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Cephalosporium leaf stripe – an emerging threat to wheat crops in short rotations

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CONTENTS

1. Abstract

Cephalosporium gramineum is a fungus which causes distinct yellow stripes on the upper foliage of cereal crops and can also lead to the production of stunted white ears in crops. The fungus is thought to be carried on the trash of preceding crops. It has been recognised on cereals crops and grass weeds. The aims of this project were to establish if all wheat varieties were equally at risk from the disease, the effect on yield of the disease, the ability of seed treatment chemicals to control the fungus and the effect of cultivation methods on disease levels. Field and laboratory experiments were used to look the effects of these different factors on disease levels.

Field experiments with the winter wheat Recommended List varieties showed that there are differences in susceptibility to the fungus. Resistance ratings were calculated from the data generated in the project. The disease was shown to have a negative effect on yield in winter wheat in field trials although the strength of this interaction varied from year to year. Trial results indicated that leaf stripe symptoms are greater in winter wheat than barley or oats.

In order to detect the presence of the fungus in crop, seed and soil samples, a new molecular diagnostic was developed. The test showed the presence of the fungus in leaves and in seed but no fungus was detected in soil samples taken from the field trial site. The fungus was detected in seed from the trial site and from samples collected across the UK. Movement in seed may be a possible way in which the fungus can spread to new areas.

Pure colonies of the fungus were produced from barley and wheat leaves sampled in the first year of the field trial. Growth of the fungus was reduced by the fungicides used in seed treatments in a laboratory bioassay. This implies that seed treatments may be exerting some control of the pathogen. However initial glasshouse tests to measure this effect proved inconclusive.

The effect of cultivation techniques on the disease development was studied in a series of field trials. The results were inconclusive as in one year the ploughed plots had more disease and the following year it was the plots which had minimum cultivation. Research from North America suggests that the disease favours minimum cultivation as this leaves more trash from previous crops on the soil surface. This trash acts as a source of inoculum to infect the following crop.

The effect of soil quality on disease levels in the field trials in the second growing season was measured. Damaged roots have been suggested as another cause of increased disease levels. Poor soil structure could contribute to root damage. No clear correlation between observed disease and soil moisture or structure quality was observed. However, testing did show that minimum cultivation plots had poorer structure than the ploughed plots and this may have contributed to higher levels of disease in the 2010 season.

In conclusion, the disease has been shown to be higher in wheat but resistance is available through variety selection. The efficacy of seed treatment chemicals is yet to be established. Cultivation may also affect disease levels and monitoring of the disease spread via infected plants would be appropriate.

2. Introduction

Cephalosporium leaf stripe is caused by the fungus *Hymenella cerealis*, (*Cephalosporium gramineum)*. Affected tillers appear randomly through the crop and show a distinct yellow stripe which extends to the leaf sheath.

Figure 1. Cephalosporium leaf stripe on winter wheat

Figure 2. Cephalosporium leaf stripe in winter barley

The fungus has a wide geographical range and has been reported in Germany, Italy, Netherlands, USA, Poland, Japan and UK (Martynuik, 1995). Symptoms are more pronounced in crops grown in short rotations. Wheat is the major economic host, but other cereal hosts include oats, barley, rye, triticale and grass hosts including *Bromus* sp*, Dactylis glomerata* and *Agropyron repens* (Howell & Burgess, 1969). The causal fungus is a slow-growing fungus in the soil but it is favoured by wet soil conditions and continuous cereal cropping. The soil borne fungus enters plants via the roots during winter and early spring. There is evidence that the fungus can be transmitted by seed (Murray 2006), but there is no information on the impact fungicide seed treatments have on the disease at these early stages. Once inside the plant, the fungus moves up the plant causing blockage at the nodes, distinctive leaf symptoms and stunting. At harvest, the fungus returns to the soil in the trash. Removing straw, ploughing and, where permitted, burning are the most effective ways to prevent a build-up of the problem (Christian & Miller 1984). If straw removal is not practical, then deeper ploughing to remove the straw from the root zone may help. In the USA, yield losses of up to 80% have been recorded in susceptible varieties (Quincke *et al.* 2012). In the UK yield losses have been estimated to be in the region of 0.5 t/ha but there is no measure of yield loss in replicated field trials. The AHDB Cereals & Oilseeds/BASF Encyclopaedia of cereal diseases states that “the disease is common at low levels in the UK and does not cause economic loss”. Outbreaks on farms in

Northumberland, the Lothians and Perthshire contradict this statement. This implies that the problem is more widespread and also highlights the need to understand more about the biology of a pathogen which can cause economic losses in winter wheat grown in short wheat rotations.

Spring sown cereals and the resultant trash are also potential carriers, but symptoms are rare. This wide host range causes issues with planning crop rotations for fields where disease levels have built up and there is little data to assist growers make decisions on crop rotations once the disease is established. There is little data on how long the fungus can survive in the trash or soil and this limits the knowledge required to give advice on the length of cereal breaks required following an outbreak.

Removing wheat trash and maintaining a rotation with non-hosts is the best method to reduce the disease. Continual wheat growers are reluctant to take this action since it breaks the take-all decline, leading to an increase in take-all when wheat is re-sown. In the USA, take-all fungus and cephalosporium leaf stripe can compete with each other in a field and, over a five year period, yield losses ranged between 26–65% for cephalosporium and 52–91% for take-all (Bockus *et al.* 1994).

In the USA, a break from cereals for 2 years would be recommended where the disease has increased to a level to cause economic concern. Where this is not possible, a break using a spring cereal will help to reduce the disease, but it can take longer. In the USA, some wheat varieties show tolerance but none are resistant. There is no data regarding tolerance in European varieties. In the UK, this disease occurs even under traditional ploughing cultivations, but it has become a major problem on farms sowing wheat under reduced tillage, or where straw is incorporated on close crop rotations.

Specific Issues

Little data exists on methods to manage the disease in the UK. This project will provide new information on the tolerance and resistance of Recommended List varieties in high disease pressure situations over three seasons.

The importance of agronomic management including the impact of minimum tillage, depth of straw incorporation by ploughing, straw removal or straw burning (where permitted) on levels of cephalosporium in the root zones will be measured. The potential to use fungicide seed treatments will be measured on the basis of a seed treatment trial and by screening the fungus against common fungicides. Practical advice on disease management through crop rotation will be determined using farm records from high risk fields. This will provide information on the success of different rotations at reducing the disease.

The overall aim of the project is to provide growers with practical advice to manage cephalosporium leaf stripe in winter wheat, w. barley and w. oats on high risk farms.

The specific objectives of the project are:

1. Provide information on varietal yield loss in wheat, resistance and tolerance to cephalosporium leaf stripe in the major cereal crops
2. Provide information on the impact of seed treatments on the fungus and symptoms
3. Understand the impact of cultivation and straw management on the survivability of the fungus
4. Develop practical guidelines on crop rotations to manage the disease

3. Materials and methods

3.1. Field Experimentation

A series of field trials were carried out at a high disease pressure site, Woodhead farm in East Lothian (Grid ref NT528677), to assess varietal susceptibility to cephalosporium leaf stripe. In 2009–11 the winter wheat Recommended List was sown in 10 m x 2 m plots. Each variety was sown in 2 replicates. In 2010–11 the winter barley and winter oats Recommended List were also sown in half plots (5 m x 2 m) at the same site. In 2010 and 2011, plots of winter wheat cv. Alchemy were sown into 10 m x 2 m plots to assess the impact of cultivation techniques on cephalosporium disease symptoms. Plots were prepared using conventional ploughing or minimum tillage.

Trial maintenance involved fertiliser, growth regulator, herbicide and fungicide applications. Details of treatments are given in Appendix One. Emergence counts were taken for all the plots in 2010. Plots were assessed for foliar disease, head blight and take all symptoms in regular intervals through the course of the trial. Winter wheat plots were taken to yield in 2009–11. Disease levels were calculated as area under the disease progress curve (AUDPC) using the trapezoidal rule. Figures were then expressed as the percentage of maximum AUDPC. Analysis was done across all the trial years.

Additional scores were made of the winter wheat RL trial in Fife in 2011 (WW2011SA136T) as cephalosporium symptoms appeared in the crop.

3.2. Cephalosporium sensitivity assays

C. gramineum was isolated from leaf samples in 2009 using the method of Stiles and Murray (1996). Briefly, leaves were surface sterilised in ethanol (30 secs) and 1.0% Sodium hypochlorite solution (90 secs) before being plated onto corn meal agar filled Petri dishes. The plates were incubated at 17 °C in darkness for 7 days. Plates were then inspected and mycelia transferred to fresh CMA plates. Microscope slides were made to confirm the spores as *C. gramineum* plates were stored in an incubator in the dark at 15–18 °C.

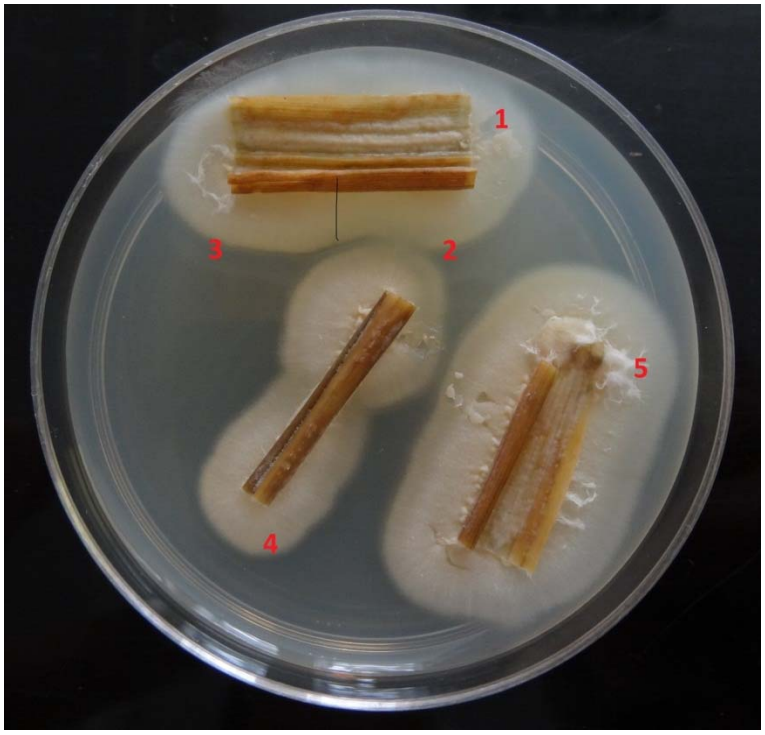
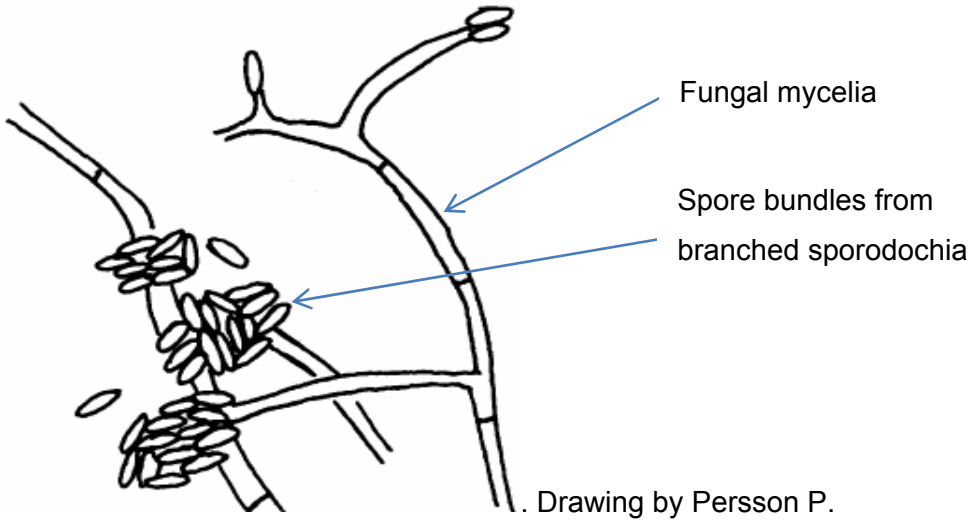


