Relationship Between the Fungal Complex Causing Fusarium Head Blight of Wheat and Environmental Conditions

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ABSTRACT

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Over 4 years, the environmental conditions and the causal agents of Fusarium head blight (FHB) disease of wheat were determined in field sites in four European countries: Hungary, Ireland, Italy, and the United Kingdom. Polymerase chain reaction-based methods were used to detect each species causing FHB and quantify its DNA (as a measurement of fungal abundance) in the samples. Canonical correspondence analysis (CCA) was used to determine the relationship of the incidence and abundance of each species with weather variables. CCA indicated that little variability in the species prevalence data was explained by the weather

variables. In contrast, a greater proportion of variability in abundance data was accounted for by the weather variables. Most samples contained two or more species and statistical analysis suggested that these species tended to coexist at field sites. CCA also indicated that there were differences in the relationships of the prevalence and abundance of the six FHB species with environmental variables. *Fusarium poae* was associated with relatively drier and warmer conditions, whereas *F. graminearum* was associated with warmer/humid conditions. *F. avenaceum* and *F. culmorum* were both associated with niches of cooler/wet/humid conditions. Two *Microdochium* species were associated with regions of relatively cool/moderate temperatures and frequent rainfalls of short duration. The results also suggested that environmental conditions differentially affect the infection and colonization processes, and the comparative abundance of the six species.

Additional keywords: community ecology, competition, interaction, synergy.

Fusarium head blight of wheat (FHB), caused by *Fusarium culmorum*, *F. graminearum*, *Microdochium nivale*, and *M. majus* (formerly var. *nivale* and var. *majus*; [10]), *F. avenaceum* and *F. poae*, can result in significant yield losses (16,24). *F. graminearum*, *F. culmorum*, *F. avenaceum*, and *F. poae* can produce a range of mycotoxins in infected grain (1), whereas it is believed that both *Microdochium* species do not produce mycotoxins.

The distribution and predominance of the pathogens is believed, to a large extent, to be determined by climatic factors, particularly temperature and moisture. In hotter regions of Europe, *F. graminearum* predominates, whereas in cooler maritime areas, *Microdochium* species are favored (21). However, recent research indicated that the incidence of *F. graminearum* has increased considerably in usually cooler regions (32,37). There is a general consensus that FHB is most severe in warm and wet conditions during anthesis (21). While *F. graminearum* is the predominant species causing FHB on wheat in the United States, in Europe FHB can be caused by a complex of head blight pathogens (9,37). The implication of such a pathogen complex on disease development and mycotoxin accumulation is not well understood. It is not possible to infer a specific fungal species that

has caused FHB from visual symptoms alone. If more than one species is present, therefore, it is difficult to determine relationships between individual species in the FHB complex and environmental conditions.

Recent advances in molecular detection and quantification of individual species that causes FHB (18) have enabled the study of community of the FHB complex at field conditions. These new molecular techniques were used in a large-scale field survey on the FHB incidence/severity and accumulation of the associated mycotoxins in Italy, Ireland, Hungary, and the United Kingdom (37,38). More than one species was detected in the majority of the sites (37). In addition, in bulk field samples, there were frequent positive correlations between the presence/absence of two or three species at a single site (37).

This paper aims to study the community of the six fungal species that cause FHB at field conditions. First, we investigated whether there was a significant positive correlation in the presence of the six species at a sampling site, especially among two or three species. Then, we used canonical correlation analysis (CCA) to investigate how the presence/abundance of the six species was related to environmental conditions. In this paper, the presence of a particular species was determined by a polymerase chain reaction (PCR)-based diagnostic technique whereas species abundance was measured by the amount of DNA of each species within each sample quantified using competitive PCR assays.

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MATERIALS AND METHODS

Field sampling. Field sampling was as described by Xu et al. (37) and therefore only a brief summary is given here. A total of 213 sites were sampled for FHB over the period of 2001 to 2004 across four countries. Species prevalence and two/three-species association at the sampling site scale were reported previously, based on the data from 2001 to 2002 (37). In 2001, there were 15, 12, 19, and 20 sites for Hungary, Ireland, Italy, and the United Kingdom, respectively; in 2002, the corresponding number of sites was 15, 13, 19, and 20. In 2003 and 2004, there were 10 sites in each country in each year. These sites were chosen to represent a wide range of climatic conditions and located in important winter wheat production areas in Europe where FHB can cause serious damage. In addition to climatic conditions, these sites also differed in other agronomic characteristics, such as variety, tillage, cropping history, and soil type. Temperature (°C), rainfall (mm), relative humidity (%) and leaf surface wetness were recorded electronically at intervals of 2 h or less. For sites sampled in Hungary, United Kingdom, and Ireland, electronic data loggers were located at each site; for Italian sites, data loggers were situated less than 1 km away from the site. For a few sites, climatic data were not available due to the malfunction of data loggers and these sites were excluded from CCA.

In each year, a sample of 200 and 400 ears was collected at each site of approximately 1 ha size at milky ripe (GS77, i.e., growth stage 77) (40) and at harvest (GS92), respectively, using a random W-shape sampling strategy. The 200 ears harvested at milky ripe were visually assessed for head blight by recording the number of spikelets infected on each ear (disease intensity). These ears were then ranked for disease intensity from the least (labeled as ear 1) to the most diseased (labeled as ear 200). Three bulk samples were then produced: bulk sample 1 for ears 1 to 30 (the 30 least diseased), bulk sample 2 for ears 86 to 115 and bulk sample 3 for ears 171 to 200 (the 30 most diseased). This was done to increase the sensitivity of PCR detection and quantification of fungal DNA. Depending on the number of ears with symptoms, sometimes only one or two bulk samples were generated. For example, if no ears had visual symptoms, then only one bulk sample of the randomly chosen 30 ears was used; if only 20 ears had visual symptoms, only two bulk samples were then generated: then bulk sample 1 consisted of 30 ears randomly selected from the 180 apparently healthy ears whilst bulk sample 2 consisted of the 20 ears with symptoms and 10 randomly chosen healthy ears. Ears harvested at milky ripe were freeze-dried and then milled. Ears collected at harvest were threshed and the grains were milled.

