frequently detected. In Hungary, *F. graminearum* and *F. avenaceum* were also common, while only *F. avenaceum* also occurred frequently in Italy. Both *F. culmorum* and *M. nivale* var. *nivale* were detected only in a few samples in Hungary and Italy. In the UK, apart from the dominant species (*F. poae*), all other pathogens except *F. culmorum* were also frequently detected, especially the two *M. nivale* varieties ($\geq 80\%$ at harvest) in 2002. Logistic regression analysis showed that the incidence of each FEB pathogen at harvest differed significantly between countries ($P < 0.01$). *Fusarium graminearum* and *F. avenaceum* were the most frequently detected in Ireland (44% and 51%, respectively), significantly ($P < 0.01$) greater than their incidences in Hungary and Italy. Incidence of *F. poae* was greatest in the UK (95%), significantly ($P < 0.01$) greater than in Italy (61%) and Hungary (74%), which in turn were significantly ($P < 0.01$) greater than in Ireland (32%). At harvest, the incidence of *F. culmorum* was significantly ($P < 0.01$) greater in Ireland (68%) than in the other three countries. Incidences of *M. nivale* var. *nivale* and var. *majus* were significantly greater in Ireland and the UK than in Italy and Hungary.

Table 2 shows the number of FEB pathogens detected within each sample over the 2 years. Clearly, number of samples with more than one FEB pathogen increased over time. About 26%, 52% and 74% of samples with FEB pathogens detected contained at least two pathogens at GS69, GS72 and at harvest, respectively. All six pathogens were detected in three samples from Ireland. Combined over the 2 years, 17 out of all possible 45 pair-wise combinations of pathogens showed significant interactions in assessments at three sampling times, with eight from harvest samples (Table 3). In general, there were close agreements between the log-linear model and Fisher's exact test. Only combinations with both tests significant at 5% were considered to be significant in this study. All these 17 cases, apart from the *F. poae–F. culmorum* interaction at harvest, were synergistic interactions: i.e., the presence of one pathogen tending to favour the presence of the other. In general, because of the low incidence at anthesis, log-linear models could not be applied to assess many pair-wise interactions at that stage (Table 3). Overall, *F. graminearum* showed fewest interactions with other pathogens; at harvest it was not associated with any other pathogens.

Over the three sampling times, positive interactions between *F. avenaceum* and *F. culmorum*, and between *F. poae* and *M. nivale* var. *nivale* were all significant. Table 4 shows two examples of such positive associations at harvest for these two pairs. In the *F. avenaceum–F. culmorum* example, the number of sites with both present or absent was nearly 17 more than expected under the assumption of independence. Similarly, the number of sites with both *F. poae* and *M. nivale* var. *nivale* present or absent was 14 more than expected under the assumption of independence. The negative association of *F. poae* with *F. culmorum* is also shown in Table 4, in which the number of sites with both species present or absent was nearly 10 less than expected. Positive interactions between *F. avenaceum* and *M. nivale* var. *majus*, and

Table 2. Number of sampling sites with number of FEB pathogens detected by a diagnostic PCR technique in four countries over 2 years (2001 and 2002)

Country	Anthesis (GS69)					Milky ripe stage (GS77)					Harvest				
	0	1	2	3	>3	0	1	2	3	>3	0	1	2	3	>3
Hungary	22	6	2	0	0	13	13	3	0	1	4	11	12	2	1
Ireland	22	2	0	1	0	1	8	7	5	4	0	3	6	5	11
Italy	27	7	3	1	0	24	5	3	3	3	12	10	12	3	1
UK	16	19	4	1	0	0	20	3	10	7	0	6	12	11	11

Table 3. Results of log-linear analysis on two-pathogen interactions for pathogens capable of causing Fusarium ear blight in four European countries. Presence of a FEB pathogen was determined by diagnostic PCR