Figure 3. Isolation plate of *Cephalosporium gramineum*



. Drawing by Persson P.

http://www.vaxteko.nu/html/sll/slu/vaxtskyddsnotiser/VSN82-1/VSN82-1G.HTM

Figure 4. *C. gramineum* spores

Profuse sporulation occurs on the senescent tissue during cool, wet periods in the autumn. Unicellular conidia are produced on masses of conidiophores called sporodochia on the infected crop residue (Bruehl, 1968; Wiese and Ravenscroft, 1978). Sporodochia are flat and black when dry and raised and yellow-brown when moist. Conidia are the primary inoculum source. They are hyaline, oval shaped, non-septate and very small (4–7 µm x 2–3 µm).

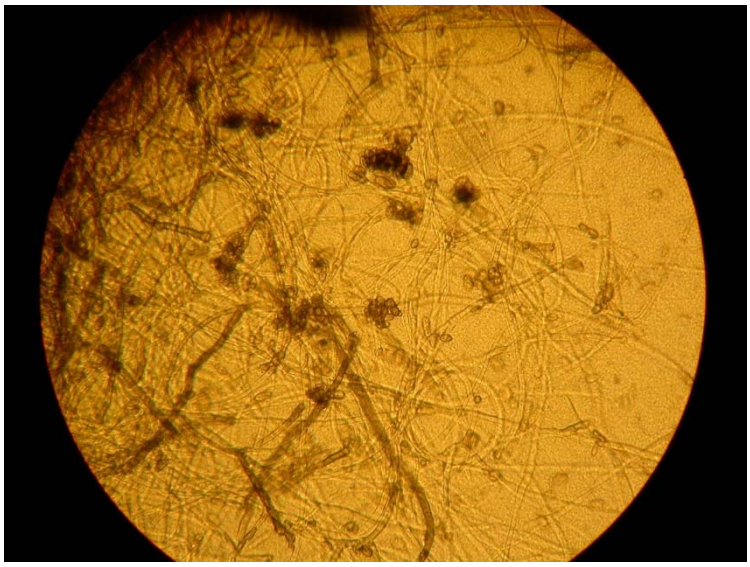


Figure 5. Spores and mycelia of *C. gramineum* (x 40 magnification)

C. gramineum presence was confirmed using PCR primers (Ceph F1 Ceph R1; Gorniak & Havis, unpublished). DNA from fungal culture was extracted using microLYSIS®-Plus (Microzone, Haywards Heath, UK) following manufacturer's instructions. DNA was amplified using MegaMix-Royal (Microzone) and the reaction carried out in Biometra thermocycler. To a total reaction volume of 25µl, 0.1µM of each of the primers (Table 1.) was added and 1.0µl of template DNA. The PCR conditions included an initial denaturation step of 95°C for 5 min; 29 cycles of denaturation at 94°C for 30 seconds, annealing at 53°C for 30 seconds and elongation at 72°C for 1 min; then a final elongation step at 72°C for 10 min. Amplified products were run on a 1.2% agarose gel containing GelRedTM Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA) at a constant 125 V for approximately 40 min.

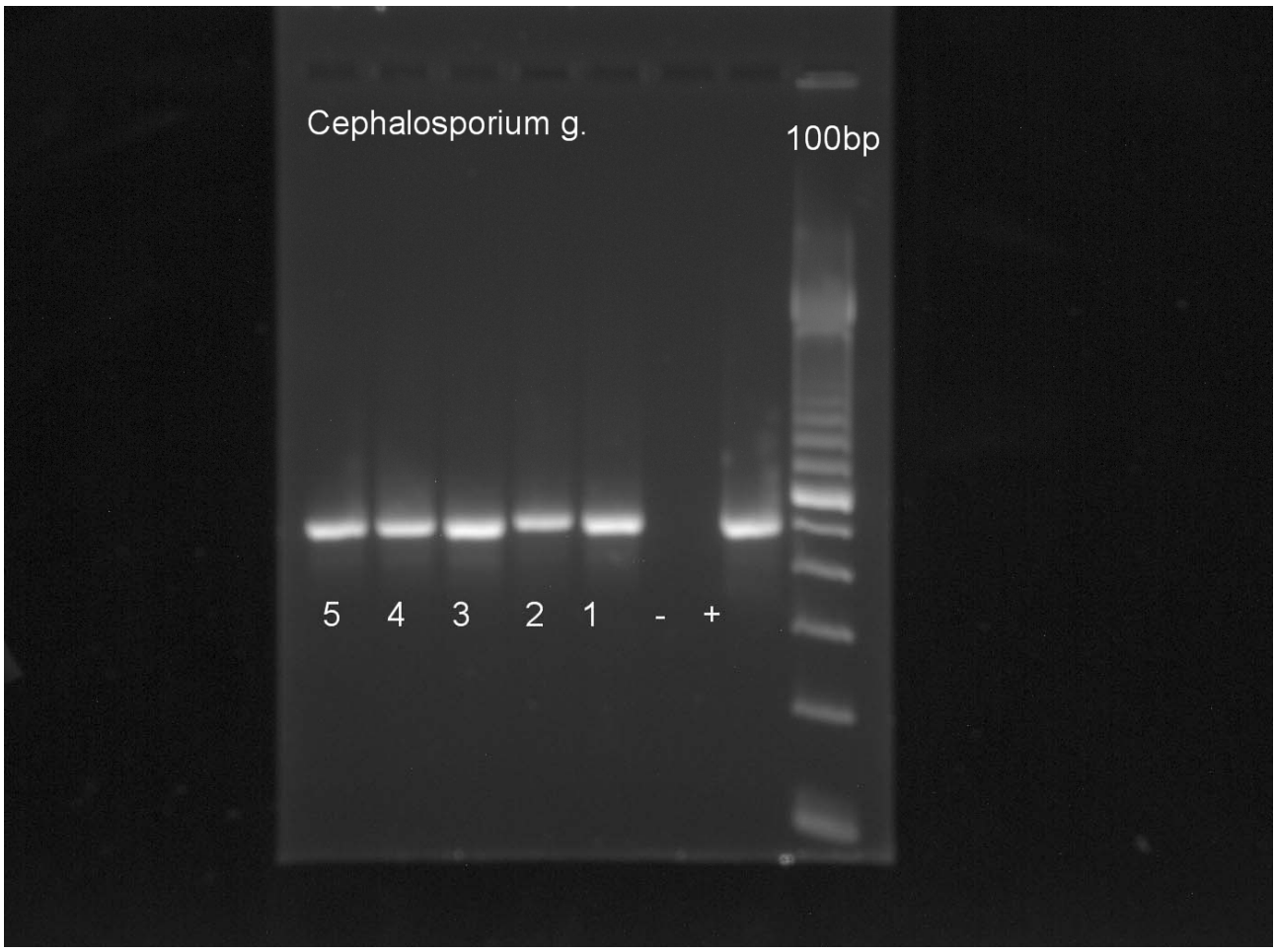


Figure 6. Conventional PCR gel used to confirm presence of fungus on isolation plate

Table 1. Primers used in conventional and qPCR

Oligonucleotide name Oligonucleotide type sequences (5′–3′) and labeling
1406f forward primer TGYACACACCGCCCGT
3126r reverse primer ATATGCTTAAGTTCAGCGGGT
CephF1 forward primer TGATGTCTGAGTACTATATAATAG
CephR1 reverse primer GTTATAATGACGCTCGAA
CephP1 TaqMan®probe FAM-ATCTCTTGGTTCTGGCATCG-BHQ1[a]
CeHcF forward primer CTGATCATCACGATGTAAGCTC
CeHcR reverse primer ATCGATGCCAGAACCAAGAG

[a] Probe labelled with 6-carboxy fluorescein (FAM) and Black Hole Quencher 1 (BHQ-1)

To produce spores for sensitivity assays isolates were grown in Potato Dextrose Broth at 20 ºC for 7 days and a spore suspension produced by sonicating the culture for 1 minute and vortexing for 2 minutes. Sensitivity assays were carried out on a 98 well plate with wells containing 20 µl of spore suspension and 180 µl of fungicide amended media. Each concentration was tested in triplicate and plates were placed on an orbital shaker at 20 ºC for 7 days. Plates were read at 400 nm

absorbance on a plate reader (Omega, BMG,Germany) and EC50 values calculated using the MARS data software.

Table 2. Seed treatment chemicals tested in sensitivity assays

Active ingredient	Trade Name
Carboxin	Kinto
Fludioxonil	Beret Gold
Fluquinconazole	Jockey
Prochloraz	Prelude
Prothioconazole	Redigo
Silthiofam	Latitude
Triticonazole	Kinto
Epoxiconazole	Jockey

3.3. Cepahlosporium Diagnostic Development

3.3.1. DNA Extraction

Pure cultures

Cephalosporium gramineum cultures were grown on corn meal agar in 9 cm petri dishes incubated at 20°C (Murray 1988). Fungal material was harvested, freeze-dried and finely ground. DNA was extracted from fungal mycelium (CABI 80179) using the method of a Nucleon® PhytoPure Plant DNA kit (GE Healthcare UK Ltd, Buckinghamshire, UK). The DNA concentration was determined using a spectrophotometer NanoDrop (Thermo Scientific, Wilmington, USA).