Detecting and quantifying fungal DNA. For each sample, DNA of all six FHB causing species was extracted from approximately 4 g of milled, freeze-dried spikelets or grains. Quantification of DNA was done by the competitive PCR (qPCR) method described by Simpson et al. (26). Diagnostic PCR (dPCR) was carried out following the protocols described by Nicholson et al. (18) and Xu et al. (37).

The species-specific PCR primer pairs used to detect *Microdochium* and *Fusarium* species were those described in Nicholson et al. (17) and Nicholson et al. (18), respectively. Reaction components and amplification conditions for qPCR were the same as those for diagnostic PCR except for the addition of the relevant competitor template. Following amplification, PCR products were separated by electrophoresis and fungal, and competitor PCR products were quantified using Molecular Analyst software (Bio-Rad Laboratories, UK). The ratio of target and competitor PCR product was estimated and the amount of fungal DNA derived by reference to a standard curve generated using purified DNA of the relevant target species. Standard negative, positive, and competitor control reactions were included to each PCR run.

Statistical methods. Statistical analyses were carried out to (i) determine whether there were interactions in species presence between two or three species, (ii) determine whether species presence is correlated among the six species, and (iii) study the presence and abundance of the six species in relation to environmental conditions. For samples with insufficient fungal DNA to quantify but with positive diagnostic PCR results, pathogen DNA was set to 0.001% of the total DNA in the sample as the limit of quantification (LOQ) was about 0.001%. The amount of fungal DNA was averaged over three (in some cases two) replicates to obtain an average fungal DNA of each species at each site. For GS77 samples, a species was deemed to be present at a site if it was detected in any of the bulk samples and its DNA concentration was averaged over all the bulk samples.

Association among two/three species. Log-linear models were used to test the independence between two or three species in the FHB complex within each sampling site (37). In the log-linear model, each count in the individual cell of a joint counts table was treated as a Poisson response variable, which was regressed on independent factors, representing the presence or absence of each species. Because of the limited number of sites used and very small counts in some cells, species interactions of high order (>3) were not tested. To assess interactions between two species, represented by F1 and F2, the following model, $\ln(y) = u + F1 + F2$, was fitted to the 2×2 contingency table. In the model, 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 species. This model will have a residual deviance with 1 degree of freedom (df) for the interaction between the two species. The significance of the interaction can be determined as this deviance statistic has a chi-square distribution with 1 df. Similarly, the significance of interactions between three species was assessed based on the residual deviance (with 1 df) from the following model: $\ln(y) = u + F1 + F2 + F3 + F1 \times F2 + F2 \times F3 + F1 \times F3$.

Association among the six species. A variance ratio test was used to determine whether the presence of the six species at the same site was positively correlated among themselves; the principle of this method is the same as the commonly-used variance-mean ratio test for a Poisson variable (14). This test of mutual independence among the six species is analogous to the index of association used in genetics for testing an overall allelic association in haploid organisms (3,34). If there is mutual independence among the six species, occurrence of any one species at a site is not related to presence/absence of one or more other species at the same site. For this analysis, there were six binary data at each site at each growth stage (milky ripe or harvest), indicating the presence/absence of the six species. Samples from a single country at the same growth stage within the same year were treated as a group. Under the assumption of independence, the expected variance of the number of species present at a sampling site is $V_{\text{exp}} = \sum_{i=1}^6 p_i(1-p_i)$ where the p_i is the observed incidence of species i in a given group (i.e., a single year/growth stage/country combination) of the samples and the summation is over the six species. The following regression model was then used to relate the observed variance (V_{obs}) in the number of species present at a site within a given group of samples to the expected (V_{exp}) variance under the assumption of mutual independence: $\ln(V_{\text{obs}}) = a + b \ln(V_{\text{exp}})$. If either a is significantly greater than 0 or b is significantly greater than 1, then positive correlation in the presence of the six species at a single site is indicated. Both log-linear modeling and regression analyses were carried out using Genstat version 6.1 (22).

Environmental variables. Weather data were summarized in two periods: from the start of anthesis to 7 days after the end of anthesis (anthesis period), and from the start of anthesis to harvest (harvest period). Weather data for periods prior to anthesis were not used because of the lack of the data for many sites during

these periods. The anthesis period data were used for analysis of milky ripe samples and harvest period data for harvest samples. Preliminary analysis (results not shown) indicated that using two non-overlapping periods (anthesis period and post-anthesis to harvest) did not improve results of statistical analyses. For each period, 16 weather variables were derived from the recorded data (Table 1). There was a single value for each of 16 derived variables for each combination of site/year/country during either the anthesis or harvest period. Because they were measured on differ-

ent scales (units), all weather variables were mean-centered and then scaled by division by their standard deviations before statistical analysis, to equalize their variances. Standardization can also aid interpretation of CCA results (12).