	F. avenaceum				*F. poae*				*F. culmorum*				*M. nivale* var. nivale				*M. nivale* var. *majus*			
	2001	2002	All	Year[a]	2001	2002	All	Year	2001	2002	All	Year	2001	2002	All	Year	2001	2002	All	Year
Sampling during the anthesis (GS69)																				
F. graminearum	NS[b]	–	NS	–	NS	–	NS	–	NS	–	NS	–	NS	–	NS	–	–	–	NS	–
F. avenaceum					NS	NS	NS	NS	** (+)[c]	** (+)	** (+)	NS	NS	NS	NS	–	–	NS	NS	–
F. poae									* (+)	NS	NS	** (+−)	NS	** (+)	* (+)	* (+−)	–	NS	NS	–
F. culmorum													NS	NS	NS	–	–	NS	NS	–
M. nivale var. *nivale*																	–	NS	NS	–
Sampling during the milky ripe stage (GS77)																				
F. graminearum	NS	** (+)	** (+)	NS	NS	NS	NS	NS	NS	NS	NS	NS	–	NS	**(+)	–	NS	* (+)	** (+)	NS
F. avenaceum					NS	NS	NS	NS	**(+)	NS	*(+)	*(+−)	–	NS	NS	–	*(+)	NS	**(+)	NS
F. poae									NS	NS	NS	NS	–	** (+)	* (+)	–	NS	NS[d]	NS	NS
F. culmorum													–	NS	NS	–	*(+)	NS	NS	NS
M. nivale var. *nivale*																	–	**(+)	**(+)	–
Sampling during the harvest																				
F. Graminearum	NS	NS	NS	NS	NS	NS[d]	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
F. Avenaceum					NS	NS	NS	NS	NS	**(+)	**(+)	NS	NS	*(+)	*(+)	NS	NS	**(+)	**(+)	**(−+)
F.Poae									*(−)	NS	*(−)	NS	NS	**(+)	**(+)	NS	NS	NS	NS	NS
F. culmorum													NS	**(+)	**(+)	*(+−)	NS	**(+)	**(+)	**(−+)
M. nivale var. *nivale*																	NS	**(+)	**(+)	NS

[a]Significance of the two-pathogen interactions with year over the four countries.
[b]Models cannot be fitted due to low incidence of pathogen presence, NS, * and ** not significant and significant at $P = 0.05$ and $P = 0.01$, respectively.
[c]The sign in the bracket indicates the type of interaction: +, synergistic and −, competitive; the first and second signs in the bracket under the year column indicate the type of interaction in 2001 and 2002, respectively, where they are different between 2 years.
[d]Significant at $P = 0.05$ using Fisher's exact test.

Table 4. Three examples in which there were significant two-pathogen interactions for pathogens capable of causing Fusarium ear blight in four European countries over the 2 years. Samples were taken at harvest

F. avenaceum	*F. culmorum*		*F. poae*	*M nivale* var. *nivale*		*F. poae*	*F. culmorum*	
	Absent	Present		Absent	Present		Absent	Present
Absent	85[a] (76.4[b])	10 (18.6)	Absent	40 (32.8)	2 (9.2)	Absent	29 (33.8)	13 (8.2)
Present	22 (30.6)	16 (7.4)	Present	64 (71.2)	27 (19.8)	Present	78 (73.2)	13 (17.8)
Significance level		$P < 0.01$			$P < 0.01$			$P < 0.05$

[a]Number of sampling sites in which the specific combination of the two pathogens were observed.
[b]Number of sampling sites in which the specific combination of the two pathogens would be expected under the assumption of independence.

between *M. nivale* var. *nivale* and *M. nivale* var. *majus* were significant in two out of three assessments. For all other interactions, they were significant at only one of the sampling times; these include combinations of *F. graminearum* with *F. avenaceum, M. nivale* var. *nivale* and *M. nivale* var. *majus, F. poae* with *F. culmorum, F. avenaceum* with *M nivale* var. *nivale*, and *F. culmorum* with *M. nivale* var. *nivale*, and *M. nivale* var. *majus* (Table 3). Similarly, there were many combinations of two pathogens with significant interactions within each year (Table 3). There were no significant interactions for four pairs of pathogens over all samples in either year: *F. poae–F. graminearum*, *F. poae–F. avenaceum*, *F. poae–M. nivale* var. *majus* and *F. culmorum–F. graminearum* (Table 3).

In only six cases were there significant differences in the interactions of two pathogens between years (Table 3). In all these cases, positive associations were observed in one year but negative associations in the other year, although such relationships may not be significant when analysed separately within each year as shown in Table 3. One example of such differences in the interaction of *F. culmorum* with *M. nivale* var. *majus* between 2 years is given in Table 5: they were negatively associated in 2001 but positively in 2002. Only in six combinations of two pathogens did the two-pathogen interactions differ significantly between the four countries over the 2 years: *F. poae–F. graminearum, F. poae–F. avenaceum, F. culmorum–F. graminearum, F. graminearum–F. avenaceum, M. nivale* var. *majus–F. graminearum* and *M. nivale* var. *majus–F. culmorum*. However, because of the low incidence in some categories (< 3) of the $2 \times 2 \times 4$ contingency tables, these results may not be very reliable. For instance, the interaction between *F. graminearum* and *F. culmorum* was positive in Italy but negative in Ireland. However, the differences between the observed and expected under the assumption of independence were all small (< 2) (Table 6).