Plant material

Genomic DNA was extracted from 100 seeds by powdering the air dried samples using the mixer mill Retsch MM200. One gram of finely ground material was used in each DNA extraction. Using the method of Fraaije *et al.* (1999), 4ml of extraction buffer[1] was added to each sample, except that the DNA extraction buffer was amended with 5mM 1,10-phenanthroline monohydrate and 2% (w/v) polyvinylpyrrolidone. After incubating the mixture for 20 min at 70 °C, 400µl ice-cold ammonium acetate (7.5M) was added to the samples and the total suspension kept on ice for 30 min. After centrifugation at 14.000rpm for 10 min, an equal volume of cold (– 20 °C) isopropanol was added to the supernatant and the extract stood at room temperature for 15 min. After centrifugation at

[1] Extraction Buffer (1litre) 475mls of T.E.N Buffer [T.E.N Buffer (500mls), 31.52 g Tris-base (C4H11NO3)·HCl (400nM), 14.1g NaCl, 11.9g of EDTA (50nM), Adjust to ph 8.0 with Sodium Hydroxide, Make up to 500mls with Sterile Distilled Water] 475mls of 2% Sodium Dodecyl Sulfate solution (Lauryl Sulfate), 0.8g Phenanthropholine (equivalent to 5mM), 20g Polyvinylpyrrolidone)

14.000rpm for 5 min, DNA pellets were washed with ice-cold 70% ethanol. Samples were air dried for 15 min and dissolved in 200µl sterile distilled water. For every sample, the DNA concentration was measured with the NanoDrop (Thermo Scientific, Wilmington, USA).

3.3.2. Amplification and Cloning

Conventional PCR was carried out in a Biometra thermocycler using GoTaq® Green Master Mix (Promega Corporation, Madison, UK) in a 50µl reaction volume, 1ng template DNA was added to the reaction mixture containing 500nM universal 16S/18S rRNA forward primer 1406f (TGYACACACCGCCCGT) (Fisher, 1999) labelled with TET, 500nM 28S rRNA reverse primer 3126r (ATATGCTTAAGTTCAGCGGGT) (Ranjard *et al.,* 2001). These primers target the last 120bp of 18S rRNA gene, ITS region 1 (ITS1), 5.8S rRNA gene and ITS2, with the 3126r primer matching the 5′ end of the 28S rRNA gene (DeRito & Madsen, 2009).The PCR conditions were 94 °C for 2 min, and then cycled 34 times through 30 sec at 94 °C, 30 sec at 56°C, and 1 min at 72 °C. The PCR was terminated with a DNA extension for 10 min at 72 °C (Borneman & Hartin, 2000). Gel electrophoresis was used to analyse PCR amplified products; PCR products in a 10 µl sample were separated on a 1.2% (w/v) agarose gel containing GelRed™ Nucleic Acid Gel Stain, 10,000X in DMSO (Biotium, Inc., Hayward, CA) run in Tris-borate-EDTA (TBE) buffer (89mM Tris base, 89mM boric acid, 2mM EDTA, pH 8.0) and exposed to UV light to visualise DNA fragments. PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostic GmbH, Mannhelm, Germany). The ligation of purified samples was carried out using pGEM®-T Easy Vector (Promega Corp., Madison, WI). Plasmids were transformed into competitive *Escherichia coli* cells JM109 cells (Promega Corp.) using the methods of (Sambrook *et al.* 1989). Plasmid DNA was extracted and purified by using the Wizard® Plus SV Minipreps DNA Purification System (Promega Corp). Purified plasmids quantity and quality was measured on Nanodrop (Promega Corp.). Nucleotide sequences were determined at Durham University Sequence Centre (Durham, UK) by using the dideoxy chain termination method. Resulting F-ARISA fragment lengths in each sample were analysed using a Geneious software (Biomatters Ltd, Auckland, New Zealand).

3.3.3. Primers and Probe design

Forward CephF1 (5’-TGATGTCTGAGTACTATATAATAG-3’) and reverse CephR1 (5’-GTTATAATGACGCTCGAA-3’) primers were designed by Sigma-Aldrich Co. LLS. Primer forward corresponded to the region from base 316 to 339 of the sequence (KF053537) and primers reverse to the region from 504 to 522 of the same sequence.
Hydrolysis probe CephP1 (Table 1.) designed by Sigma (described previously) was labelled at the 5’ end with the reporter dye FAM (6-carboxy-fluorescein) and with BHQ-1 dye (Black Hole Quencher 1) at the 3’ end.

The specificity of primers and probe was checked using the National Centre for Biotechnology Information BLAST server. At the time of primer design there were no matching sequences however, over time more sequences were added and the primers no longer gave a signal for Cephalosporium alone. The eyespot fungus, *Tapesia yallundae,* also gave a band with the primers when a conventional PCR was carried. In order to validate the test results, the primer set designed by Klos *et al.* (2012) were used on the extracted samples

For detection of *C. gramineum* using the internal transcribed spacer (ITS) region as a target, primers forward CGF (5′-GTCGTTCGTGTAGGGGCAA-3′) and revers CGR (5′-CTGATCCGAGGTCAACCTGT-3′) were designed. The specificity of the primers used in this assay was checked using the National Center for Biotechnology Information BLAST programme. A 496-bp fragment was amplified as specific region of *Cephalopsorium*. Detection level of genomic DNA occurred at the 100fg/µl.

PCR reaction was carried out in the TProfessional TRIO Thermocycler (Biometra GmbH, Goettingen, Germany) in 25µl reaction volume using GoTaq®Green Master Mix (Cat No. M712,Promega Corporation, Madison, UK). The PCR conditions were 95°C for 2 min; 32 cycles of 95°C for 30 sec, 55°C for 30 sec and 73°C for 1 min; with the final extension of 73°C for 5 min. Primers CGF and CGR were used in concentration of 1200nM. Positive control contained 25ng DNA template where seeds samples were used undiluted. 496-bp PCR products were separated on 1.2% agarose gel stained with GelRed in TBE buffer. AlphaImager HP (Cell Biosciences, Inc., Santa Clara, CA) was used to visual PCR products.

3.3.4. Real-Time PCR Analysis

Amplification reactions were performed in total volume of 25µl and were run on the Agilent MxPro - Mx3000P (Agilent Technologies UK Limited, Stockport, UK) under the following conditions: 1 cycle at 95°C for 4 min followed by 50 cycles 95°C for 20 sec, 56°C for 40 sec; with a final extension at 72°C for 30 sec. The optimum PCR concentrations of primers and probe were experimentally determined for each of the primers forward and reverse (Table 1) at 300nM and hydrolysis probe (Table 1.) at 100nM. The real-time PCR reactions were carried out using Brilliant III Ultra-Fast qPCR Master Mix (Agilent Technologies UK Limited, Stockport, UK), 5µl DNA template (20ng), 30nM ROX reference dye (Agilent Technologies UK Limited, Stockport, UK), sterilised distilled water and primers/probe, as descried previously.
All qPCR reactions were carried out in duplicate for each DNA extract. For negative controls, sterile distilled water was used as no template control. The crossing point value (Cq), which refers

to the cycle number where the sample's fluorescence significantly increases above the background level, was calculated automatically by the MxPro – Mx3000P software (version 4.10) as the first maximum of the second derivative of the curve.

3.3.5. Standard curve preparation and spike test

Standard curves were obtained using five-fold dilution series (from 10ng to 0.128pg) by plotting known amounts of target genomic DNA against Cq values. All standard curve samples were run simultaneously with test samples in each real-time PCR experiment. The amplification efficiency, *E*, was calculated from the slope of the standard curve using the following formula (Bustin *et al*., 2009):

$E = 10^{-1/slope}$

% Efficiency = (E – 1) x 100

Varying amount of fungal genomic DNA (0.01, 0.1 and 1ng) were spiked into a fixed amount of plant genomic DNA (1, 5 and 20ng) and used as template in the qPCR assay. The correlations between Cq values and the concentration of input template was then determinate. For all experiments conducted in this study, standard curves with R2 values >0.98 were obtained.

3.4. Glasshouse Testing of Seed treatment Chemicals

Three winter wheat seed samples with high levels of cephalosporium from the 2010 harvest were treated with 5 different seed treatment chemicals. Sample C21 was cv. KWS Santiago (13.8pg), sample C32 was cv. Relay (37.4 pg) and sample C33 was cv. Horatio (62.6 pg)

Table 3. Seed treatment fungicides used in glasshouse experiment

Active ingredient	Product	Application Rate
Metalaxyl	Anchor	500 mls/100 kg seed
Fludioxonil	Beret Gold	200 mls/100 kg seed
Fluquinconazole	Jockey	150 mls/100 kg seed
Silthiofam	Latitude	200 mls/100 kg seed
Prochloraz + Triticonazole	Kinto	200 mls/100 kg seed

Seeds were sown in 30 cm trays and emerging leaf layers assessed for visual symptoms and then harvested for DNA extraction and *C. gramineum* DNA quantified as described previously.

3.5. Sampling from field experiment

A number of sample points were selected from the trial site in each year, based on the location of the previous seasons plots, in order to test the maximum number of rotation variations in the field oil samples were taken from 1–15 cm and the 16–30 cm. Samples were taken and used for isolation of *C. gramineum* and also extraction of DNA and quantification of *C. gramineum* DNA. The selection of points in the site enabled a number of rotational options to be examined.

Table 4. Rotations at Humbie Trial site 2008–13.

Harvest Year	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**
Commercial field	Wheat	W. Barley	OSR	OSR	W. Wheat	S. Barley
Trial Site One	W. Wheat	W. Wheat	OSR	OSR	W. Wheat	S. Barley
Trial Site Two	W. Wheat	W. Barley	W. Wheat	OSR	W. Wheat	S. Barley
Trial Site Three	W. Wheat	W. Barley	OSR	W. Wheat	W. Wheat	S. Barley

3.5.1. Soil Processing

A corer was used for soil sampling. Samples were stored at 4 °C prior to testing. Samples were dried then sieved through a 2 mm seive to remove all stones and clods. After a thorough mixing, a 60g sub sample was placed in a Retsch Planetary ball mill along with 120 mls sterile CTAB-EDTA buffer (1 litre of buffer; 20g CTAB, 20m mls of 1mM Tris-HCl (pH 8.0), 40 mls of 0.5M EDTA, 280 mls of 5mM NaCl, 30 g of polyvinylpyrrolidone-40, 1.0g of Dithiothreitol and 580 mls of sterile distilled water) and 15 ball bearings. Soil was milled at 300 rpm for 5 minutes then 4 x 1.5 ml aliquots were taken from each sample and placed in individual Eppendorf tubes. (Bowls and balls were cleaned with ethanol and sodium hydroxide before reuse).

3.5.2. DNA Extraction from soil samples

Samples were centrifuged at 6,000 rpm for 5 minutes. Supernatant was removed with a pipette and placed in a fresh 5 ml tube. In the fume hood 0.9 mls chloroform were added prior to vortexing twice and spinning at 13,000 rpm for 4 mins. 0.9 mls of the aqueous upper phase was removed and placed into new tubes. 90 µl of 3M sodium acetate (204.15 grams sodium acetate trihydrate in

500 mls sterile distilled water) and 900 µl isopropanol were added to the tubes. Samples were vortexed and incubated for 1 hour at room temperature. Tubes were then spun at 13,000 rpm for 4 mins. The supernatant was removed with a pipette and the pellet washed by the addition of 150 µl 70% ethanol and centrifuging at 13,000 rpm for 2 mins. The ethanol was removed and the pellet dried for 10 mins before being resuspended in 100 µl of 1 x TE buffer. Samples were vortexed and left over night at 4 °C to dissolve.