Distribution/abundance of pathogen species. CCA was used to analyze the distribution and abundance of the six FHB causing species in relation to environmental conditions. CCA (27,28) is a method of direct gradient analysis. The main axes of a CCA are constrained to be a linear combination of environmental variables,

TABLE 1. Descriptions of 16 climatic variables during the anthesis or the harvest period

Variable	Description
T	Average daily temperature (°C)
RH	Average daily relative humidity (%)
VPD	Average daily vapor pressure deficit (Pa), derived from T and RH
Rain	Average daily total rainfall (mm)
RT	Number of rainfall events (there must be a minimum of 2-h dry period between two rainfall events)
XR	Maximum rainfall of a single rain event (mm)
RD	Average daily duration of rainfall (h)
Wet	Average daily duration of wet period (h). Wet was defined as either recorded rainfall during the scan interval is >0.1 mm or VPD is <1.1 Pa. These threshold values were determined empirically from the observed relationship between rainfall, VPD, and wetness as recorded by electronic sensors. We only used latter in the analysis since electronic wetness sensors were not available at some Italian sites
RW	Average length of wet periods initiated with rainfall (there must be a minimum 2-h dry period between two wet periods) (h)
TRW	Average temperature within all rainfall-initiated wet periods (°C)
LRW	The length of the rain-initiated wet period when the product of the duration of this rain-initiated wet period with the corresponding average temperature is maximum among all the rain-initiated periods within the period (anthesis or harvest) concerned (h), i.e., this is the length of wetness duration where the degree.wet.hour (similar to the common degree day measurement) is the maximum
TLW	Average temperature within the rain-initiated wet period when the product of the duration of this rain-initiated wet period with the corresponding average temperature is maximum among all the rain-initiated periods within the period (i.e., anthesis or harvest) concerned (°C), i.e., this is the average temperature during the wet duration where the degree.wet.hour (similar to the common degree day measurement) is the maximum
LWTW	The maximum product of the duration of a rain-initiated wet period with the corresponding average temperature among all the rain-initiated periods within the period (i.e., anthesis or harvest) concerned (LWTW = LRW * TLW), i.e., this is the maximum degree.wet.hour (similar to the common degree day measurement)
LV	Average daily duration of high moisture (h); a high moisture condition was defined empirically as VPD <2.2 Pa
OT	Average daily duration of hours when temperature is between 15 and 25°C (h)
LVOT	Average daily duration of hours when VPD is less than 2.2 Pa and temperature is between 15 and 25°C (h)

TABLE 2. Proportion of sites with detectable polymerase chain reaction (dPCR) and quantifiable (qPCR) amounts of DNA of the six *Fusarium* head blight species (n = 213)

Fungal species	dPCR		qPCR	
	Milky ripe	Harvest	Milky ripe	Harvest
<i>F. avenaceum</i>	0.32	0.36	0.23	0.25
Hungary	0.10	0.28	0.08	0.20
Ireland	0.67	0.78	0.51	0.69
Italy	0.24	0.14	0.14	0.00
UK	0.35	0.37	0.22	0.22
<i>F. culmorum</i>	0.18	0.32	0.14	0.11
Hungary	0.08	0.16	0.04	0.04
Ireland	0.33	0.60	0.22	0.16
Italy	0.05	0.07	0.3	0.00
UK	0.28	0.50	0.24	0.23
<i>F. graminearum</i>	0.35	0.47	0.23	0.18
Hungary	0.34	0.48	0.20	0.12
Ireland	0.51	0.62	0.33	0.40
Italy	0.14	0.28	0.09	0.02
UK	0.47	0.58	0.31	0.23
<i>F. poae</i>	0.48	0.54	0.30	0.14
Hungary	0.42	0.34	0.26	0.06
Ireland	0.38	0.47	0.20	0.09
Italy	0.31	0.53	0.14	0.03
UK	0.78	0.80	0.57	0.33
<i>M. majus</i>	0.29	0.38	0.22	0.21
Hungary	0.00	0.06	0.00	0.00
Ireland	0.73	0.78	0.56	0.51
Italy	0.16	0.28	0.12	0.17
UK	0.33	0.50	0.26	0.20
<i>M. nivale</i>	0.15	0.25	0.10	0.02
Hungary	0.00	0.08	0.00	0.00
Ireland	0.16	0.38	0.02	0.00
Italy	0.16	0.16	0.14	0.09
UK	0.28	0.42	0.22	0.00

which makes it comparable to linear regression. An overall measure of the CCA fit is given by the trace of the underlying correspondence analysis and by the proportion of variance in the species' data that is explained by each canonical axis. The trace is the total variance in the species data (i.e., the sum of all eigenvalues). In CCA, data for all species are analyzed jointly in relation to environmental conditions in order to reveal similarities as well as differences in the species-environment relationship between species. In addition, CCA is appropriate to deal with data sets that contain many zeros, as in this study.

Inference from CCA relies on several postulates about the underlying probability model for the data (12), e.g., Gaussian response curves, species having equal ecological tolerance and equal maxima along canonical axes. Although violation of these postulates may theoretically invalidate its use, Ter Braak (28) argues, as well as supported by simulation research (20), that the method is still robust when such postulates are violated.

CCA was performed using the CANOCO program (version 4.5 for Windows) (30). Initially, variable selection was primarily based on the forward selection method implemented in CANOCO; the α value was set to 0.05. To produce the most parsimonious ordinations, each of highly correlated variables and those weakly associated with only the first two axes, was then removed in turn from the full model derived from the forward selection. The main criterion of removing a variable from the full model was that its removal would not result in more than a 2% reduction in the total variation accounted for by the first two CCA axes. Random permutation, as implemented in CANOCO, was used to determine the significance of each axis. To test significance of axes other than the first, sample scores in constrained ordination as the linear combination of environmental variables of the lower canonical axes, were used as covariates. To test the significance of each axis, 500 random permutations were used. CCA was applied separately to the presence and abundance data at milky ripe and harvest.

RESULTS

Overall prevalence and abundance. Table 2 shows the overall incidence of each FHB causing species detected by the PCR techniques at milky ripe and harvest stages. All six species were frequently detected; the overall incidence was highest for *F. poae* (0.51) and lowest for *M. nivale* (0.20). The proportion of sites with the species detected increased from milky ripe to harvest, and varied greatly between the four countries (Table 2). In 53 and 65% of samples at milky ripe and harvest, respectively, more than one FHB-causing species was detected (Table 3). There were more sites in Ireland (27 out of 45) and the United Kingdom (23 out of 60) with at least four species present, than in Hungary (2 out of 50) and Italy (6 out of 58).