Significant interactions were found in nine combinations of three pathogens (Table 7). Only in the combination of *F. graminearum*, *F. avenaceum* and *F. poae* was there a significant interaction in both years as well as in the overall data pooled over the 2 years. *F. poae* and *F. graminearum* featured in seven and six out of the nine combinations, respectively, whereas *F. culmorum*

Table 5. An example where two-pathogen (*F. culmorum* and *M. nivale* var. *majus*) interactions differed significantly ($P < 0.01$) between the 2 years. Samples were taken at harvest

2001			2002		
F. culmorum	*M nivale* var. *majus*		*F. culmorum*	*M nivale* var. *majus*	
	Absent	Present		Absent	Present
Absent	41[a] (43.8[b])	16 (13.2)	Absent	35 (32.2)	15 (17.8)
Present	6 (3.2)	3 (5.8)	Present	1 (3.8)	16 (13.2)

[a]Number of sampling sites in which the specific combination of the two pathogens in each year were observed.
[b]Number of sampling sites in which the specific combination of the two pathogens would be expected in each year under the assumption of independence.

Table 6. An example where two-species interactions varied. significantly ($P < 0.01$) with four countries. Samples were taken during the milky ripe stage (GS77)

Country	*F. graminearum*	*F. culmorum*	
		Absent	Present
Hungary	Absent	26[a] (26.1[b])	2 (1.9)
	Present	2 (1.9)	0 (0.1)
Ireland	Absent	9 (10.3)	5 (3.7)
	Present	10 (8.7)	1 (2.3)
Italy	Absent	33 (31.2)	0 (1.8)
	Present	3 (4.8)	2 (0.2)
UK	Absent	29 (29.5)	3 (2.5)
	Present	8 (7.5)	0 (0.5)

[a]Number of sampling sites in which the specific combination of the two pathogens were observed in each country.
[b]Number of sampling sites in which the specific combination of the two pathogens would be expected in each country under the assumption of independence.

featured only once. For all the nine combinations, the significant interactions resulted from the fact that pathogen 2 and 3 (Table 7) were positively associated in the absence of pathogen 1 but negatively associated in the presence of pathogen 1. Table 8 shows an example illustrating such interactions. Only in one combination, did the three-pathogen interactions differ significantly between years ($P < 0.05$). Similarly, it is possible that there may have been interactions among four pathogens, but it as not possible to test this in the present study because of low incidence in some cells of $2 \times 2 \times 2 \times 2$ contingency tables. However, the existence of such high-order interactions may be difficult to interpret.

Discussion

The frequency of FEB pathogens increased significantly from anthesis to the milky-ripe stage and to harvest. Pathogen predominance differed significantly between countries. All six pathogens were detected most frequently in Ireland and least frequently in Hungary and Italy. Factors responsible for this might include the differences in varietal susceptibilities environmental conditions, and cultural practices between countries.

Overall, *F. poae* was the most common species found in all countries except Italy. Indeed, *F. poae* was detected in almost all samples at harvest in the UK. As expected, both *M. nivale* varieties were most frequently detected in cooler UK and Irish regions whereas they were rarely detected in Hungary and Italy. The overall dominance of *F. poae* in the UK, Ireland and Hungary agrees with other recent studies. *Fusarium poae* was the dominant FEB pathogen in the UK in the early 1990s and again in 2001 (Polley et al., 1991; Anon, 2004). Walsh et al. (1998) reported that *F. poae* was commonly found in Ireland. Surveys in mid-European countries identified *F. poae* and *F. avenaceum* as the most important species (Lukanowski and Sadowski, 2002). Exact reasons for the predominance of *F. poae* are not clear. *Fusarium poae* is generally believed to be less pathogenic and aggressive than other FEB pathogens (Pettersson and Olvång, 1995; Brennan et al., 2003).

