HGCA

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INVESTIGATION OF FUSARIUM INFECTION AND MYCOTOXIN LEVELS IN HARVESTED WHEAT GRAIN (1998)

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INVESTIGATION OF FUSARIUM INFECTION AND MYCOTOXIN LEVELS IN HARVESTED WHEAT GRAIN (1998)

by

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This is the final report of a seven month project which started in November 1998. The work was funded by a grant of £13,812 from HGCA (Project No. 2081).

CONTENTS

FINAL REPORT

PROJECT NUMBER: 2081 **REPORTING YEAR :** 1998/1999

TITLE OF PROJECT: Investigation of Fusarium Infection and Mycotoxin Levels in Harvested Wheat Grain (1998)

LEAD PARTNER: Central Science Laboratory
SCIENTIFIC PARTNERS: John Innes Centre

SECTION A

The MAFF-funded winter wheat disease survey of 1998 indicated that the level of fusarium ear blight was the highest since national monitoring of ear diseases began in 1987. A rapid response enabled grain samples to be collected from 53 of the most severely affected crops. Trichothecene mycotoxin and polymerase chain reaction (PCR) analysis were undertaken on each grain sample to try and relate the presence of particular *Fusarium* species with mycotoxin contamination of grain. These data were also analysed against agronomic factors collected during the national disease survey.

The level of mycotoxin detected in the grain samples was generally low. The predominant species responsible for the outbreak was *M. nivale*, a non-mycotoxin producer. It is the predominance of this species that probably accounted for the low levels of mycotoxin detected. Even so, four per cent of samples contained 1 ppm DON, a level equivalent to the tolerance limit suggested for the EU. A strong correlation (0.68) was seen between the amount of *F. culmorum* and *F. graminearum* DNA detected in the sample by PCR and the production of DON and NIV. This suggests that had the outbreak been dominated by a mycotoxin producing species, such as *F. culmorum* or *F. graminearum*, much higher levels of mycotoxin would have been present in grain samples. In 1998 the level of *F. graminearum* detected was unusually high (40% of samples infected). Increased incidence of *F. graminearum* has been indicated in countries where maize has been increased in closed rotation with wheat. However, there was no evidence from the survey to suggest that the increased incidence of *F. graminearum* in 1998 was linked with maize as a previous crop.

Regional differences in disease levels were shown with the most severely affected crops grown in the South West and Wales. PCR analysis indicated that the more aggressive species, *F. culmorum* and *F. graminearum*, were more abundant in regions with the highest disease severity. These regions are, in general, warmer than the rest of the UK. As *F. culmorum and F. graminearum* prefer warmer conditions (optimum growing temperature 20-25°C) this may account for their increased presence.

Although no cultivar is totally resistant to fusarium ear blight, the survey has shown differences in disease levels between cultivars. Indicating that the choice of cultivar needs to be considered if trying to reduce the risk from fusarium.

The rapid collection of grain following the 1998 harvest and the information gathered has provided valuable and unique data on the epidemiology of the species involved. The use of PCR analysis has provided accurate data on the relative contribution of the species involved to the disease levels recorded and to mycotoxin production.

Previous experimental work funded by the HGCA (project report N°. 143) indicated that high humidity conditions during anthesis could lead to significant mycotoxin contamination of grain in the UK. This project has confirmed that this occurs in the field in wet seasons and has generated data, which will be useful in the development of disease forecasting schemes and assessing future risks of mycotoxin contamination of grain in the UK.

SECTION B

OVERALL AIM

To investigate the outbreak of fusarium ear blight (FEB) in 1998, to identify the causal species and to determine the subsequent level of mycotoxin contamination in harvested grain.

METHOD

The 1998 MAFF funded Winter Wheat Disease Survey highlighted an outbreak of fusarium disease on the ear at the milky-ripe growth stage (GS 75) and indicated a potential risk for mycotoxin contamination at harvest. Surveyed sites were selected where in excess of 30% of plants showed fusarium ear blight symptoms (53 sites in total). From each site selected, 3 kg of grain was sampled at or immediately prior to harvest and sent to the Central Science Laboratory (CSL), where 300g sub-samples were taken for polymerase chain reaction (PCR) analysis at the John Innes Centre and trichothecene mycotoxin analysis at CSL. The remaining grain was stored at CSL at –30°C. The use of PCR analysis provided quantitative data on the relative amount of *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium avenaceum*, *Fusarium poae* and *Microdochium nivale* vars. *nivale* and *majus* present in the grain sample. The presence of trichothecene mycotoxin was analysed by gas chromatography/mass spectroscopy. The trichothecenes tested for included deoxynivalenol (DON), nivalenol (NIV), 3-O-acetyldeoxynivalenol (3AcDON), fusarenon X (FUS X), T2-toxin (T2), HT2-toxin (HT2), diacetoxyscirpenol (DAS) and 15-O-acetyl-4-deoxynivalenol (15AcDON). This range of toxins covers the majority of toxins likely to be produced by the *Fusarium* species found in the UK. Analysis for zearalenone was carried out using enzyme-linked immunosorbent assay (ELISA) test kits. Analyses of all data were carried out to relate species incidence and mycotoxin contamination, with climatic and agronomic factors.

RESULTS

The outbreak of fusarium ear blight in 1998 was the highest since national monitoring of ear diseases began in 1987 (Figure 1). Disease incidence at GS 75 exceeded 60% of crops affected with an average of 12% of ears showing symptoms. Additionally, one in six of the affected crops showed disease levels in excess of 30% ears affected and three crops showed 100% infection. Disease severity was also high, with an average of 1% of the total ear area affected. PCR analyses of the 53 crops surveyed (Figure 2) indicated that *M. nivale* var. *majus* was the predominant ear blight pathogen present, affecting 94% of samples, whilst *F. avenaceum*, *F. poae*, and *F. culmorum* affected 43%, 34% and 21% of samples respectively. *Fusarium graminearum*, a species not usually common in the UK, affected 40% of samples.