3.5.3. DNA purification

Once DNA was fully dissolved the samples were passed through micro Bio-spin columns loaded with 15mm polyvinylpyrrolidone (PVP) to remove humic acids. The columns were primed by the addition of 150 µl HPLC water and then spun at 5,000 rpm for 3 mins. 150 µl of water was then added to each column prior to another centrifugation at 5,000 rpm for 3 mins. The surface of the PVP was roughened with a pipette tip to prevent DNA going down the side of the tube prior to the addition of the total DNA sample. The tubes were spun at 5000 rpm for 4 minutes to elute the DNA. The purified eluate was transferred to a new sterile 0.5 ml eppendorf pipette and stored at -20 °C

DNA was quantified on a nanodrop spectrophotometer and 100 ng added to the reaction mixture in the real time PCR assay described above.

3.6. Seed Testing

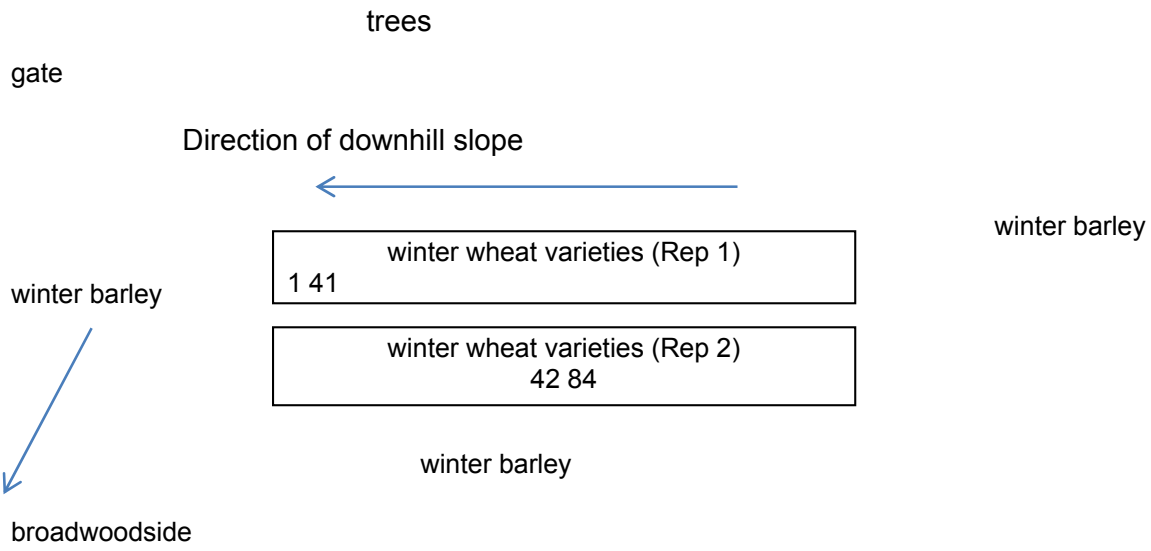
Seed samples from the winter wheat 2009 trial were tested for the presence of *C. gramineum* DNA. 200 seeds were milled into a fine powder and then 1.0 g sub samples were taken and DNA extracted using the C-TAB method described above. DNA in samples was quantified on a nanodrop spectrophotometer and 100ng of template added to the reaction mixture. Samples were obtained for testing from the Scottish Agronomy Winter Wheat Trial which was scored in 2011. In addition, wheat samples from diverse sources were tested in 2011. Extracted DNA was double checked for presence of *C. gramineum* with PCR primers from Klos (2012).

3.7. Soil Analysis

Visual Soil Structure Analysis

A visual survey of the soil structure was carried out in the wheat plots and the Alchemy ploughed and minimum tillage plots in 2010 following the method of Ball *et al.,* 2007.

Field Plan 2010



Aeration was found to be poor in some plots and soil moisture content analysis was carried out on the upper 20 cm of a number of the wheat plots.

4. Results

4.1. Field experiments

Disease information for the winter wheat trials was recorded in AHDB Cereals & Oilseeds log books. In general, highest cephalosporium levels were observed in 2009 and the lowest levels in 2011. Disease levels in the winter barley and winter oats and minimum tillage and ploughed plots are included in the appendix.

4.1.1. Resistance Ratings

Data from the three years of trials at Humbie with the winter wheat Recommended List was used to calculate provisional resistance ratings to cephalosporium leaf stripe (full figures see Appendix). The highest disease incidence recorded was used as a fixed point of 4 and the lowest disease incidence as a fixed maximum resistance of 7.5.

Table 5. Winter wheat varieties from Recommended List with cephalosporium resistance ratings

Variety	Resistance Rating	Variety	Resistance Rating	Variety	Resistance Rating
Denman	4	Gallant	6	Duxford	7
Timber	4	Invicta	6	Gravitas	7
Torch	4	JB-Diego	6	KWS-Gator	7
Crusoe	5	KWS-Solo	6	KWS-Podium	7
Horatio	5	KWS-Target	6	KWS-Santiago	7
Oakley	5	Monterey	6	KWS-Sterling	7
Alchemy	6	Scout	6	Relay	7
Beluga	6	Solstice	6	SY-Epson	7
Cocoon	6	Stigg	6	Tuxedo	7
Conqueror	6	Warrior	6	Viscount	7
Cordiale	6	Claire	7	Chilton	8
Einstein	6	Delphi	7	Grafton	8
				Panorama	8

4.1.2. Winter wheat analysis

Only twenty two varieties were common in all three years of the winter wheat trials. Each trial was analysed independently initially.

Table 6. Summary statistics for variety means for 2009 trial

	GS	Date	Min	Max	Mean	Median
Yield (t/ha)			5.0	7.4	6.4	6.50
Winter hardiness	14	20 Mar 2009	7.0	9.0	8.8	9.0
Septoria	64	11 June 2009	3.5	11.0	6.6	6.0
Septoria	67	25 June 2009	2.5	7.5	4.1	4.0
Cephalosporium	64	11 June 2009	3.0	23.5	12.5	13.0
Cephalosporium	67	25 June 2009	2.5	20.0	9.3	8.5
Cephalosporium	73	15 July 2009	1.0	37.5	12.2	11.5
Flag leaf green area(%)	73	15 July 2009	32.5	96.5	74.5	80.0
F1 green area	73	15 July 2009	22.5	85.0	64.2	70.0
F2 green area	73	15 July 2009	22.5	60.0	23.7	22.5
Head fusarium	73	15 July 2009	0.1	20.0	5.3	4.5
Small tillers(%)	73	15 July 2009	3.5	17.5	9.4	8.5
AUDPC			70.5	652.5	324.9	332.5
AUDPC % of max			0.2	1.9	1.0	1.0

Table 7. Pearson correlations between response variables for variety means in 2009 trial

	Yield (t)	ceph64	ceph67	ceph73	AUDPCT	sept64	sept67
Yield (t)	1.000						
ceph64	-0.462	1.000					
ceph67	-0.355	0.494	1.000				
ceph73	-0.782	0.511	0.366	1.000			
AUDPCT	-0.692	0.771	0.788	0.822	1.000		
sept64	0.290	-0.621	-0.470	-0.360	-0.576	1.000	
sept67	0.293	-0.270	-0.319	-0.171	-0.311	0.414	1.000

ceph is % cephalosporium leaf stripe.

Sept is % septoria disease symptoms.

Figures indicate the growth stage at which assessment took place.

Strong positive interactions were shown between cephalosporium levels and the AUDPC figures. There were strong negative interactions between late season cephalosporium and yield and also between cephalosporium and mid-season septoria.

Table 8. Pearson correlations between response variables for variety means in 2009 trial

	Yield	Flag leaf green	F1 green	F2 green	WH	Headfus 73	Small tillers (%)
Yield (t)	1.000						
Flag leaf green	0.397	1.000					
F1 green	0.424	0.931	1.000				
F2 green	0.365	0.594	0.688	1.000			
WH (t)	0.342	-0.145	-0.070	0.123	1.000		
Headfus 73	-0.734	-0.573	-0.624	-0.448	-0.103	1.000	
Small tillers (%)	-0.537	-0.248	-0.248	-0.070	0.163	0.319	1.000

WH is whiteheads per variety.
Headfus is Fusarium % on the heads.
F1 green is the %green leaf area on the F1 leaf layer, F2 green is % green leaf area on the F2 leaf layer.

Strong negative correlations were detected between head fusarium and the number of small tillers and yield. There was also a strong correlation between head fusarium and late season green leaf area.

Table 9. Pearson correlations between response variables for variety means in 2009 trial

	ceph64	ceph67	ceph73	AUDPC ()	headfus73	Small tillers (%t)	F1 green area (t)
ceph64	1.000						
ceph67	0.494	1.000					
ceph73	0.511	0.366	1.000				
AUDPC	0.771	0.788	0.822	1.000			
headfus73	0.508	0.397	0.919	0.794	1.000		
Small tillers (%/t)	0.197	0.402	0.408	0.449	0.319	1.000	
F1 green area	-0.389	-0.206	-0.678	-0.550	-0.624	-0.248	1.000

ceph is % cephalosporium leaf stripe.
Figures indicate the growth stage at which assessment took place.
Headfus is Fusarium % on the heads

Cephalosporium and head fusarium at GS 73 (positively correlated with each other) were strongly correlated with yield.

Table 10. Summary statistics for variety means for 2010 trial

	GS	Date	Min	Max	Mean	Median
Yield (t/ha)			4.8	7.2	6.4	6.4
Winter hardiness			8.0	9.0	8.9	8.9
Septoria	60	16 June 2010	4.5	9.0	6.1	6.0
Septoria	71+	14 July 2010	2.5	7.0	4.1	4.0
Cephalosporium	32	18 May 2010	0.0	4.5	1.3	1.0
Cephalosporium	60	16 June 2010	1.0	6.5	3.4	3.0
Cephalosporium	65	24 June 2010	3.5	26.5	14.3	15.0
Cephalosporium	71	7 July 2010	0.6	21.5	6.9	5.0
Cephalosporium	71+	14 July 2010	0.0	4.0	1.1	1.0
Flag leaf green area (%)	71	7 July 2010	82.5	98.5	94.5	95.0
F1 green area	71	7 July 2010	32.5	94.0	75.8	77.5
Take-all sev (%)	71	7 July 2010	53.3	71.3	60.7	60.0
Small tillers (%)	71	7 July 2010	0.5	6.5	2.5	2.0
AUDPC			78.9	391.0	212.5	196.2
AUDPC % of max			0.2	1.2	0.6	0.6
Emergence count	24	8 April 2010	20.4	28.6	24.0	24.3
Vigour	24	8 April 2010	5.0	10.0	8.4	8.5

Take all sev is the % of Take all severity.