However, it was worth stressing that the present results were only about the presence of FEB pathogens at each sampling site not disease

Table 7. Results of log-linear analysis on three-pathogen interactions for pathogenic fungi capable of causing Fusarium ear blight in four European countries. Presence of a FEB pathogen was determined by diagnostic PCR

Pathogen 1	Pathogen 2	Pathogen 3	2001	2002	All	Year
F. graminearum	*F. poae*	*M. nivale* var. *nivale*	–[a]	*(2)[b]	NS	NS
F. graminearum	*F. poae*	*M. nivale* var. *majus*	NS	**(2)	**(2)	NS
F. graminearum	*M. nivale* var. *majus*	*M. nivale* var. *majus*	–	*(2)	*(2)	NS
F. avenaceum	*F. poae*	*M. nivale* var. *nivale*	–	*(2)	NS	NS
F. avenaceum	*F. poae*	*M. nivale* var. *majus*	NS	*(2)	NS	NS
F. graminearum	*F. avenaceum*	*F. poae*	*(3)	*(3)	**(3)	NS
F. graminearum	*F. avenaceum*	*M. nivale* var. *majus*	**(3)	NS	NS	**(3)
F. graminearum	*F. poae*	*F. culmorum*	*(3)	NS	NS	NS
F. avenaceum	*F. poae*	*M. nivale* var *majus*	NS	*(3)	NS	NS

[a]Models cannot be fitted due to low disease incidence; NS, * and ** not significant and significant at $P = 0.05$ and $P = 0.01$, respectively.
[b]Number in the bracket indicates the sampling time (2 – milky ripe stage and 3 – harvest).

Table 8. One example where there were significant ($P < 0.01$) three pathogen interactions over the 2 years in four countries. Samples were taken at harvest

F. graminearum	*F. avenaceum*	*F. poae*	
		Absent	Present
Absent	Absent	21[a] (17.5[b])	35 (38.5)
	Present	6 (9.5)	16 (12.5)
Present	Absent	6 (9.5)	33 (29.5)
	Present	9 (5.5)	7 (10.5)

[a]Number of sampling sites in which the specific combination of the three pathogens were observed.
[b]Number of sampling sites in which the specific combination of the three pathogens would be expected under the assumption of independence.

severity. Using disease severity to study pathogen association is not possible as we cannot determine the contribution of each pathogen to the visual disease severity/incidence at each site. In contrast, the presence of a specific pathogen can be determined, as done in this study, by molecular methods.

In regions with cooler summer (UK and Ireland), our results indicated that *F. graminearum* appeared to have recently increased in its occurrence whereas the occurrence of *F. culmorum* was less frequent compared to previous reports (see review by Parry et al., 1995). *F. graminearum* is conventionally associated with regions in warmer climates. However, in this study, it was the second most common species detected in all countries. Indeed its incidence was not significantly different among the four countries at harvest. The increase of *F. graminearum*, especially in the UK, appears to have been at the expense of *F. culmorum*. This observed change of relative predominance of these two species agrees with other recent reports in Europe (Obst et al., 2002; Waalwijk et al., 2003; Anon, 2004). Several reasons are possible for this change in species prevalence. First, recently there has been an increased production of maize in rotation with wheat in Europe (Logrieco et al., 2002; Fox, 2004). It is well known that in regions where *F. graminearum* is present, incidence and severity of FEB are normally significantly greater when wheat followed maize than when wheat followed other crops (Dill-Macky and Jones, 2000; Cromey et al., 2002). Recently it was also reported that the numbers of arbuscular mycorrhizal fungi spores and species in the field samples were highest in the grasslands, lower in the low- and moderate-input arable lands, and lowest in the lands with intensive continuous maize mono-cropping (Oehl et al., 2003). Second, *F. graminearum* may have gradually adapted to cooler regions. This possibility is enhanced by the fact that, unlike other FEB pathogens, *F. graminearum* regularly produces ascospores in addition to asexual conidia, hence increasing its ability to generate more variability for adaptation. *F. graminearum* is homothallic, hence ascospores can be produced wherever conditions are suitable for production of perithecia. Third, due to possible climate change cooler regions may have gradually become warmer. Fourth, the profile of FEB disease complex may also be significantly affected by cultivars used. Most breeding programmes rely heavily on natural disease development in field conditions and thus any resulting resistant varieties would be expected to be resistant against those predominant FEB pathogens at the time. Therefore, new cultivars released in northern Europe may show greater resistance to *F. culmorum*, the previously dominant species, than to other FEB pathogens, although resistance to FEB could also be broad spectrum: resistance against *F. culmorum* was found to be also effective against *M. nivale* (Van Eeuwijk et al., 1995). Fifth, the FEB pathogen complex and their relative prevalence are affected by fungicide application regimes since fungicides have differential effects against different FEB pathogens (Pirgozliev et al., 2003). Finally, direct competition between *F. culmorum* and *F. graminearum* may also contribute to this shift in FEB complex in countries such as UK. All these factors are likely to interact and identifying the exact causes of such species shifts would be very difficult. As a result, the relative prevalence of FEB pathogens can vary greatly between consecutive years (Anon, 2004). Similar variation was also observed for the stem-based *Fusarium* complex between years and cultivars (Parry, 1990).