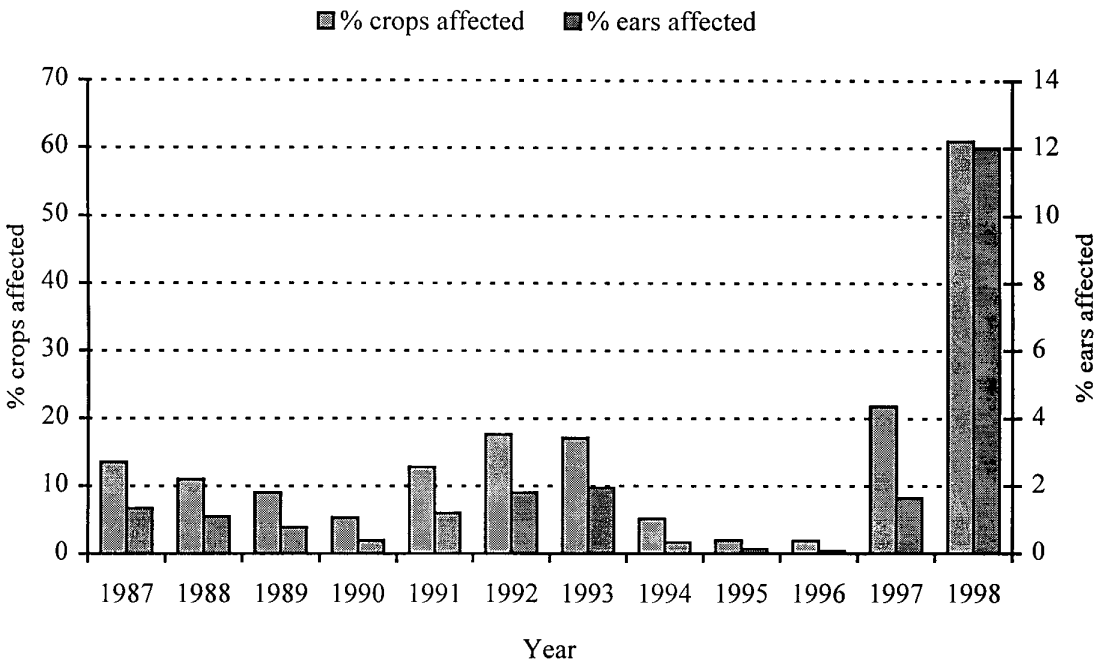

Figure 1. Incidence of fusarium ear blight 1987-1998 as indicated by the national survey.

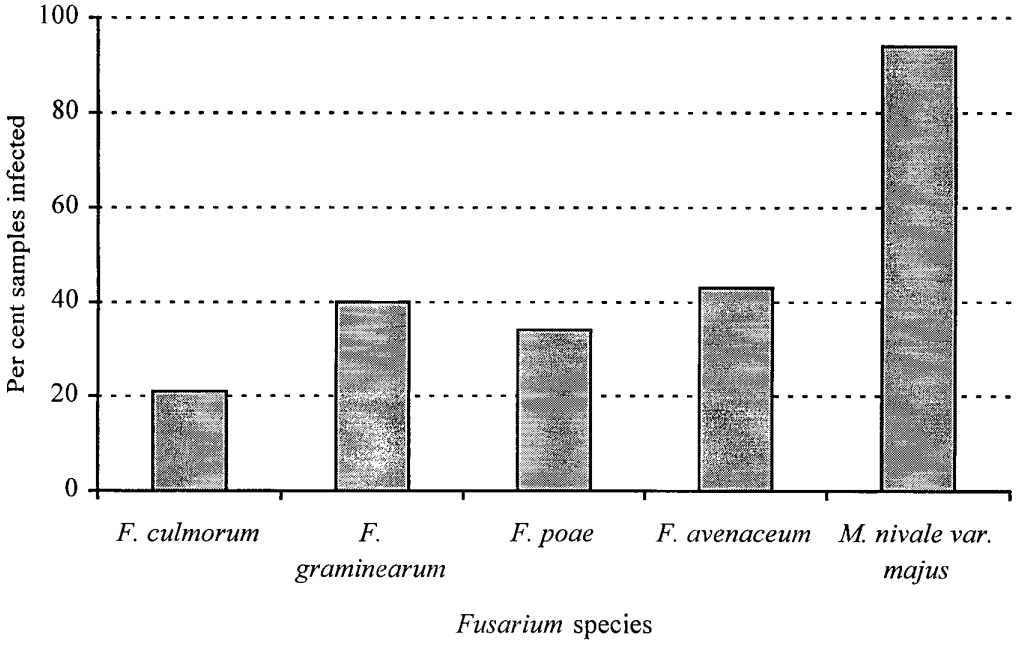

Figure 2. *Fusarium* species identified from PCR analysis of grain samples from the 53 crops surveyed at harvest.

Analysis of data from the national survey indicated regional differences in disease levels (Figure 3). The most severely affected crops were grown in the South West and Wales with an average of 23% and 15% of ears affected respectively. Crops in the north were the least affected with 34% of crops and 5% of ears showing symptoms. Data from the PCR analysis (Figure 4) confirmed the regional distribution of the disease from the national survey with the highest levels of *Fusarium* species found in regions with the highest incidence. No samples were obtained from the northern region as no sites from the national survey had greater than 30% ears showing fusarium symptoms.