In many samples, a species was detected by diagnostic PCR but the amount of its DNA was below its LOQ (Table 2). For example, *F. graminearum* DNA was detected and quantified in respective 47 and 18% of samples at harvest, indicating that *F. graminearum* DNA was over the limit of detection but less than its LOQ in 29% of the samples. The amount of fungal DNA was extremely skewed with a long right tail (Table 4) (i.e., most samples where a species was detected had very low levels of fungal DNA).

Within a site, the amount of *F. graminearum*, *F. avenaceum*, and *M. majus* DNA at milky ripe was correlated positively with the amount of that species in the corresponding harvest samples ($r = 0.52, 0.87$, and 0.50 , respectively; $P < 0.01$). There were correlations in the amount of quantified fungal DNA between *F. avenaceum* and *F. culmorum* at milky ripe ($r = 0.29, P < 0.01$) and at harvest ($r = 0.50, P < 0.01$), and between the two *Microdochium* species at milky ripe ($r = 0.82, P < 0.01$). At milky ripe, as expected, quantified fungal DNA was significantly more in the batch of 30 most diseased ears than in that of 30 least diseased ears.

Association among two or three species. At both sampling times, 10 out of all possible 15 pair-wise combinations of the six

TABLE 3. Number of sites with specific number of Fusarium head blight (FHB) species detected by the diagnostic polymerase chain reaction technique ($n = 213$)

Sampling time	No. of FHB species detected				
	0	1	2	3	>3
Milky ripe	50	51	48	32	32
Hungary	18	18	13	1	0
Ireland	1	9	8	14	13
Italy	31	13	4	4	6
UK	0	11	23	13	13
Harvest	31	43	45	36	58
Hungary	11	17	15	5	2
Ireland	2	4	6	6	27
Italy	17	17	13	5	6
UK	1	5	11	20	23

TABLE 4. Summary statistics of the competitive polymerase chain reaction (qPCR) results (% of the total DNA) for Fusarium head blight species in those samples with positive diagnostic PCR (dPCR) tests ($n = 213$)

Fungal species	Milky ripe						Harvest					
	dPCR		Competitive PCR data ($\times 10^{-4}$)				dPCR		Competitive PCR data ($\times 10^{-4}$)			
	# Samples >LOD ^a	# Samples >LOQ ^b	Minimum	Mean	Median	Maximum	# Samples >LOD	# Samples >LOQ	Minimum	Mean	Median	Maximum
<i>F. avenaceum</i>	68	26	3	339	9	11,767	79	57	10	1,103	20	29,650
<i>F. culmorum</i>	39	10	3	675	7	8,848	69	31	10	210	10	3,637
<i>F. graminearum</i>	74	51	3	100	7	3,457	101	50	10	102	10	1,100
<i>F. poae</i>	101	39	3	82	7	3,967	115	32	10	77	10	4,100
<i>M. majus</i>	62	19	3	246	7	3,346	84	51	10	1,024	14	10,490
<i>M. nivale</i>	32	6	3	132	7	1,025	55	4	10	215	10	480

^a Number of samples with positive dPCR, LOD = limit of detection.

^b Number of samples with positive competitive PCR, LOQ = limit of quantification. For the Milky ripe samples, the minimum DNA is 3×10^{-4} instead of the LOQ (1×10^{-3}) because it was averaged over three subsamples.

species showed significant interactions in their presence at a single site (Table 5). All these 20 cases were the type of synergistic interactions: i.e., the presence of one species tending to favor the presence of the other. Furthermore, species association was consistent between two sampling times: eight of the 10 pair-wise combinations with significant interactions at milky ripe were the same as those at harvest (Table 5). Overall, the presence of *M. majus* appeared to be most strongly related to presence of other species. For example, in the *F. avenaceum*–*M. majus* case at both sampling times, the number of sites with both present or absent was nearly 20 more than expected under the assumption of independence. In only three cases were there significant interactions between three species: *F. graminearum*–*F. poae*–*F. culmorum* and *F. graminearum*–*M. nivale*–*M. majus* at milky ripe, and *F. graminearum*–*F. poae*–*F. avenaceum* at harvest.

Association among the six species. A variance ratio test was used to determine whether the presence of the six species were correlated within sites. Log-linear models well described the relationship of the observed variance in the number of FHB causing species present at each site with the corresponding expected variance, under the assumption of species independence. A common intercept was found for the regression models for the two growth stages, but the estimated slopes differed ($P < 0.01$). For the milky ripe samples, the fitted model was $\ln(V_{\text{obs}}) = 0.610 + 1.256\ln(V_{\text{exp}})$ and for the harvest samples it was $\ln(V_{\text{obs}}) = 0.610 + 2.019\ln(V_{\text{exp}})$. The standard error of the parameter estimates were 0.086 (for the intercept), 0.322, and 0.137 (for the two slope estimates), respectively.

Figure 1 shows the observed data together with the two fitted models, which explained about 77% of the total variation in $\ln(V_{\text{obs}})$. Since the intercept estimate (0.610) was greater than 0 ($P < 0.01$) and slope estimates greater than 1.0, the observed variance in the number of species present at each site was significantly greater than expected under the assumption of mutual independence. Thus, the presence of one species was significantly correlated positively with the presence of other species. Further-

more, the correlation was greater for the harvest samples than for the milky ripe samples.

Climatic conditions. Environmental conditions varied greatly between sampling sites as well as between countries and years. Table 6 gives summaries of the 16 climatic variables across all sampling sites. Average temperature during the anthesis period

Fig. 1. Natural logarithms of observed variance in the number of Fusarium head blight (FHB) species present at a single site plotted against the natural logarithms of variance expected under the assumption of independence among the six FHB species in their presence. Samples from a single country at the same growth stage within the same year as a group, giving a total of 16 points (year/country combinations) at the milky ripe (open circle) and harvest stage (solid circle). The dotted and solid lines are fitted models for the milky ripe and harvest samples, respectively.