In many cases, there were significant interactions between two pathogens, particularly towards the later sampling. Generally, two-pathogen interactions were most frequently detected for *F. avenaceum, F. culmorum* and two *M. nivale* varieties. All two-pathogen interactions were synergistic except between *F. culmorum* and *F. poae* and were generally consistent between the 2 years. In no cases were significant but opposite

interactions observed in the 2 years for the same pair of pathogens. Significant interactions may arise from either direct or indirect interactions, or both. Given the nature of bulk samples used in the present study, direct interaction between two pathogens in the same ear tissue is far less likely than indirect interactions mediated by other factors. Several factors may have resulted in these observed positive interactions. First, it is generally accepted that FEB is most severe in cereals where warm and wet conditions occur during the anthesis period (Bai and Shaner, 1994; Parry et al., 1995; Miller, 2002; Pirgozliev et al., 2003; Xu, 2003) though the exact relationship between disease development and environmental conditions may differ between FEB pathogens (Rossi et al., 2001, 2002). Thus, provided that inoculum is present at a given site, conditions conducive to one FEB pathogen are also likely to favour others. Second, current varieties may have similar resistance/susceptibility to a pair of pathogens. Finally, fungicides used in previous seasons may also have similar activity on a pair of species. One notable exception is the negative interaction between *F. culmorum* and *F. poae*. We do not know whether this is due to direct competition between the two species or differential effects of fungicides applied historically at these sites on the two species.

Overall, *F. graminearum* was involved in the smallest number of significant interactions with other FEB pathogens. This scarcity of interactions involving *F. graminearum* results probably from two biological causes. Unlike other FEB pathogens *F. graminearum* has a sexual stage. Its ascospores, in addition to conidia, are an important source of inoculum (Fernando et al., 1997; Scholz and Steffenson, 2001) . Production and dispersal of ascospores might require different weather conditions from those for conidia. *Fusarium graminearum* is probably the most aggressive FEB pathogen (Marin et al., 1998; Velluti et al., 2000). It may rapidly colonise the plant tissues under favourable conditions during wheat anthesis, which may preclude the attack by other pathogens. However, if weather conditions are not favourable for *F. graminearum* (or inoculum concentration is low), other species may cause FEB, as secondary invaders, among which *F. poae* is the most frequent. These characteristics may be partially responsible for a recently reported negative interaction between *F. graminearum* and other *Fusarium* spp. (Kosiak et al., 2004). Lack of negative interactions between *F. graminearum* and other *Fusarium* pathogens in the present study spp. may be the outcome of complex interactions with other factors such as climatic conditions, inoculum concentration and varietal susceptibility.

There are nine combinations in which three-pathogen interactions were significant. The general pattern is very similar. The most common species involved were *F. poae* and *F. graminearum*, which is in contrast to the two-pathogen interactions. Hence, it may be misleading to conduct investigations on interactions between only two pathogens. However, caution should be exercised when interpreting these results since only one three-pathogen interaction was significant in both years. Further research is needed on the consequence of FEB pathogen interactions on FEB severity and production of associated mycotoxins, which is unlikely to be obtained from field samples. Currently, experiments are under way in controlled environment conditions at East Malling Research to obtain such information.