Table 11. Pearson correlations between response variables for variety means in 2010 trial

	Yield (t)	ceph32	ceph60	ceph65	ceph71	ceph71plus	AUDPCT
Yield (t)	1.000						
ceph32	-0.090	1.000					
ceph60	0.310	0.007	1.000				
ceph65	-0.036	0.062	0.280	1.000			
ceph71	0.064	0.045	0.270	0.541	1.000		
ceph71plus	0.174	-0.163	0.221	-0.062	-0.014	1.000	
AUDPCT	0.116	0.188	0.503	0.607	0.901	0.095	1.000

ceph is % cephalosporium leaf stripe at GS 64.

Figures indicate the growth stage at which assessment took place

The only strong interactions observed here are between individual cepahalosporium assessments and AUDPC figures.

Table 12. Pearson correlations between response variables for variety means in 2010 trial

	Yield (t)	WH (t)	Flag leaf	F1 green area	Take-all	Small tillers (%)	emercnt	vigour
Yield (t)	1.000							
WH (t)	0.089	1.000						
Flag leaf	0.093	-0.147	1.000					

F1 green area	0.006	-0.098	0.513	1.000				
Take-all	0.104	-0.143	0.050	0.060	1.000			
Small tillers (%)	-0.078	0.132	-0.490	-0.579	-0.254	1.000		
emercnt	0.468	0.177	-0.047	-0.072	0.108	0.063	1.000	
vigour	0.224	0.122	-0.188	-0.130	-0.120	0.199	0.279	1.000

WH is the % of whiteheads in each variety.

Emercnt is the emergence count for each variety (i.e. no of seedlings/plot area)

A strong positive correlation was observed between emergence counts and yield and flag and F1 green leaf area. A negative correlation was observed between the percentage of small tillers and green leaf area in the top two leaf layers in the crop.

Table 13. Pearson correlations between response variables for variety means in 2010 trial

	ceph32	ceph60	ceph65	ceph71	ceph71plus	AUDPC (t)	Take-all	Small tiller (%)
ceph32	1.000							
ceph60	0.007	1.000						
ceph65	0.062	0.280	1.000					
ceph71	0.045	0.270	0.541	1.000				
ceph71plus	-0.163	0.221	-0.062	-0.014	1.000			
AUDPC (t)	0.188	0.503	0.607	0.901	0.095	1.000		
Take-all	-0.230	0.033	0.016	-0.130	0.383	-0.172	1.000	
Small tiller %	0.048	0.126	0.458	0.850	-0.109	0.789	-0.254	1.000
emercnt	-0.025	0.033	-0.161	0.036	0.161	0.081	0.108	0.063

ceph is % cephalosporium leaf stripe.

Figures indicate the growth stage at which assessment took place.

Emercnt is the emergence count for each variety (i.e. no of seedlings/plot area)

A very strong positive correlation was observed between the percentage of small tillers and late cephalosporium levels and also between the small tillers and cephalosporium AUDPC figures.

Table 14. Pearson correlations between response variables for variety means in 2010 trial

	ceph32	ceph60	ceph65	ceph71	ceph71plus	AUDPCT	Flag leaf	F1 green area
ceph32	1.000							
ceph60	0.007	1.000						
ceph65	0.062	0.280	1.000					
ceph71	0.045	0.270	0.541	1.000				
ceph71plus	-0.163	0.221	-0.062	-0.014	1.000			
AUDPC	0.188	0.503	0.607	0.901	0.095	1.000		
Flag leaf	-0.276	0.118	-0.433	-0.417	0.178	-0.419	1.000	
F1 green area	-0.072	-0.056	-0.443	-0.498	0.164	-0.484	0.513	1.000
vigour	0.067	0.270	-0.115	0.197	0.119	0.209	-0.188	-0.130

ceph is % cephalosporium leaf stripe.

Figures indicate the growth stage at which assessment took place.

In 2010 yield was reasonably correlated with emergence counts but not with cephalosporium. However, stepwise regression for yield fits emergence count and then cephalosporium at GS 60. Nevertheless, the percentage variance accounted for in yield by these two terms together was only 27%. In 2010 both F1 green and flag leaf at GS 71 were reasonably negatively correlated with cephalosporium at the same GS of 71.

Table 15. Summary statistics for variety means for 2011 trial

Variate	GS	Date	Min	Max	Mean	Median
Yield (t/ha)			7.2	8.6	10.5	8.6
Septoria	37	19 May 2011	5.0	11.0	7.6	7.3
Septoria	60	16 June 2011	4.5	14.5	7.7	7.5
Cephalosporium	37	19 May 2011	0.0	2.5	0.6	0.3
Cephalosporium	60	16 June 2011	1.5	11.0	3.3	2.5
Mildew	37	19 May 2011	0.0	0.3	0.01	0.0
Mildew	60	16 June 2011	0.0	0.0	0.0	0.0
AUDPC			20.3	155.2	52.3	43.9
AUDPC % of max			0.6	0.5	0.2	0.1

For 2011 mildew was only found in one plot (0.5%) at the first assessment date and was not found at all at the second assessment date.

In the 2011 trials DM% for Cocoon were 63.2% and 65.35% for reps 1 and 2, respectively. This was very low indeed but consistent across replicates.

Table 16. Pearson correlations between response variables for variety means in 2011 trial

	Yield (t)	sept37	sept60	ceph37	ceph60	AUDPCT
Yield (t)	1.000					
sept37	0.076	1.000				
sept60	0.046	0.022	1.000			
ceph37	0.110	-0.180	-0.090	1.000		
ceph60	-0.059	0.021	-0.444	0.273	1.000	
AUDPCT	-0.014	-0.042	-0.410	0.568	0.947	1.000

ceph is % cephalosporium leaf stripe.

Sept is % septoria disease symptoms.

Figures indicate the growth stage at which assessment took place.

No associations between yield and disease were found in the 2011 trial. AUDPC for cephalosporium correlated with cephalosporium disease levels at each assessment and especially at GS 60 but this might be expected as AUDPC is calculated from observed disease levels. Septoria and cephalosporium at GS 60 were negatively correlated. However, septoria at GS 37 and GS 60 are not correlated with each other.

4.1.3. Winter barley analysis

In general, cephalosporium levels were lower in the winter barley than winter wheat. Levels of cephalosporium were low in 2010. No correlation was observed between emergence, vigour and early season cephalosporium.

Winter barley trials were only yielded in 2011. Analysis of the impact of cephalosporium and other foliar diseases on winter barley yields showed that strongest interaction was a negative interaction between mid-season cephalosporium and yield. There was also a positive correlation between late season rhynchosporium and mid-season mildew.

Table 17. Summary statistics for Winter Barley variety means for 2011 trial

	GS	Date	Min	Max	Mean	Median
Yield (t/ha)			5.9	8.4	7.3	7.2
Cephalosporium	57	19 May 2011	0.0	1.0	0.2	0.0
Cephalosporium	76	16 June 2011	0.0	3.5	0.6	0.5
Mildew	57	19 May 2011	0.0	2.5	0.6	0.5
Mildew	76	16 June 2011	0.5	6.0	2.7	2.5
Rhynchosporium	57	19 May 2011	0.0	3.0	0.8	0.5
Rhynchosporium	76	16 June 2011	0.0	5.0	1.8	2.0
Ramularia	76	16 June 2011	0.0	0.0	0.0	0.0
AUDPC			0.0	49.0	11.5	7.0
AUDPC % of max			0.0	1.8	0.4	1.5

Table 18. Pearson correlations between response variables for variety means in 2011 trial

	Yield	ceph57	ceph76	cephaudpct	rhyncho57	rhyncho76
Yield	1.000					
ceph57	-0.510	1.000				
ceph76	-0.224	0.149	1.000			
cephaudpct	-0.371	0.468	0.943	1.000		
rhyncho57	-0.001	0.063	0.120	0.128	1.000	
rhyncho76	-0.205	0.176	0.005	0.063	0.269	1.000

ceph is % cephalosporium leaf stripe.
rhyncho is % rhynchosporium disease symptoms.
igures indicate the growth stage at which assessment took place

Yield was negatively correlated to cephalosporium levels at GS 57, although symptom levels in the trial were very low. Cephalosporium levels at GS 76 were positively correlated to the ceph AUDPC.

4.1.4. Winter oat analysis

Levels of cephalosporium were very low during the trials. Levels reached a maximum of just over 2 % in 2010 but symptoms were only recorded on 3 varieties early in the growing season. As with winter barley there was no correlation between early cephalosoporium and emergence. In 2011 leaf stripe was observed in 5 varieties but again levels did not increase above 2% in any variety.

4.1.5. Minimum tillage analysis

Conflicting results were produced by the minimum tillage vs ploughed experiments over 2 seasons. In 2010 cephalosporium levels were much higher in the ploughed plots compared to the min till plots (GS 61 ploughed 19% min till 13.5 %, GS 75 ploughed 12% min till 2.5 %). In contrast, results from 2011 (a lower disease year) showed disease levels were twice as high in the min till plots (GS 60 ploughed 3% min till 6.6 %). These differences were not statistically different. Despite the higher disease levels in the ploughed plots in 2011, crop yield levels were still higher in the ploughed plots (8.9 t/ha compared to 5.8 t/ha). In this year the ploughed and minimum tillage plots were in separate banks in the field plot layout and this may have affected disease levels and yield results.

4.2. Sensitivity assays

A number of isolates were successfully isolated from plant material in 2009. 20 isolates were produced from the winter wheat but an equal number were produced from the winter barley crop surrounding the trial plots. Isolates were tested for their sensitivity to the major fungicides used in seed treatments.