TABLE 5. Results of the log-linear analysis on two-species interaction for pathogenic species causing Fusarium head blight in four European countries during 2001 to 2004; presence of a species was determined by a diagnostic polymerase chain reaction (dPCR) technique

Fungal species	<i>F. avenaceum</i>	<i>F. poae</i>	<i>F. culmorum</i>	<i>M. nivale</i>	<i>M. majus</i>
<i>F. graminearum</i>	* (NS) ^a	NS (NS)	** (**)	NS (*)	** (**)
<i>F. avenaceum</i>		* (NS)	** (**)	* (**)	** (**)
<i>F. poae</i>			NS (NS)	* (**)	NS (**)
<i>F. culmorum</i>				NS (NS)	** (**)
<i>M. nivale</i>					** (**)

^a NS, *, and **: not significant and significant at 5 and 1%, respectively; the text in the bracket indicates the significance for samples at harvest and the other for samples at milky ripe.

TABLE 6. Summary of 16 climatic variables during the anthesis and harvest period: minimum, average, maximum, and coefficient of variation (CV)

Variable ^a	Anthesis period				Harvest period			
	Minimum	Mean	Maximum	CV (%)	Minimum	Mean	Maximum	CV (%)
T	12.3	16.3	22.9	14.2	13.0	17.6	23.4	13.8
RH	54.0	75.0	89.0	9.5	59.0	74.7	90.0	10.2
VPD	2.1	5.5	11.2	38.0	2.0	6.2	12.0	41.1
Rain	0.0	1.7	5.8	66.1	0.0	1.8	6.0	48.8
RT	0.0	9.2	27.0	60.9	0.0	32.3	83.0	54.1
XR	0.0	12.4	49.5	67.7	0.0	25.1	220.2	84.6
RD	0.0	1.5	5.7	71.0	0.0	1.5	4.6	60.2
Wet	0.4	6.1	14.2	49.0	0.3	6.4	14.4	50.5
RW	0.0	6.9	25.0	63.0	0.0	7.8	17.4	40.8
TRW	0.0	13.1	22.3	26.0	0.0	14.2	20.1	12.7
LRW	0.0	14.2	49.0	66.5	0.0	25.2	68.0	54.3
TLW	0.0	13.1	29.7	27.7	0.0	14.9	24.1	14.4
LWTW	0.0	187.3	674.2	64.0	0.0	371.7	1003.0	53.3
LV	0.8	9.3	16.6	33.9	2.0	9.3	15.9	38.0
OT	5.7	11.7	19.0	25.9	7.4	12.0	18.3	17.3
LVOT	0.0	1.7	7.7	88.8	0.4	2.4	6.7	44.3

^a Refer to Table 1 for description of each variable.

ranged from 12.3 to 22.9°C, average relative humidity from 54 to 89%, and average daily rainfall from 0.0 to 5.8 mm. As expected, sampling sites in Ireland and the United Kingdom generally experienced cooler and wetter conditions than the Hungarian and Italian sites. Average temperature during the anthesis period was 14.2, 14.9, 18.3, and 17.9°C for the Irish, United Kingdom, Hungarian, and Italian sites, respectively, and the corresponding average humidity was 82, 78, 71, and 70%. Because of higher temperatures, on average it took only one week from the start to the end of wheat anthesis for Hungarian and Italian sites, compared with two weeks for Irish and United Kingdom sites. Overall, there was greater rainfall during the anthesis period in 2004 than in the other 3 years; average daily rainfall was 1.5, 1.6, 1.6, and 2.4 mm for 2001, 2002, 2003, and 2004, respectively. There was also considerable variability in weather conditions between sampling sites of the same country within each year. For example, average temperature during the anthesis period ranged from 12.4 to 15.8°C, average daily rainfall from 0 to 5.1 mm for the United Kingdom sampling sites in 2004. As expected, there were highly significant correlations between many pairs of environmental variables (data not shown).

CCA. CCA was used to investigate how the presence/abundance of the six species was related to environmental conditions.

The dPCR data (species presence). Environmental variables accounted for little variation in the fungal presence data. The five extracted axes accounted for 12.2 and 14.7% of the total variation in pathogen presence at milky ripe and harvest, respectively; only the first two axes were statistically significant ($P < 0.01$, Table 7). CCA biplots of the dPCR data are shown in Figure 2, whilst the relationship between environmental variables and the first two axes is given in Table 8. The first CCA axis mainly represented the dry-moist gradient (Fig. 2 and Table 8). At milky ripe, axis 1 was highly correlated positively with T and VPD and axis 2 was, to a lesser extent, correlated positively with VPD. Both axes were negatively correlated with the average daily duration of rainfall (RD) and the average daily duration of high moisture (LV). Thus, at milky ripe *F. graminearum* appeared to occur more frequently in warmer/wetter conditions, *F. poae* in warmer/drier conditions, *M. nivale* in cooler/drier conditions, *M. majus* in cooler/wetter conditions, and *F. avenaceum* and *F. culmorum* were between these extremes (Fig. 2A).