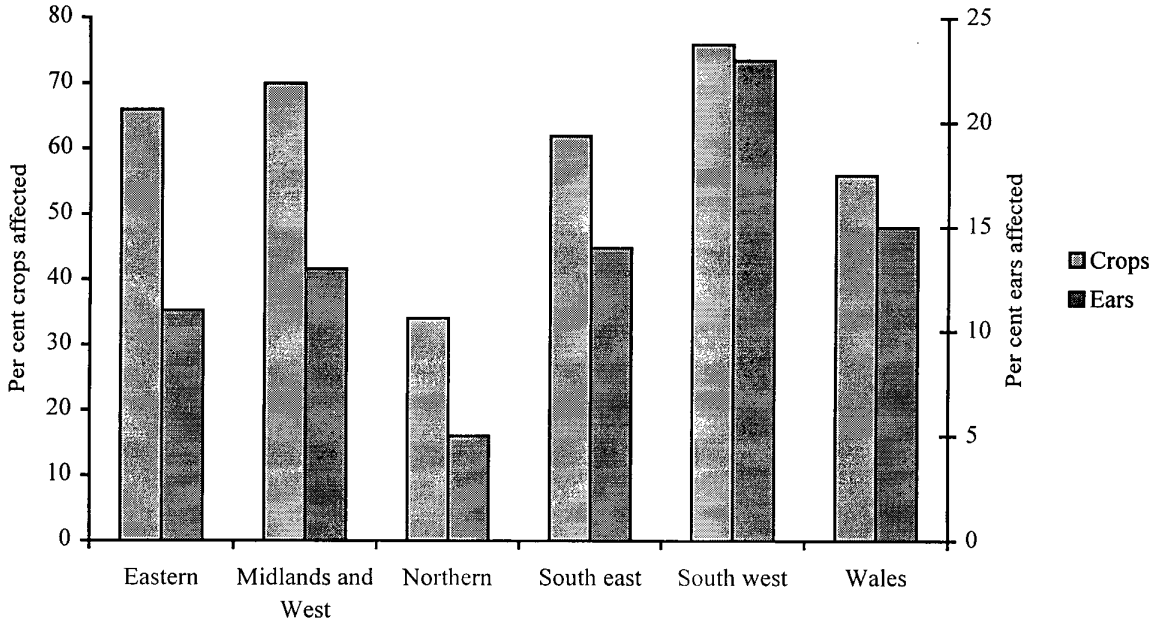

Figure 3. Regional incidence of fusarium ear blight in 1998 from the national survey.

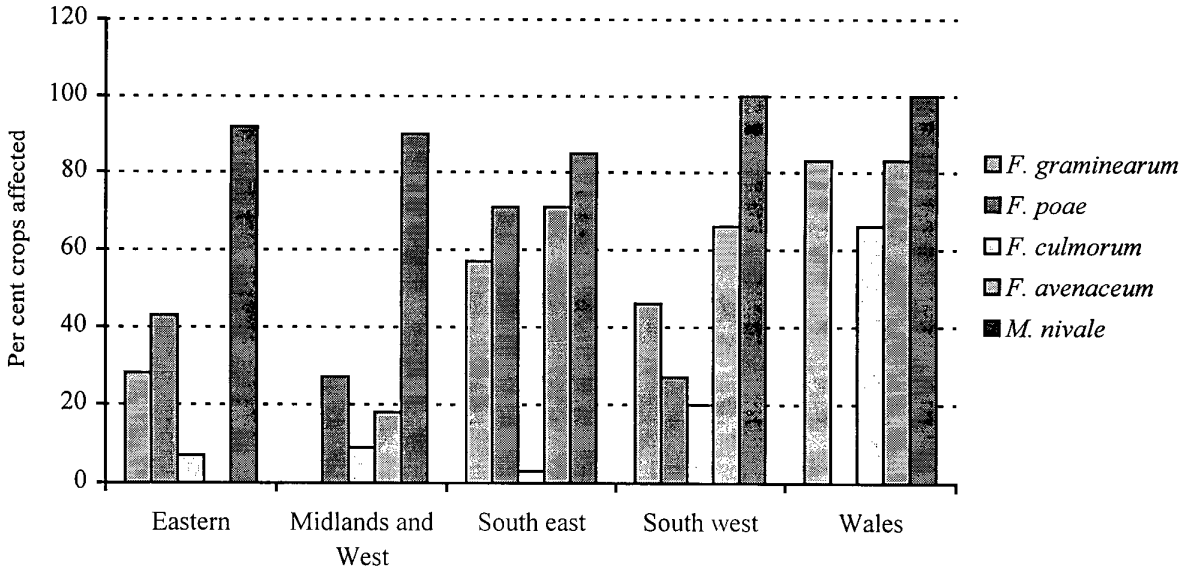

Figure 4. Regional distribution of *Fusarium* species as indicated by PCR analysis.

PCR analysis indicated regional differences in the species encountered, with *F. graminearum* being particularly prevalent in the South West, South East and Wales and *F. culmorum* being most common in Wales. There were no differences in the level of *M. nivale* across the regions sampled.

The influence of a range of agronomic factors on disease incidence and severity was examined. Cultivar was shown to be an important factor in determining the level of infection (Figure 5). Equinox was the most severely affected of the cultivars grown in 1998 with 93% of crops and 30% of ears affected. Of the seven most popular cultivars grown (Riband, Consort, Brigadier, Hereward, Reaper, Hussar and Buster), Reaper and Buster were the most severely affected with 76% and 64% of crops affected respectively. Riband was the least affected cultivar with 50% of crops and 6% ears showing ear blight symptoms.