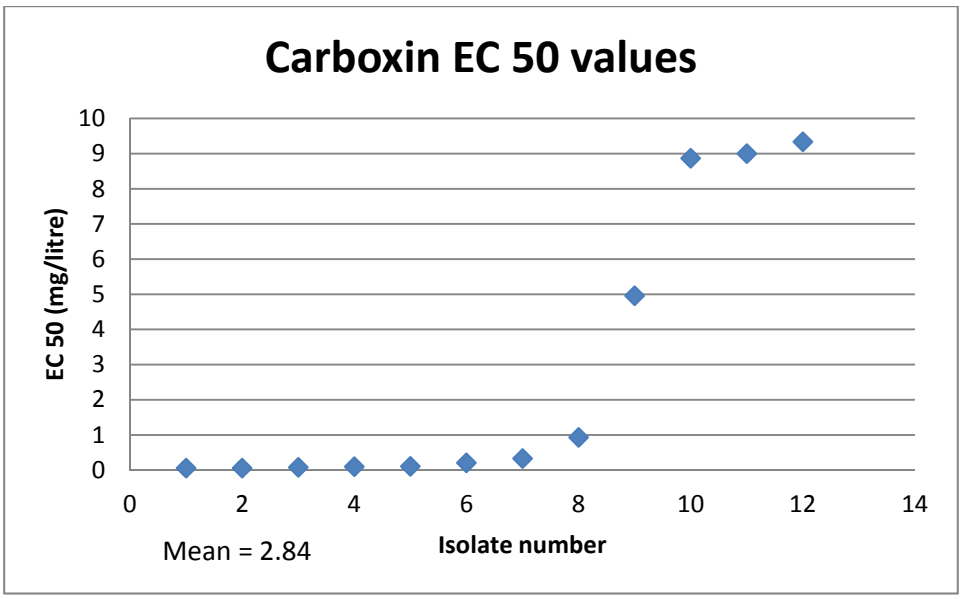


Figure 7. Carboxin sensitivity – Scottish isolates 2009

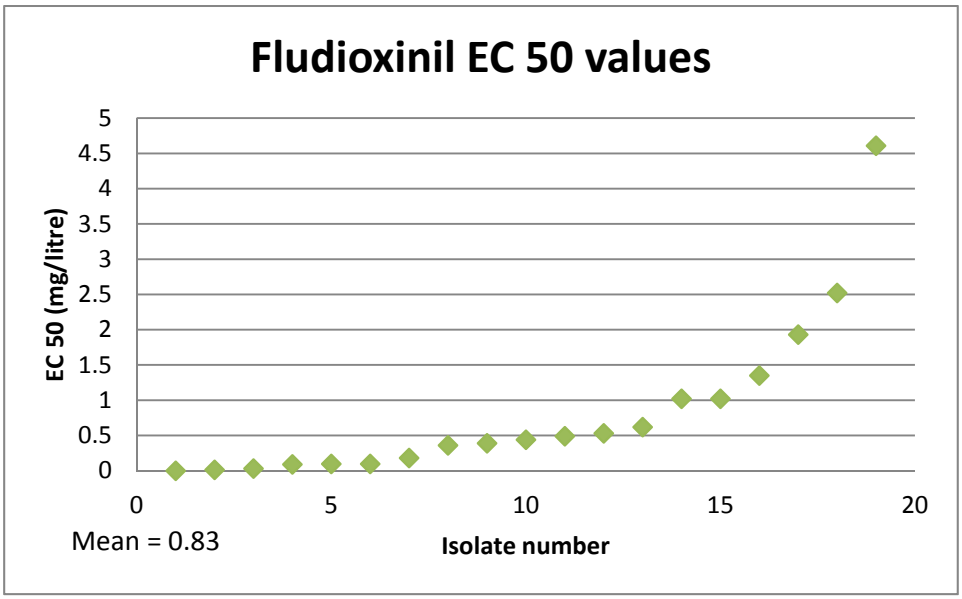


Figure 8. Fludioxonil sensitivity – Scottish isolates 2009

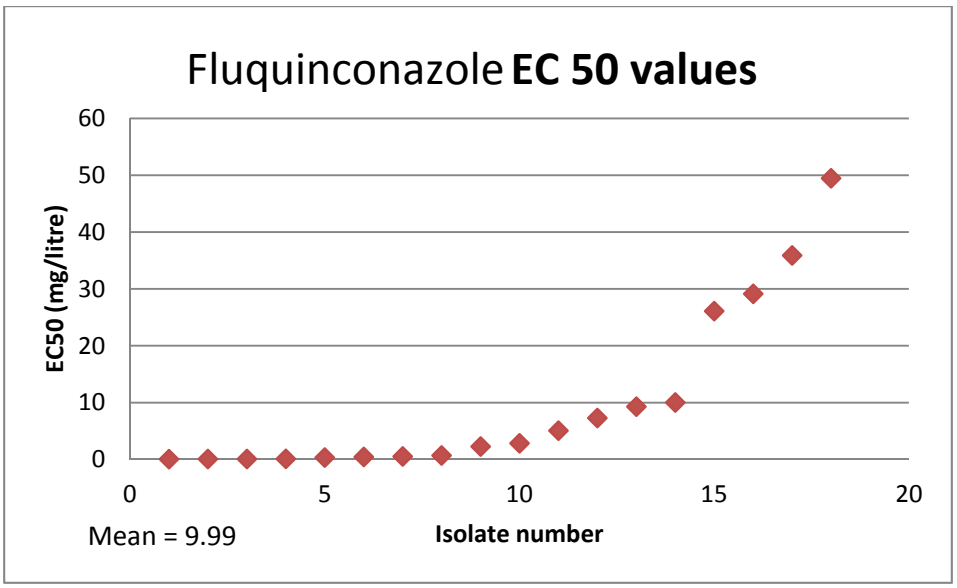


Figure 9. Fluquinconazole sensitivity – Scottish isolates 2009

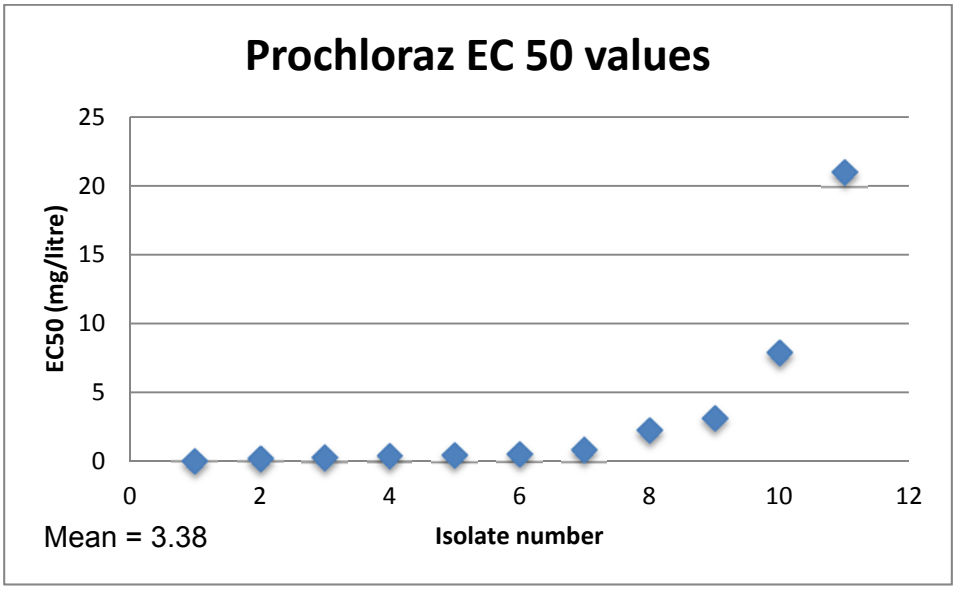


Figure 10. Prochloraz sensitivity – Scottish isolates 2009

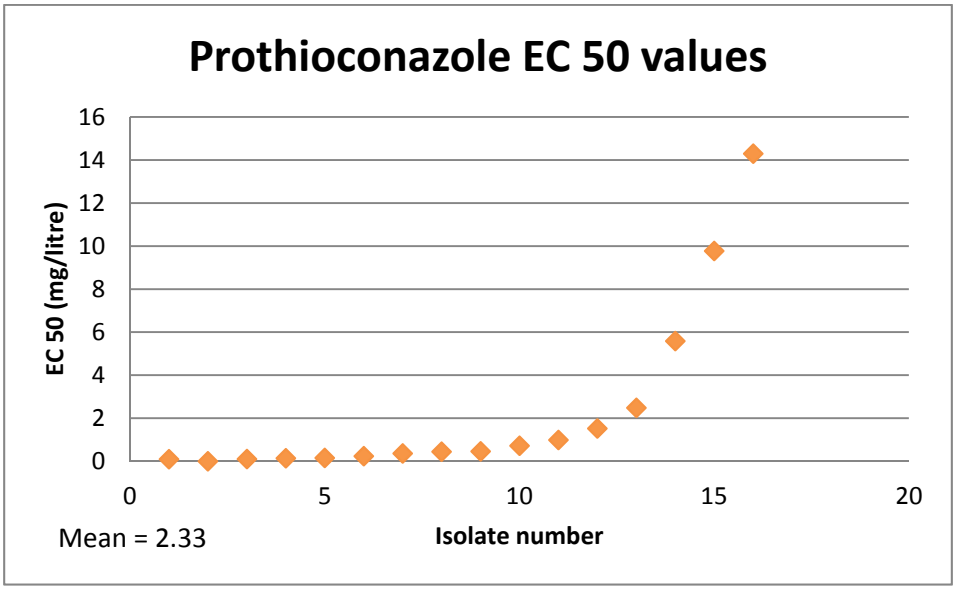


Figure 11. Prothioconazole sensitivity – Scottish isolates 2009

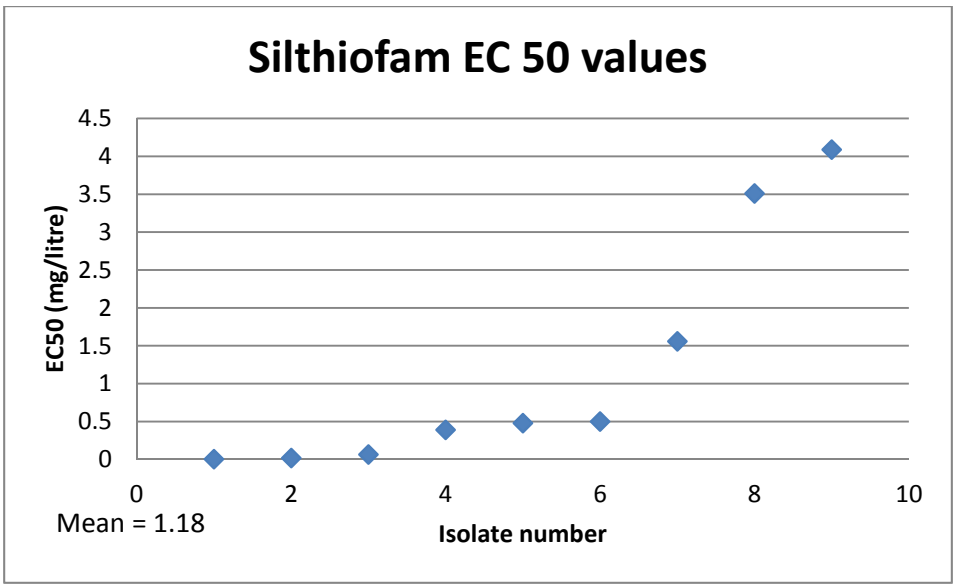


Figure 12. Silthiofam sensitivity – Scottish isolates 2009

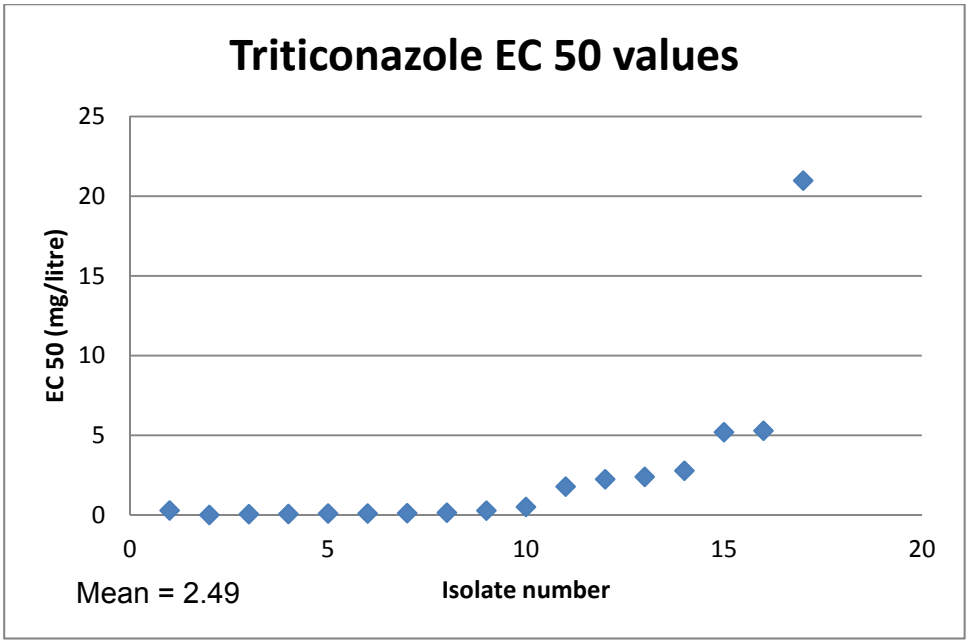


Figure 13. Triticonazole sensitivity – Scottish isolates 2009

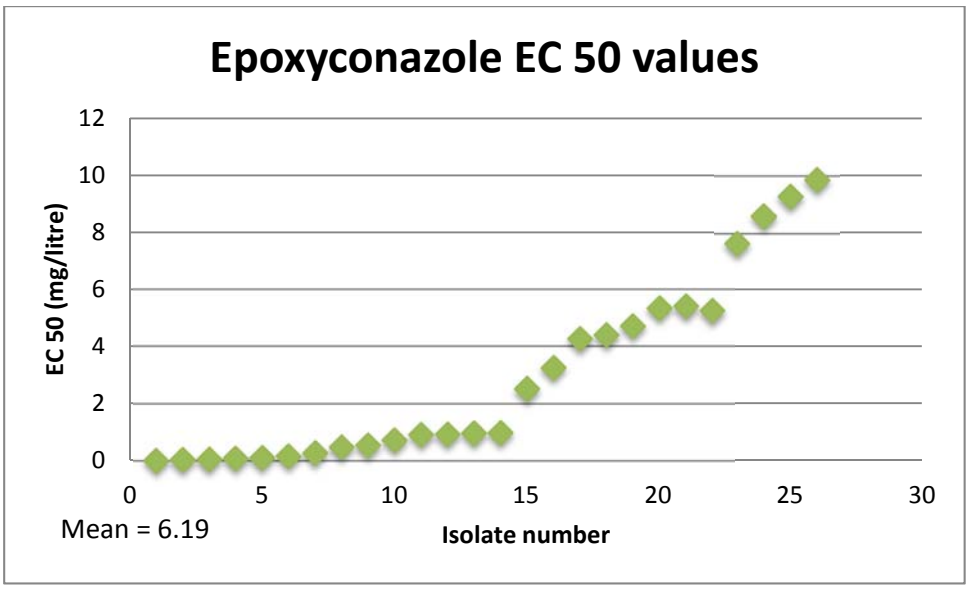


Figure 14. Epoxiconazole sensitivity – Scottish isolates 2009

Only a limited number of the isolates tested gave EC50 values with the Mars software. The results can be summarised in a table.

Table 19. Sensitivity of *C. gramineum* isolates to the major seed treatment chemicals

Active Ingredient	Seed Treatment	EC_{50} Range (ppm)	EC_{50} Mean (ppm)
Carboxin	Anchor ®	0.06 – 9.34	2.84