Similarly, at harvest the first two axes mainly reflected the contrast of dry-moist conditions (Fig. 2B and Table 8). However, the correlation of individual weather variables with the two axes was weaker than that at milky ripe (Table 8). The relative configurations of the six species in relation to environmental

TABLE 7. The characteristics of the five axes of the canonical correspondence analysis (CCA) for the diagnostic and the competitive polymerase chain reaction (PCR) data of the six pathogens causing Fusarium head blight at the milky ripe and harvest in four European countries; of the total 213 sites, CCA were only applied to 148 sites since there were either no species present at all or no weather data were available for the remaining sites

Axis	Diagnostic PCR								Competitive PCR							
	Milky ripe				Harvest				Milky ripe				Harvest			
	λ^a	%U ^b	%C ^b	ρ^c	λ	%U	%C	ρ	λ	%U	%C	ρ	λ	%U	%C	ρ
1	0.089**	6.2	50.9	0.51	0.086**	7.8	53.4	0.54	0.557**	17.0	39.6	0.81	0.493**	17.8	51.8	0.78
2	0.061**	10.5	85.6	0.42	0.061**	13.4	91.2	0.48	0.429**	30.1	70.2	0.70	0.262**	27.3	79.5	0.62
3	0.018	11.9	95.7	0.24	0.008	14.2	96.9	0.19	0.322**	39.9	93.1	0.63	0.110**	31.3	91.0	0.47
4	0.008	12.2	100.0	0.18	0.005	14.6	99.3	0.16	0.092*	42.7	99.8	0.41	0.078*	34.1	99.0	0.42
5	0.000	12.2	100.0	0.18	0.001	14.7	100.0	0.10	0.002	42.8	100.0	0.11	0.007	34.4	100.0	0.15

^a λ – the eigenvalue associated with each axes, indicating the amount of the variation the axis accounted for in the original data; ** and * indicate significant at the $P = 1$ and 5% level, respectively, based on 500 random permutation tests.

^b %U – accumulated percentage of the total variation in the original fungal presence/abundance data explained by CCA axes; %C – accumulated percentage of the total explained variation by CCA axes.

^c ρ – the correlation between each axis and site species scores, usually called species-environment correlation.

Fig. 2. Canonical correspondence analysis (CCA) biplot of the fungal presence of the six Fusarium head blight (FHB) species determined by the diagnostic polymerase chain reaction (dPCR) technique. Details on the relationships of environmental variables with the two CCA axes are given in Table 8.

conditions at harvest were similar to those observed at milky ripe (Fig. 2), with two noticeable differences. First, relative to the other four pathogens, both *F. avenaceum* and *F. culmorum* occurred on sites with relatively more humid/moist conditions at harvest than at milky ripe. Second, *F. graminearum* appeared more likely to occur in relatively drier conditions at harvest than at milky ripe, relative to the other species.

The qPCR data (species abundance). Environmental variables accounted for a greater proportion of the total variation in the fungal abundance data than the presence data. The five extracted axes accounted for 43 and 34% of the total variation at milky ripe and harvest, respectively. Most of the variation explained was attributed to the first two axes: 70 and 80% for the milky ripe and harvest qPCR data, respectively (Table 7). The first four axes were all statistically significant (axes 1 to 3 at the 1% level and axis 4 at the 5% level). Figure 3 shows the CCA biplots of qPCR data whilst Table 9 gives a summary of the relationships between environmental variables and the first two CCA axes.

For the milky ripe qPCR data, the first CCA axis was highly correlated negatively with Wet ($r = -0.61$) and LV ($r = -0.57$), reflecting the dry/moist gradient. Axis 2 mainly reflected the

gradient of atmospheric drying power. The first axis mainly separated *F. poae* and the two *Microdochium* species from the others and the second axis mainly differentiated *F. poae* and *F. graminearum* from the others (Fig. 3A). The two *Microdochium* species were located close together in relatively drier regions compared with the others. *F. graminearum* correlated positively with warmer/wetter conditions and *F. poae* with warmer/drier conditions. Warmer conditions were indicated by both high vapor pressure deficit (VPD) and LVOT; the latter is a function of the length of time when temperature was between 15 and 25°C. Again, both *F. avenaceum* and *F. culmorum* appeared to be adapted to humid (low VPD)/wetter conditions (Fig. 3A).

More environmental variables were included in the CCA for the harvest qPCR data than for the milky ripe data (Table 9). The first axis accounted for more than 50% of the total explained variability and was highly correlated with the average daily duration of rainfall (RD) (Table 9). The first axis was also correlated positively with VPD and the maximum degree.wet.hour for a single rain-initiated wet period (LWTW), and negatively with LV. This axis mainly separated *F. avenaceum* and *F. culmorum* from the others (Fig. 3B), suggesting that both *F. avenaceum* and *F. culmorum* were associated with niches with relatively drier condi-

TABLE 8. Canonical correspondence analysis (CCA) results of the diagnostic polymerase chain reaction (dPCR) data for pathogens capable of causing of Fusarium head blight: canonical coefficients and intra-set correlation of environmental variables with the first two axes of CCA. All environmental variables were standardized before CCA by subtracting their means and then dividing by their standard deviations

Milky ripe					Harvest				
Canonical coefficients ^b		Correlation			Canonical coefficients		Correlation		
Variables ^a	Axis 1	Axis 2	Axis 1	Axis 2	Variables ^a	Axis 1	Axis 2	Axis 1	Axis 2
T	1.63**	-2.56**	0.90	0.03	T	-1.43	5.95**	0.46	0.09
VPD	-0.21	1.31**	0.79	0.42	RH	3.81**	-6.19**	-0.34	-0.23
RD	-0.14	-0.63**	-0.63	-0.49	VPD	3.75**	-8.08**	0.42	0.16
LV	0.20	-0.63	-0.69	-0.55	RD	-0.31	0.01	-0.42	-0.12
LVOT	-0.74**	1.27**	0.34	-0.11	LV	-1.94*	3.35**	-0.40	-0.22
					LVOT	0.19	-1.87**	0.04	-0.30

^a T – average temperature (°C), RH – average relative humidity (%), VPD – average vapor pressure deficit (Pa), derived from T and RH, RD – average daily duration of rainfall (h), LV – average daily duration of high moisture (h); a high moisture condition was defined as VPD <2.2 Pa, LVOT – average daily duration (h) when VPD <2.2 and 15°C < T ≤ 25°C.