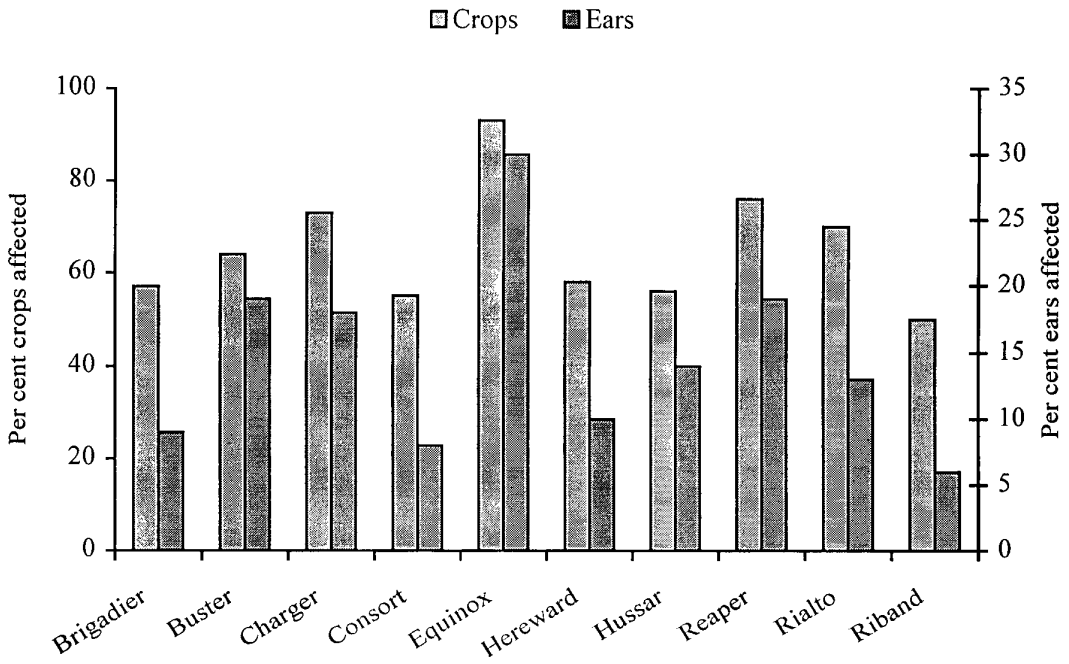

Figure 5. Influence of cultivar on the incidence of FEB in 1998.

Other factors such, as fungicide use, were also investigated but no clear trends were observed due to the small sample sizes involved.

Analysis for trichothecene mycotoxin showed that all but one of the 53 samples examined at harvest contained at least one mycotoxin. Deoxynivalenol (DON), nivalenol and HT2 were the most common toxins, found in 96%, 87% and 38% of the samples respectively. Although trichothecene contamination of the grain samples was widespread, the levels encountered were generally low (Figure 6). However, four per cent of the samples tested contained DON at 1 ppm. Levels of zearalenone were also low, with no sample containing concentrations of toxin greater than 0.2 ppm

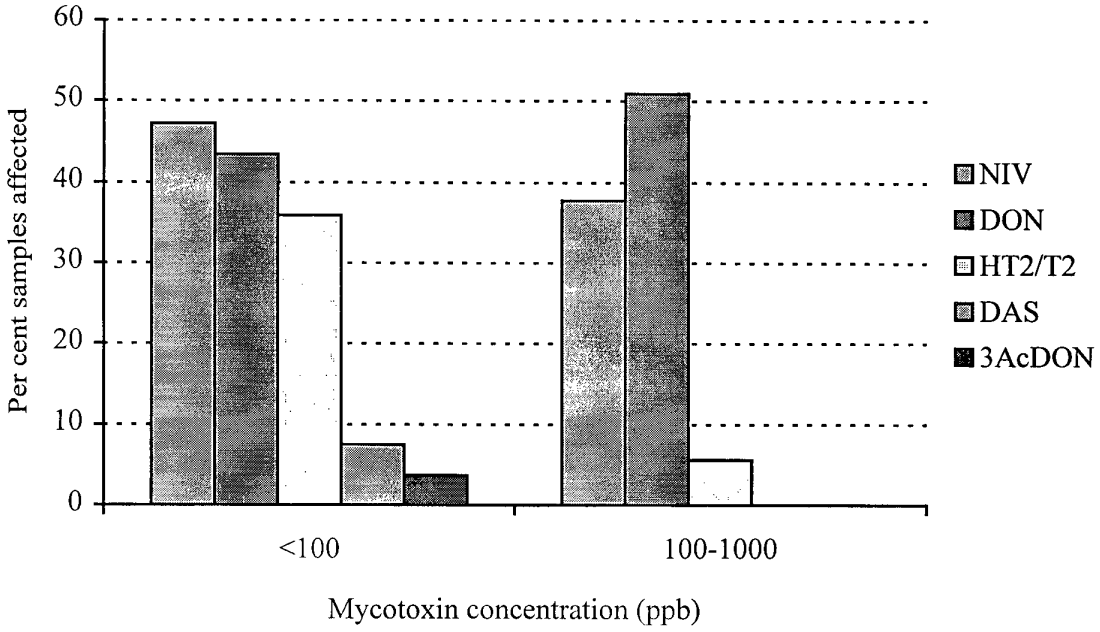

Figure 6. Occurrence of Fusarium mycotoxins in 1998 grain samples.

Correlation analyses indicated the contamination of grain with trichothecene mycotoxins was related to the presence of particular *Fusarium* species with a strong correlation (0.68) between the amount of *F. culmorum* and *F. graminearum* DNA detected in the sample by PCR and the production of DON and NIV.

CONCLUSIONS AND IMPLICATIONS FOR LEVY PAYERS

Nationally, a warmer spring than usual coupled with the fourth wettest June this century (data from the Met. Office) produced conditions conducive to the development of a significant outbreak of FEB.

Analysis of national survey data from the MAFF winter wheat disease survey indicated regional differences in disease levels. These differences are most likely linked to regional differences in meteorological conditions. Analysis of PCR data showed that differences in disease levels were linked to differences in *Fusarium* species incidence, with the more aggressive species, *F. culmorum* and *F. graminearum*, being more abundant in the South East, South West and Wales; regions with the highest disease severity. These regions are in general warmer than the rest of the UK. *F. culmorum and F. graminearum* prefer warmer conditions for growth (optimum growing temperature 20-25°C) this may account for their increased presence.