Fludioxonill	Maxim XL ®	0.001 – 4.61	0.83
Fluquiconazole	Jockey ®	0.047 -49.5	9.99
Epoxiconazole	Tracker ®	0.002 – 9.86	2.94
Prochloraz	Kinto ®	0.01 – 21.04	3.38
Prothioconazole	Redigo ®	0.099 -14.3	2.33
Silthiofam	Latitude ®	0.005 – 4.09	1.18
Triticonazole	Kinto ®	0.29 – 20.98	2.49

4.3. Cephalosporium detection by DNA

Harvested seed from the 2009 harvest was tested for the presence of cephalosporium DNA. Results are shown below. Fungal DNA was detected in the seed of all the varieties grown in 2009.

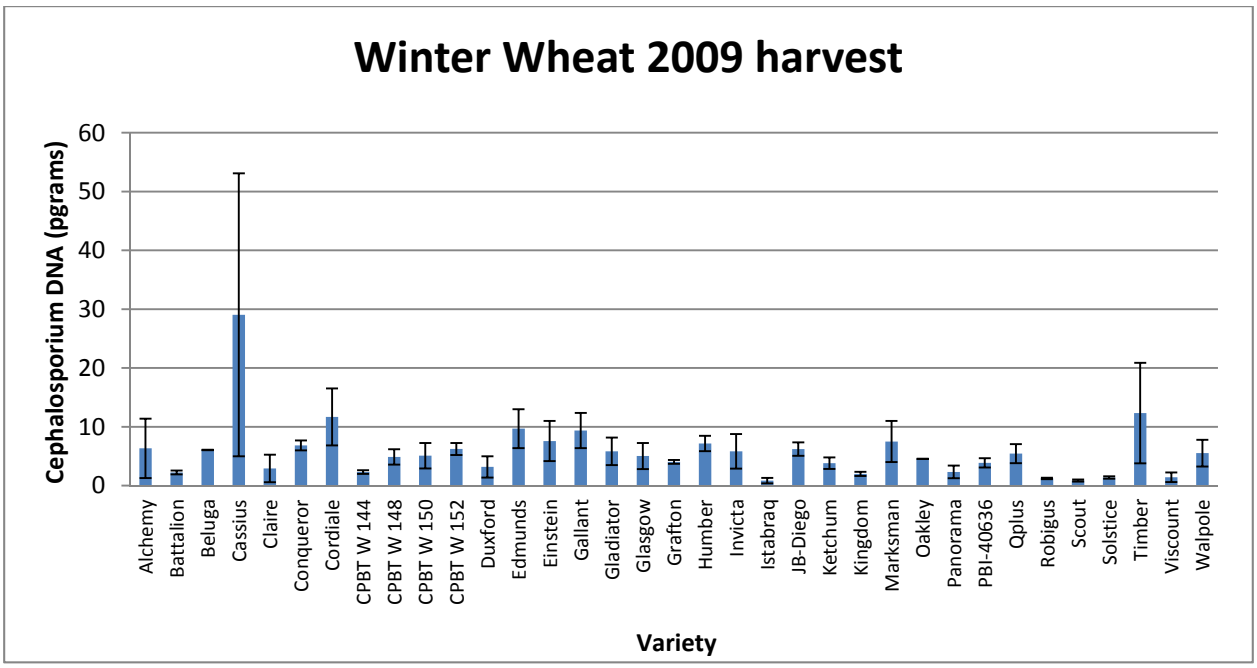


Figure 15. Cephalosporium DNA levels in harvested grain in 2009. Error bars indicate standard error from the mean.

Seed levels ranged from 29 to 0.86 pgrams. Cassius had the highest levels with Scout the lowest.

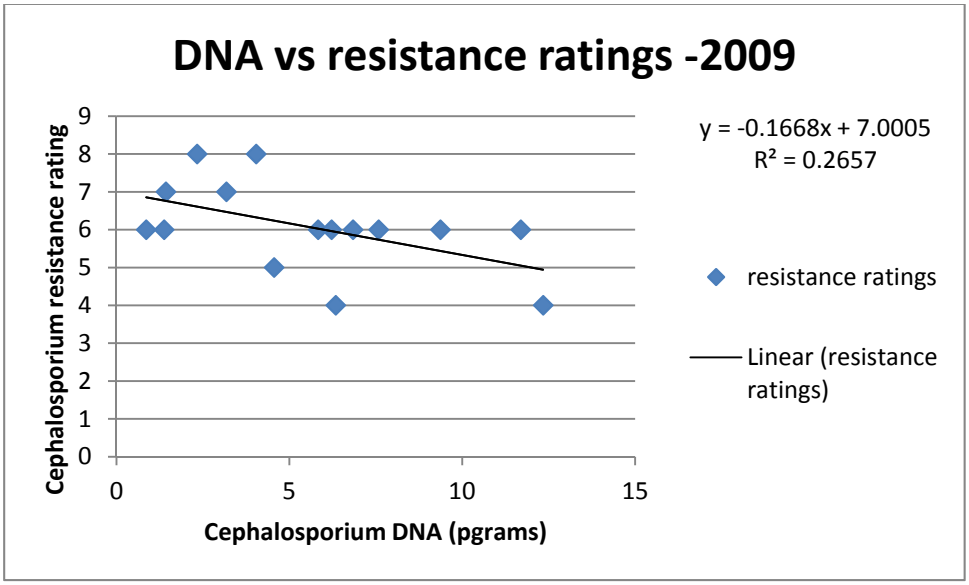


Figure 16. Cephalosporium Resistance ratings vs DNA.

Cephalosporium DNA levels were correlated with resistance ratings and a small correlation observed.

In addition, harvest samples were collected from the winter wheat RL trial at the Scottish Agronomy site in Fife in 2010 and tested for the presence of *C. gramineum*.