^b ** and * significant at the 1 and 5% level, respectively, on a *t* test; however, it should be noted that this *t* test is only of indicative value because the *t* values for the canonical coefficients do not follow the Student distribution.

Fig. 3. Canonical correspondence analysis (CCA) biplot of the fungal abundance of the six *Fusarium* head blight (FHB) species measured as % of the total DNA by the competitive polymerase chain reaction (PCR) technique. Details on the relationships of environmental variables with the two CCA axes are given in Table 9.

tions, because CCA indicated that these two species were associated with relatively shorter length RD and lower LWTW, which is a function of wetness duration. The second axis mainly reflected the wet-dry/warm comparison (Table 9 and Fig. 3B) and separated *M. nivale* and *F. poae* from the other four species, indicating *M. nivale* and *F. poae* were more likely to be abundant in regions of warmer/drier conditions than the others.

Overall there were considerable differences in pathogen–environment relationships between milky ripe and harvest, particularly for *F. graminearum*, *F. culmorum*, and *M. nivale*. Relative to the other pathogens, *F. graminearum* appeared to be associated with conditions of relatively higher VPD and longer duration of wet periods at milky ripe than at harvest (Fig. 3). The opposite was true for both *F. culmorum* and *M. nivale*.

DISCUSSION

This is the first detailed study aimed to understand the relationships of the distribution and abundance of the six species that cause FHB with environmental variables. This study was made possible by recent developments in PCR-methods that allow the specific molecular detection and quantification of six individual species capable of causing FHB (18). The diagnostic PCR method was found to be more sensitive in detecting the presence of each species than a competitive PCR method. Thus, for accurate determination of fungal biomass in each sample, both diagnostic and competitive (or other quantitative) PCR methods should be used. DNA quantification based on the TaqMan system is likely to be more sensitive than the competitive PCR method used in the present study. But it has only recently been developed (33) and was not available for all the target FHB pathogens when this study was conducted. Data collected in this study have revealed that although the six species do share some common requirements of environmental conditions for their development, they may also differ considerably in their requirements.

At milky ripe and harvest, 10 out of all 15 possible pair-wise species associations were statistically significant, confirming previous findings based on the first 2 years of the same survey data (37). Furthermore, the presence of six species appeared to be positively correlated with each other; this positive correlation was greater at harvest than at milky ripe. Such positive correlations can result either from direct synergy between species on the same infection court, or from indirect association facilitated by micro-climatic conditions conducive to several species. Results from sampling at a smaller scale (0.5 × 0.5 m quadrat) within several of

the sites used in this investigation, however, suggest that direct synergy is not likely to occur (39) since there was no evidence of a significant positive correlation in the presence of species at this smaller quadrat scale. Thus, the most likely reason for observed positive association among species is “indirect interaction” facilitated by general weather conditions conducive for disease development. Fungal species causing FHB in general need a period of free moisture (water) or high humidity for infection, although they may differ in the length of period required (23). Thus, provided that inoculum is present at a given site, conditions conducive to infection and subsequent colonization for one species are also likely to favor other species to some extent. An example is the consistent positive correlation between two *Microdochium* species in their presence (37) and in their abundance as indicated by the high correlation between their DNA.

The variance-ratio test suggested that the positive correlation in the presence of the six species at a site increased from milky ripe to harvest. This is likely to result from the increased incidences of the six species from milky ripe to harvest. In general, the degree of aggregation (correlation) is expected to increase initially and then decrease as disease incidence increases from very low to very high; the turning point is often around an incidence of 0.5 (14,35,36). Although this relationship of aggregation with disease incidence is observed for aggregation within a single species, it is likely to be applicable to testing multi-species coexistence. Except *F. poae*, the incidence of all the pathogens was below 0.5 at harvest. FHB infection is most likely to take place during the anthesis period (13,21). The observed increase in the fungal incidence from milky ripe to harvest is therefore probably due to the increased probability of detection at harvest, as a result of post-milky ripe fungal colonization rather than new infections.

Much less variability in the observed species presence between sampling sites was attributable to environmental factors than for the abundance data. This phenomenon is often observed in ecological research (11,29). Even for the fungal abundance data, there is still a considerable proportion of variability in the observed data that cannot be explained by the weather variables used in this study. The large variability in environmental factors and disease severities between sites suggested that this low-to-moderate relationship of pathogen presence and abundance with environmental factors is unlikely due to the lack of variation in either environmental conditions or disease development between sites. The lack of influence of environmental conditions on fungal development, particularly species presence, may be partly due to the fact that some factors known to affect inoculum production

TABLE 9. Canonical correspondence analysis (CCA) results of the competitive polymerase chain reaction (PCR) data for pathogens capable of causing Fusarium head blight: canonical coefficients and intra-set correlation of environmental variables with the first three axes of CCA. All environmental variables were standardized before CCA by subtracting their means and then dividing by their standard deviations

Milky ripe					Harvest				
Variables ^a	Canonical coefficients ^b		Correlation		Variables ^a	Canonical coefficients		Correlation	
	Axis 1	Axis 2	Axis 1	Axis 2		Axis 1	Axis 2	Axis 1	Axis 2
VPD	-1.45**	-0.14	0.26	0.29	T	-2.63**	2.99**	0.14	0.78
Wet	-0.56*	2.94**	-0.61	-0.01	VPD	1.51**	-2.53**	0.38	0.62
RT	-0.34**	1.14**	0.16	0.23	RD	0.13	0.38*	0.58	-0.29
LV	-2.00**	-3.08**	-0.57	-0.22	RT	-0.64**	0.24	-0.02	-0.45
LVOT	0.25*	0.57**	-0.18	0.23	LV	-1.73**	-1.85**	-0.31	-0.51
					OT	-0.71**	-1.96**	0.06	0.42
					LVOT	1.57**	0.78*	-0.02	0.32
					LWTW	0.78**	-0.40*	0.34	0.28