The level of mycotoxin detected in the grain samples was generally low. The predominant species responsible for the outbreak was *M. nivale*, a non-mycotoxin producer. It is the predominance of this species that probably accounted for the low levels of mycotoxin detected. Even so, four per cent of samples contained 1 ppm DON, a level equivalent to the tolerance limit suggested for the EU. A strong correlation was seen between the amount of *F. culmorum* and *F. graminearum* DNA detected in the sample by PCR and the production of DON and

NIV. This suggests that had the outbreak been dominated by a mycotoxin producing species, such as *F. culmorum* or *F. graminearum*, much higher levels of mycotoxin would have been present in grain samples. In 1998 the level of *F. graminearum* detected was unusually high. Any further increase in incidence of this species could have future yield and mycotoxin implications. Increased incidence of *F. graminearum* has been indicated in some countries where maize has increased in closed rotation with wheat. However, there was no evidence from the survey to suggest that the increased incidence of *F. graminearum* in 1998 was linked with maize as a previous crop.

Although no cultivar is totally resistant to FEB, the survey has shown differences in disease levels between cultivars. With the indication that the choice of cultivar needs to be considered carefully if trying to reduce fusarium disease levels. The sample size for each cultivar was too small to indicate whether there were differences in toxin accumulation between cultivars, as has been suggested by workers in other countries.

The winter wheat disease survey highlighted the fusarium outbreak at GS 75 and indicated the potential risk of mycotoxin contamination at harvest. The rapid collection of grain and the information gathered has provided valuable and unique data on the epidemiology of the species involved. Unfortunately due to the nature of the disease remedial measures in terms of fungicide sprays were not possible. The use of PCR diagnostics has provided accurate data on the relative contribution of the individual species (in terms of fungal biomass) to the disease levels recorded.

Previous experimental work funded by the HGCA (project report N°. 143) indicated that high humidity conditions during anthesis could lead to significant mycotoxin contamination of grain in the UK. This project has confirmed that this occurs in the field in wet seasons and has generated data, which will be useful in the development of disease forecasting schemes and assessing future risks of mycotoxin contamination of grain in the UK.

SECTION C

METHOD

Grain samples were obtained from the 1998 winter wheat disease survey where in excess of 30% of plants showed fusarium ear blight symptoms (53 sites in total). From each site 3 kg of grain was sampled at or immediately prior to harvest sent to the Central Science Laboratory where it was stored at –30°C until required.

Mycotoxin analysis

All mycotoxin analyses were carried out at the Central Science Laboratory.

Trichothecene analysis

Preparation and extraction

From each site a 300 g sub-sample of grain was ground to flour using a Glenn Creston disk mill. A 20 g portion of the ground sample was weighed into a 250 ml round bottom flask and extracted with 100 ml acetonitrile:water (84:16, v:v) for 120 minutes on a wrist action shaker. After shaking the sample was homogenised with an Ultra Turrax blender for 1 minute. The sample extract was filtered through Whatman No. 4 filter paper.

Approximately 10 ml of filtrate was transferred to a glass culture tube. The flange end of a Mycosep #225 clean-up column (Romer Labs. Inc.) was pushed into the tube until 6 to 7 ml of the cleaned up extract had passed through the column. A Gilson pipette was used to transfer 5 ml of the extract to a vial. The clean-up column was removed and a further 10 ml of filtrate was transferred to the glass tube. The clean-up step was repeated, and a second 5 ml of cleaned –up extract was added to the vial. The 10 ml of extract collected was evaporated to dryness in small portions under nitrogen at 50°C in a 4 ml vial.

Spiked samples

To 20 g of the ground sample 200 µl of 20 µg/ml mixed trichothecene standard solution was added. The solvent was allowed to evaporate before extraction proceeded.

Derivatisation

To the dry residue 50 µl of Tri-Sil/TBT was added. The vial capped and mixed for 30 seconds using a vortex mixer. The vial was heated in an oven at 80°C for 30 minutes. After cooling to room temperature, 500 µl of hexane and 1 ml of phosphate buffer were added to the vial and mixed for 30 seconds using a vortex mixer. The phases were allowed to separate before transferring the hexane fraction to a vial containing a small amount (spatula tip) of sodium sulphate. An aliquot of extract was then transferred to an auto sampler vial.

Analysis

Analysis of the extract was by gas chromatography/mass spectroscopy (GC/MS) using selected ion monitoring for the following trichothecenes: deoxynivalenol (DON), nivalenol (NIV), 3-O-acetyldeoxynivalenol (3AcDON), fusarenon X (FUS X), T2-toxin (T2), HT2-toxin (HT2), diacetoxyscirpenol (DAS) and 15-O-acetyl-4-deoxynivalenol (15AcDON).

Zearalenone analysis

Analysis for zearalenone contamination of grain samples was carried out using veratox quantitative zearalenone test kits (Adgen Diagnostic Systems, UK). Extraction of the toxin was carried out according to the recommended procedure.

PCR Analysis

PCR analysis was carried out at the John Innes Centre.

DNA extraction

Grain samples were freeze-dried, ground to a fine powder and incubated at 65°C for 2 hours in 20 ml of CTAB buffer together with 50 µl of protease K and 30 µl of RNAase. Following incubation, an equal volume of chloroform/isoamyl alcohol (24:1) was added to the tubes, mixed and centrifuged at 2600 *g* for 15 minutes. The aqueous phase was removed to a fresh tube and two volumes of ethanol (100%) were added followed by centrifugation as above to precipitate the DNA. The pellet was washed in a 70% solution of cold ethanol and dissolved in TE buffer (10 mM Tris HCL, 1 mM EDTA).