This paper presented research findings only in one specific aspect of an overall research project on developing and validating forecasting models for FEB management in Europe. Other key aspects of this research on FEB development and accumulation of associated mycotoxins include (1) understanding the quantitative effects of weather conditions for single or mixed pathogen species in controlled environmental conditions, (2) determining the extent of interactions between pathogen species and cultivars, (3) obtaining field data on FEB development, mycotoxin production and climatic conditions, and finally (4) developing and validating forecasting models using both field and controlled environmental data. We have already developed an incidence–density relationship for a fast and reliable field assessment of FEB development (Xu et al., 2004). This paper clearly identified the increasing importance of *F. graminearum* and *F. poae* in the FEB complex in Europe. This finding will enable farmers to use correct forecasting model(s) for appropriate FEB pathogens in their region and to determine the likelihood of accumulation of specific mycotoxins associated with FEB. It also highlights the importance of frequent survey of field epidemics to identify FEB pathogen profile. Furthermore, as the incidence of FEB increased significantly from

the milky-ripe stage to the harvest, a more reliable and accurate picture of the FEB pathogen profile can be obtained by sampling at harvest time. The effects of climatic conditions on the FEB severity at each site will be dealt separately when developing and validating forecasting models.

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References

Anon (2004) http://www.csl.gov.uk/science/organ/environ/entom/fusarium/inc2.cfm. Accessed on 25/04/2004

Bai G and Shaner G (1994) Scab of wheat: prospects for control. Plant Disease 78: 760–766

Bennett JW and Klich M (2003) Mycotoxins. Clinical Microbiology Review 16: 497–516

Bottalico A and Perrone G (2002) Toxigenic *Fusarium* species and mycotoxins associated with head blight in small-grain cereals in Europe. European Journal of Plant Pathology 108: 611–624

Brennan JM, Fagan B, van Maanen A, Cooke BM and Doohan FM (2003) Studies on *in vitro* growth and pathogenicity of European *Fusarium* fungi. European Journal of Plant Pathology 109: 577–588

Cox DR and Snell EJ (1989) Analysis of Binary Data. Chapman and Hall, London

Cromey MG, Shorter SC, Lauren DR and Sinclair KI (2002) Cultivar and crop management influences on *Fusarium* head blight and mycotoxins in spring wheat (*Triticum aestivum*) in New Zealand. New Zealand Journal of Crop and Horticultural Science 30: 235–247

Dill-Macky R and Jones R (2000) The effect of previous crop residues and tillage on *Fusarium* head blight of wheat. Plant Disease 84: 71–76

Don RH, Cox PT, Wainwright BJ, Baker K and Mattick JS (1991) Touchdown PCR to circumvent spurious priming during gene amplification. Nucleic Acids Research 19: 4008–4008

Doohan FM, Brennan J and Cooke BM (2003) Influence of climatic factors on *Fusarium* species pathogenic to cereals. European Journal of Plant Pathology 109: 755–768

Fox AD (2004) Has Danish agriculture maintained farmland bird populations? Journal of Applied Ecology 41: 427–439

Goswami RS and Kistler HC (2004) Heading for disaster: *Fusarium graminearum* on cereal crops. Molecular Plant Pathology 5: 515–525

Hopwood A, Oldroyd N, Fellows S, Ward R, Owen SA and Sullivan K (1997) Rapid quantification of DNA samples extracted from buccal scrapes prior to DNA profiling. Biotechniques 23: 18–20

IARC (1993) Monographs on the evaluation of carcinogenetic risks to humans. Vol. 56. Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins. International Agency for Research on Cancer, Lyon, France

Jennings P, Turner JA, Nicholson P (2000) Overview of *Fusarium* ear blight in the UK – effect of fungicide treatment on disease control and mycotoxin production 2000 In: The BCPC Conference – Pests and Diseases, pp 707–712

Kosiak B, Torp M, Skjerve E and Andersen B (2004) *Alternaria* and *Fusarium* in Norwegian grains of reduced quality – a matched pair sample study. International Journal of Food Microbiology 93: 51–62

Li F, Luo X and Yoshizawa T (1999) Mycotoxins (trichothecenes, zearalenone and fumonisins) in cereals associated with human red intoxications stored since 1989 and 1991 in China. Natural Toxins 7: 93–97

Logrieco A, Mulè G, Moretti A and Bottalico A (2002) Toxigenic *Fusarium* species and mycotoxins associated with maize ear rot in Europe. European Journal of Plant Pathology 108: 597–609

Lukanowski A and Sadowski C (2002) Occurrence of *Fusarium* on grain and heads of winter wheat cultivated in organic, integrated, conventional systems and monoculture. Journal of Applied Genetics 43A: 69–74

Marin S, Sanchis V, Ramos AJ, Vinas I and Magan N (1998) Environmental factors, *in vitro* interactions, and niche overlap between *Fusarium moniliforme, F. proliferatum*, and *F. graminearum, Aspergillus* and *Penicillium* species from maize grain. Mycological Research 102: 831–837