^a T – average temperature (°C), VPD – average vapor pressure deficit (Pa), derived from T and RH, RD – average daily duration of rainfall (h), LV – average daily duration of high moisture (h). A high moisture condition was defined as VPD <2.2 Pa, LVOT – average daily duration (h) when VPD <2.2 and 15°C < T ≤ 25°C, RT – number of rainfall events (there must be a minimum 2-h dry period between two rainfall events), Wet – average daily duration of wet period (h). A status of wet was defined as either recorded rainfall during the scan interval is >0.1 mm or VPD is <1.1 Pa, LWTW – the maximum product of the duration of a rain-initiated wet period with the corresponding average temperature among all the rain-initiated periods within the anthesis or harvest period, OT – average daily duration (h) when 15°C < T ≤ 25°C.

^b ** and * indicate significant at the 1 and 5% level, respectively, on a *t* test; however, it should be noted that this *t* test is only of indicative value because the *t* values for the canonical coefficients do not follow the Student distribution.

and FHB development were not considered in this study. These factors include previous crop, tillage, variety susceptibility and weather conditions prior to anthesis. For example, the incidence and severity of FHB was normally significantly greater when wheat followed maize than when wheat followed other crops (4,8).

Presence of a single species, as determined by the dPCR method in the present study, was an indication of successful infection by the pathogen provided that the inoculum was present. To determine whether the inoculum is present (and its strength) for each pathogen requires further time-consuming research activities, such as using spore trapper or trapping plants, which were beyond the present research remit. The absence (or varying strength) of the inoculum may not be related to environmental conditions during the period of this study. Cultivars may be another factor that may affect pathogen abundance. Although all cultivars sampled in the present study were highly susceptible to FHB, they could still differentially affect subsequent internal colonization following successful infections. However, a recent study suggested that there were no significant differences in fungal DNA contents in grain among eight of the sampled cultivars in the present study following artificial inoculations (2).

The lack of a strong relationship in the pathogen presence/abundance with environmental conditions may also be partly caused by the difficulties in pinpointing exactly the critical stage of FHB development, often the infection period, for field data. In this study as in most others (5–7,15), weather variables data were summarized for the anthesis period, which was considered to be the most susceptible period (21). Thus, the predictive power of these summary variables is expected to be less than if conditions during the actual infection periods were used. Environmental conditions prior to the anthesis, often 7 to 14 days, are known to affect sporulation of FHB pathogens and thus subsequent FHB severity (7). However, because of the lack of climatic data prior to anthesis in many sampling sites, it was not possible to include pre-anthesis climatic data in this analysis.

Although FHB causing species share some common requirements for environmental conditions for their development, CCA also indicated that there were some differences in their exact environmental requirements. CCA provides a graphical view of the relationship between species distribution/abundance and environmental conditions, which indicates not only similarities but also differences in the species–environment relationship between six species. Because far less variation in the fungal incidence data than the abundance data was accounted for by CCA, discussions are focused on the CCA results of the abundance data. In interpreting CCA results, we focused on the relative positions among the six pathogens in relation to environmental gradients rather than on absolute relationships between fungal incidence/abundance and environmental conditions. This is particularly necessary in the present study because all climatic variables were standardized before CCA for ease of interpretation.

Of the six species, *F. poae* was associated with relatively warmer and drier conditions than the other pathogens. Turner and Jennings (31) showed that increasing humidity produced earlier disease development and increased incidence of disease for all species except *F. poae*. CCA of the incidence and abundance (milky ripe only) data suggested that the occurrence of *F. graminearum* was associated with warmer and more humid conditions, which agrees with current consensus on the environmental requirements for this pathogen (21) even though recent evidence suggested that this species has gradually adapted to cooler regions (19,32,37). *F. avenaceum* and *F. culmorum* were associated with cooler/wet/humid conditions. The two *Microdochium* species were associated with climates where the temperature was cool/moderate with frequent rains of short duration.

The overall fungal abundance–environment relationship as shown by CCA varied considerably from milky ripe to harvest; this may be because fungal infection and subsequent colonization

differ in their responses to environmental conditions. Overall, the separation of the species along the wet-dry gradient as well as the correlation of the CCA axes with wetness-related variables was weaker for the harvest data than for the milky ripe data. This can be interpreted as meaning, as expected, that wet conditions were less important for colonization than for initial infection. CCA of abundance data at harvest using all the weather variables from two separate periods (anthesis and from post-anthesis to harvest), however, did not result in clearer results than those using the weather variables from the anthesis to harvest period (data not shown). This is likely to be due partly to problems of including too many intercorrelated variables in CCA.

Variation in the relationship between fungal abundance and environment conditions between milky ripe and harvest was also species-dependent. Thus, *F. graminearum* might require relatively higher temperature and longer duration of wet period to infect than for subsequent colonization. The two *Microdochium* species appeared to occupy similar environmental niches at milky ripe but differed considerably at harvest, suggesting they might require similar conditions for infection but different conditions for subsequent colonization. *M. nivale* appeared to require higher temperature for colonization, which agrees with the recent finding that *M. nivale* on wheat competes better at higher temperature than *M. majus* (25). These differential responses add another layer of complexity in predicting fungal development.

The present analytical approach has shown that the presence/absence of the six species that cause FHB may be less influenced by the weather conditions. Ordination analysis using CCA also indicated complex relationships between fungal abundance and environmental variables. Despite the fact that this research was based on field data, CCA suggested that fungal responses to environmental conditions may differ between initial infection and subsequent colonization as well as between species. The differences between species that were determined agreed with the limited results from previous studies (32). Further research under more controlled conditions is needed to confirm this and to determine the extent to which the six species differ in their infection and subsequent colonization under different conditions.

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