PCR amplification

Amplification reactions were performed in volumes of 50 µl and contained DNA from 0.3 mg dry weight of grain material. The reaction buffer consisted of 100 µm each of dATP, dCTP, dGTP and dTTP, 100 nm each of forward and reverse primer for PCR reactions, and 0.8 units of Taq polymerase in 10 mM Tris-HCL (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCL, 100 µg/ml gelatine and 0.05% each of Tween 20 and Nonidet P-40. Primers used were those for *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium avenaceum*, *Fusarium poae* and *Microdochium nivale* vars *nivale* and *majus*. Reaction mixtures were overlaid with mineral oil prior to PCR.

Amplification was performed in a Perkin-Elmer Cetus 480 DNA thermal cycler. The cycler was programmed for a touchdown program of (94°C, 30 s; 66°C, 20 s; 72°C, 45 s) x 5 cycles, (94°C, 30 s; 64°C, 20 s; 72°C, 45 s) x 5 cycles (94°C, 30 s; 62°C, 20 s; 72°C, 45 s) x 30 cycles, with a final extension of 72°C for 5 min followed by cooling to 5°C until recovery of samples.

DNA Quantification

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) and assayed using a Fluroskan II plate reader (INC Biomedicals Ltd, UK), which measured emission at 538 nm after excitation at 485 nm. The DNA concentration was ascertained by comparison with a serial dilution (0.0 - 1.8 ng l-1) of DNA (Hind III cut DNA) included on each plate.

Analyses of all data were carried out to relate species incidence and mycotoxin contamination, with climatic and agronomic factors.

RESULTS

The outbreak of fusarium ear blight in 1998 was the highest since national monitoring of ear diseases began in 1987 (Figure 1). Disease incidence at the milky ripe growth stage (GS 75) exceeded 60% of crops affected with an average of 12% of ears showing symptoms. Additionally, one in six of the affected crops showed disease levels in excess of 30% ears

affected and three crops showed 100% infection. Disease severity was also high, with an average of 1% of the total ear area affected.

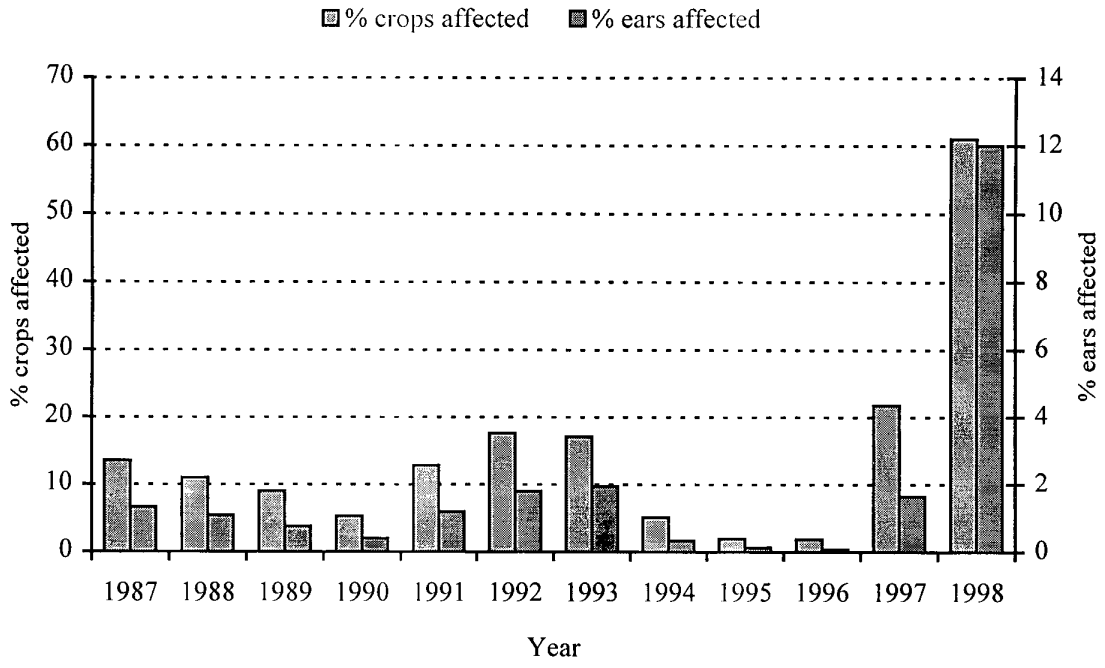

Figure 1. Incidence of fusarium ear blight 1987-1998 as indicated by the national survey.

PCR analyses of the 53 crops surveyed (Figure 2) indicated that *M. nivale* var. *majus* was the predominant ear blight pathogen present, affecting 94% of samples, whilst *F. avenaceum*, *F. poae,* and *F. culmorum* affected 43%, 34% and 21% of samples respectively. *Fusarium graminearum,* a species not usually common in the UK, affected 40% of samples.