Miller JD (2002) Aspects of the ecology of *Fusarium* toxins in cereals. In: Mycotoxins and Food Safety. 504 (pp. 19–27)

Nicholson P, Lees AK, Maurin N, Parry DW and Rezanoor HN (1996) Development of a PCR assay to identify and quantify *Microdochium nivale* var *nivale* and *Microdochium nivale* var *majus* in wheat. Physiological and Molecular Plant Pathology 48: 257–271

Nicholson P, Chandler E, Draeger RC, Gosman NE, Simpson DR, Thomsett M and Wilson AH (2003) Molecular tools to study epidemiology and toxicology of *Fusarium* head blight of cereals. European Journal of Plant Pathology 109: 691–703

Obst A, Gunther B, Beck R, Lepschy J and Tischner H (2002) Weather conditions conducive to *Gibberella zeae* and *Fusarium graminearum* head blight of wheat. Journal of Applied Genetics 43A: 185–192

Oehl F, Sieverding E, Ineichen K, Mader P, Boller T and Wiemken A (2003) Impact of land use intensity on the species diversity of arbuscular mycorrhizal fungi in agroecosystems of Central Europe. Applied and Environmental Microbiology 69: 2816–2824

Parry DW (1990) The incidence of *Fusarium* spp. in stem bases of selected crops of winter wheat in the Midlands, UK. Plant Pathology 39: 619–622

Parry DW, Jenkinson P and McLeod L (1995) *Fusarium* ear blight (scab) in small grains – a review. Plant Pathology 44: 207–238

Payne R (Eds) (2002) The Guide to GenStat® Release 6.1 – Part 2: Statistics. VSN International, Oxford

Pettersson H and Olvång H (1995) Trichothecene production by *Fusarium poae* and its ecology. In: Book of Abstracts of International Seminar on *Fusarium* – Mycotoxins, Taxonomy and Pathogenicity, Martina Franca, (pp 178–179)

Pirgozliev SR, Edwards SG, Hare MC and Jenkinson P (2003) Strategies for the control of *Fusarium* head blight in cereals. European Journal of Plant Pathology 109: 731–742

Polley RW, Turner JA, Cockerell V, Robb J, Scudamore KA, Sanders MF and Magan N (1991) Survey of *Fusarium* Species Infecting Winter Wheat in England, Wales and Scotland, 1989–1990. Home-Grown Cereals Research Report, London, 100

Rossi V, Ravanetti A, Pattori E and Giosue S (2001) Influence of temperature and humidity on the infection of wheat spikes by some fungi causing *Fusarium* head blight. Journal of Plant Pathology 83: 189–198

Rossi V, Pattori E, Ravanetti A and Giosue S (2002) Effect of constant and fluctuating temperature regimes on sporulation of four fungi causing head blight of wheat. Journal of Plant Pathology 84: 95–105

Velluti A, Marin S, Bettucci L, Ramos AJ and Sanchis V (2000) The effect of fungal competition on colonization of maize grain by *Fusarium moniliforme, F. proliferatum* and *F. graminearum* and on fumonisin B-1 and zearalenone formation. International Journal of Food Microbiology 59: 59–66

Waalwijk C, Kastelein P, de Vries I, Kerenyi Z, van der Lee T, Hesselink T, Kohl J and Kema G (2003) Major changes in *Fusarium* spp. in wheat in the Netherlands. European Journal of Plant Pathology 109: 743–754

Walsh EJ, Fanning MJ and Bannon E (1998) An evaluation of screening techniques to assess *Fusarium* head blight resistance in spring wheat. Cereal Research Communications 26: 59–66

Xu X-M (2003) Effects of environmental conditions on the development of fusarium ear blight. European Journal of Plant Pathology 109: 683–689

Xu X-M, Parry DW, Edwards SG, Cooke BM, Doohan FM, van Maanen A, Brennan JM, Monaghan S, Moretti A, Tocco G, Mule G, Hornok L, Giczey G, Tatnell J, Nicholson P and Ritieni A (2004) Relationship between the incidences of ear and spikelet infection of *Fusarium* ear blight in wheat. European Journal of Plant Pathology 110: 959–971

Zadoks JC, Chang TT and Konzak CF (1974) Decimal code for growth stages of cereals. Weed Research 15: 415–421