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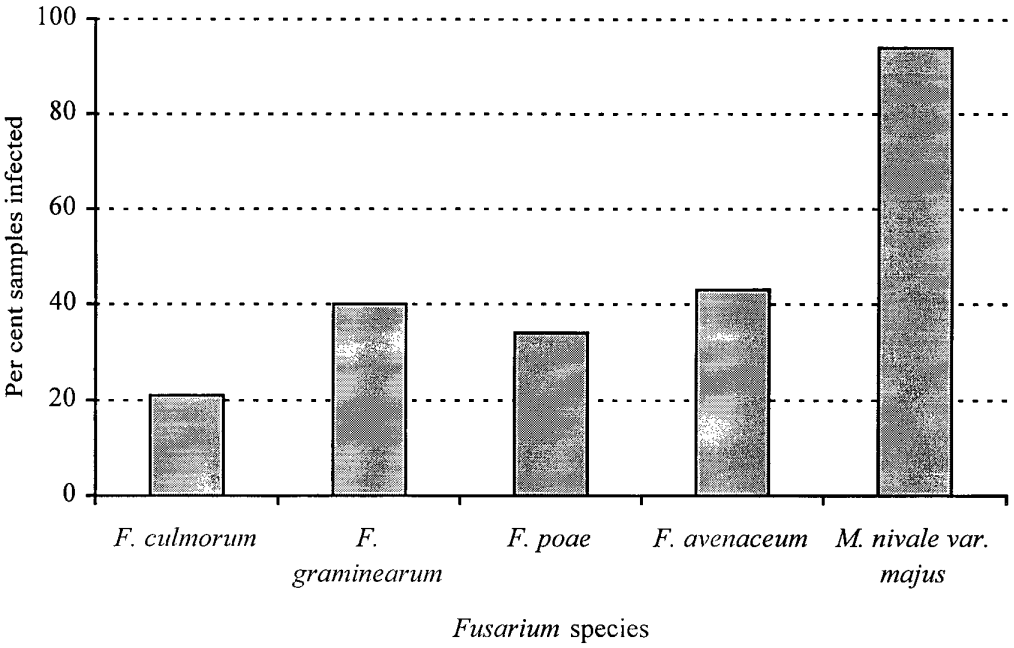

Figure 2. *Fusarium* species identified from PCR analysis of grain samples from the 53 crops surveyed at harvest.

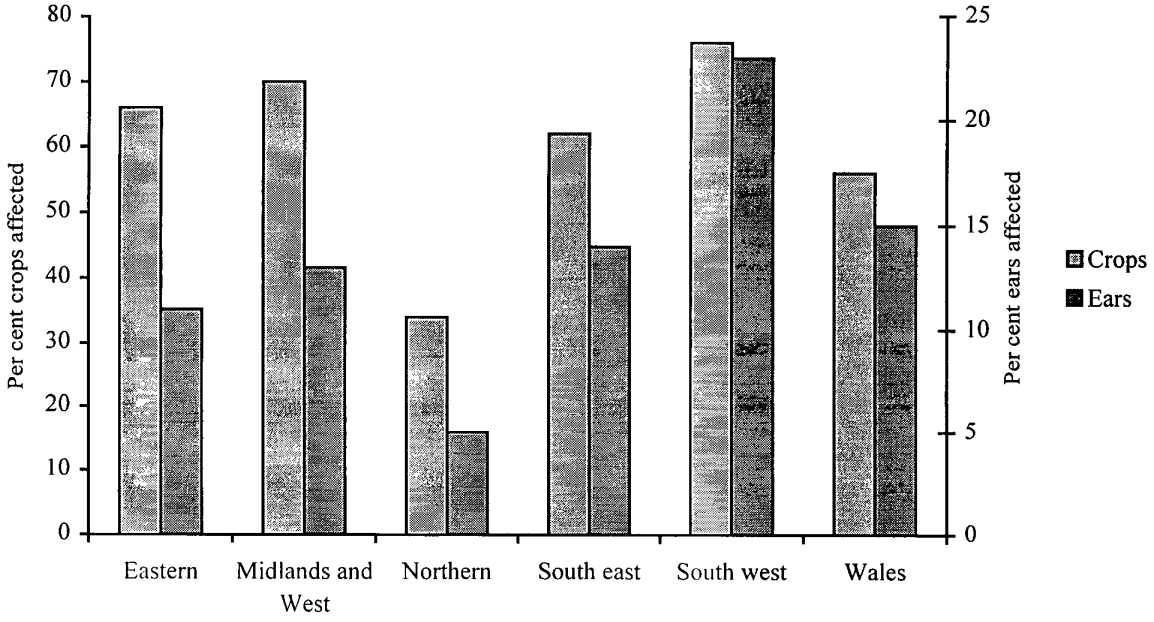

Figure 3. Regional incidence of fusarium ear blight in 1998 from the national survey.

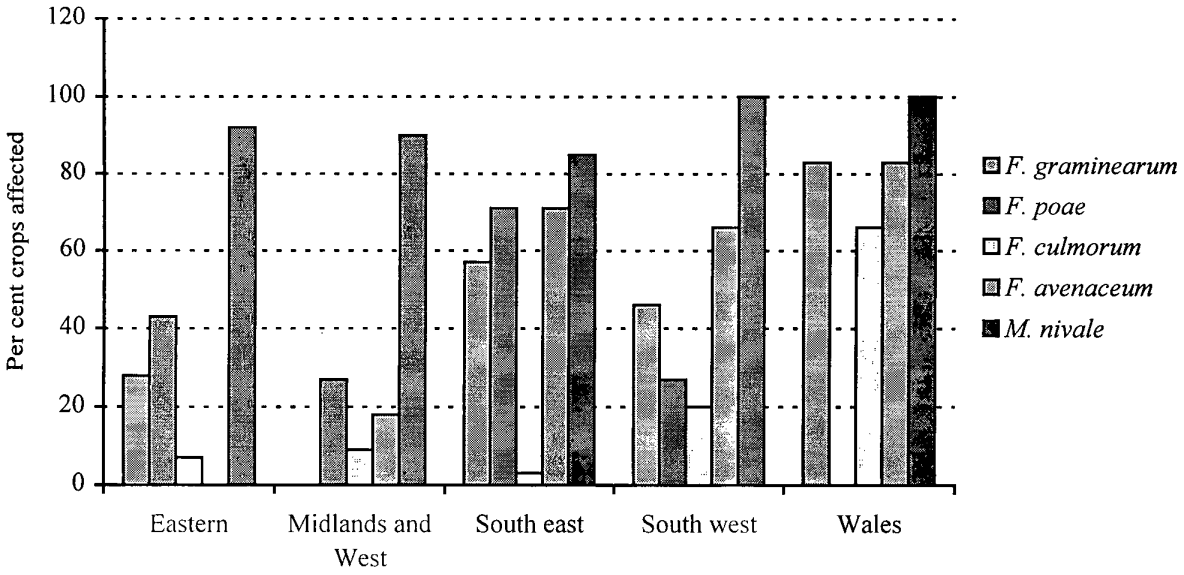

Figure 4. Regional distribution of *Fusarium* species as indicated by PCR analysis.

PCR analysis indicated regional differences in the species encountered, with *F. graminearum* being particularly prevalent in the South West, South East and Wales and *F. culmorum* being most common in Wales. There were no differences in the level of *M. nivale* across the regions sampled.

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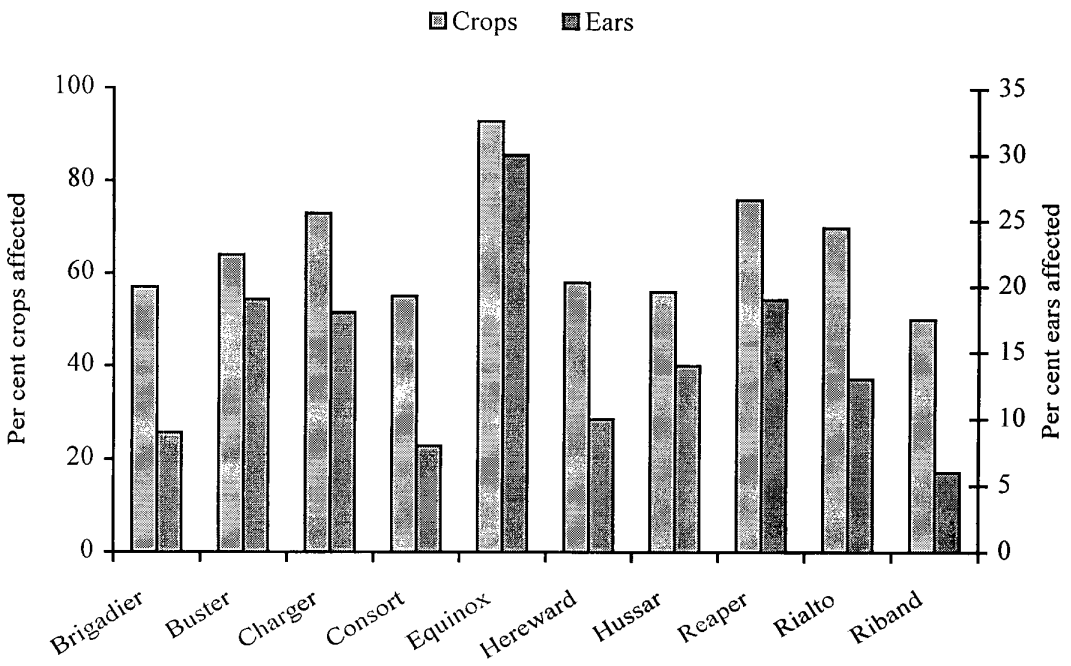

Figure 5. Influence of cultivar on the incidence of FEB in 1998.

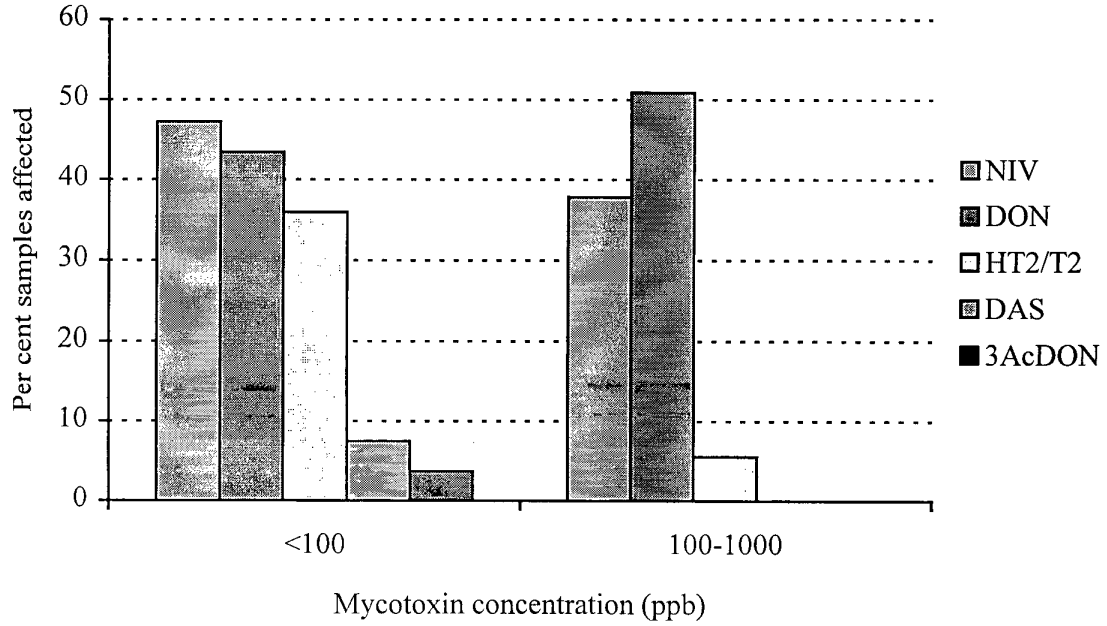

Figure 6. Occurrence of Fusarium mycotoxins in 1998 grain samples.

Correlation analyses indicated the contamination of grain with trichothecene mycotoxins was related to the presence of particular *Fusarium* species with a strong correlation (0.68) between the amount of *F. culmorum* and *F. graminearum* DNA detected in the sample by PCR and the production of DON and NIV.

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Hopwood A, Oldroyd N, Fellows S, Ward R, Owen S-A, Sullivan K, 1997. Rapid quantification of DNA samples extracted from buccal scrapes prior to DNA profiling. *Biotechniques* 23, 18-20.