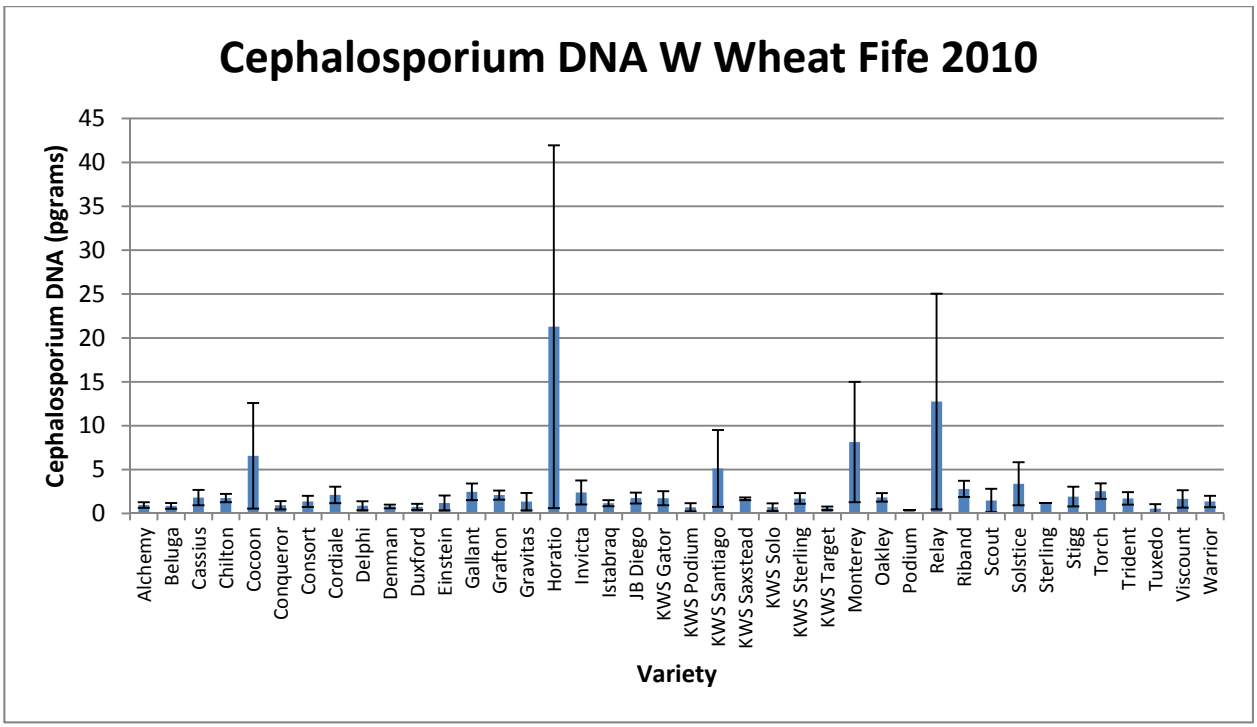


Figure 17. Cephalosporium DNA in harvested grain from winter wheat trial (Fife 2010) Error bars indicate standard error from the mean.

DNA levels ranged from 21 to 0.76 pgrams. The variety Horatio produced the highest levels with levels lowest in Duxford.

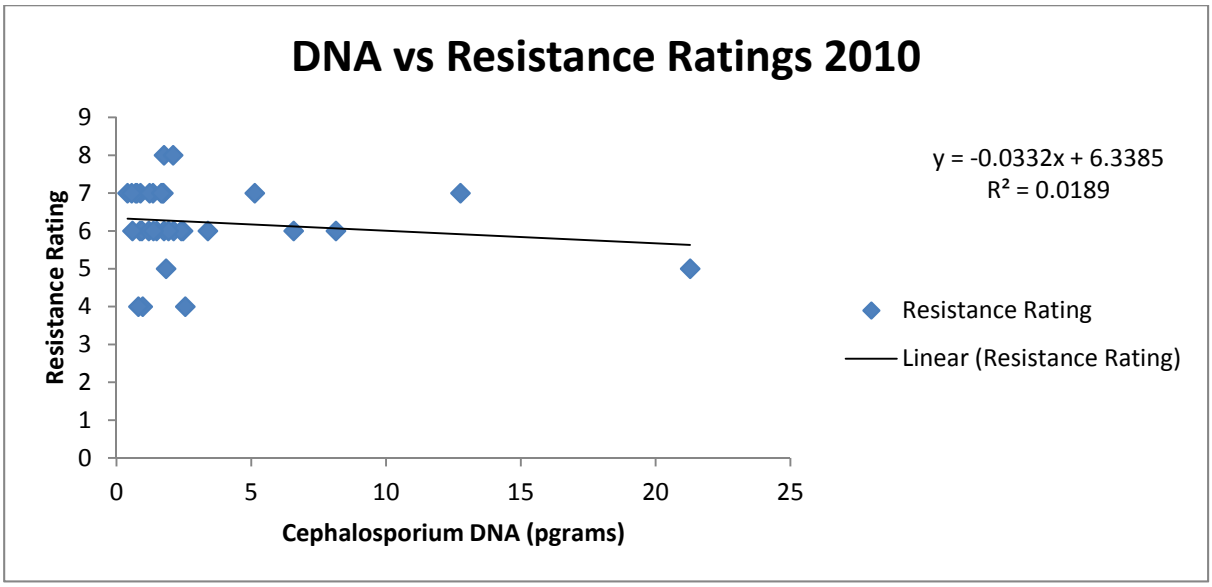


Figure 18. Cephalosporium Resistance Ratings vs DNA

No correlation was observed between resistance ratings and DNA levels in this trial.

4.4. Glasshouse testing of seed chemicals on *Cephalosporium gramineum*

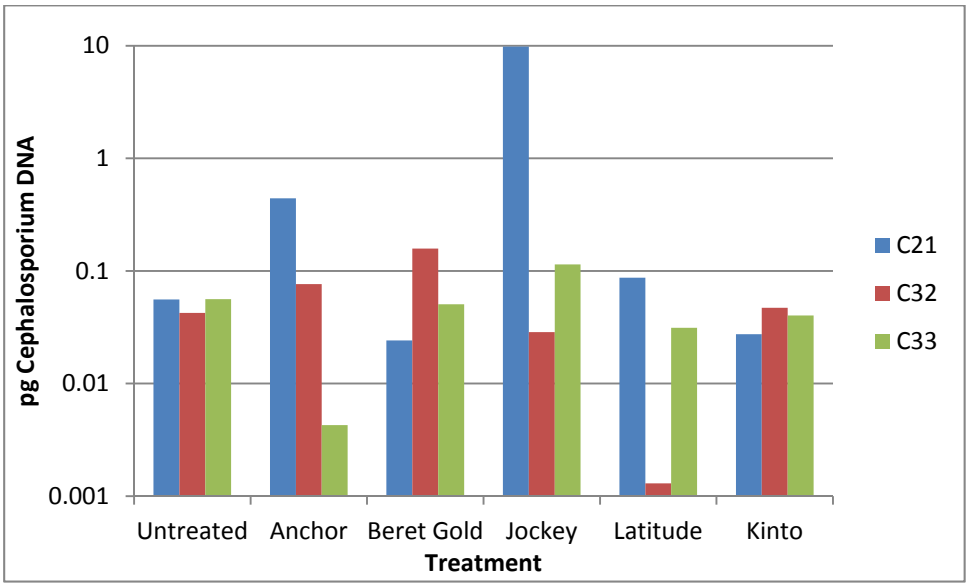


Figure 20. Cephalosporium DNA levels in leaf tissue (glasshouse experiment)

The results indicated that no consistent control of cephalosporium could be obtained by the seed treatments. Although Anchor and Latitude did give significant reductions in C33 (Horatio) and C32 (Relay), respectively, this pattern of control was not consistent. However, problems with the qPCR assay means that other fungi may be affecting the results.

4.5. Soil Testing

DNA was extracted from the soil samples collected from the trials in each year. Initial DNA quality was not of sufficient standard for qPCR assays. The protocol was refined and alternative extraction methods were also run in comparison. Good quality DNA was produced from all of the soil samples but when they were run through the qPCR, no cephalosporium levels above the minimum 0.13 pg threshold were recorded.

4.6 Seed Testing

Winter wheat samples from diverse geographical locations were obtained from the Official Seed Testing Station in Edinburgh in 2011.

As previously discussed, problems with the specificity of the qPCR were encountered. In order to check for the presence of *C. gramineum*, a conventional PCR assay was carried out on a selection of the harvested samples.

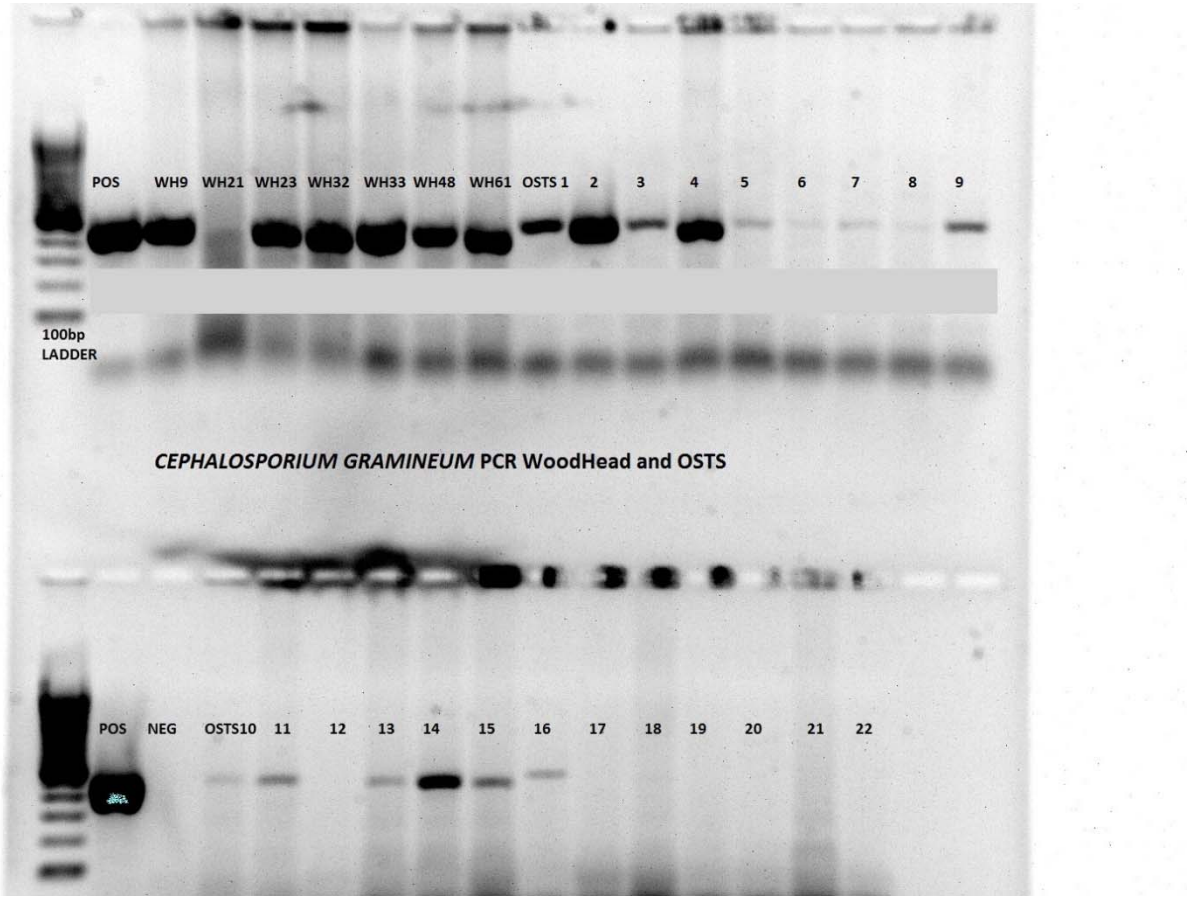


Figure 19. Gel from conventional PCR test (samples which gave positive detection with qPCR).

Name	Amount of Ceph DNA (pg) per 100ng of sample tested	Name	Amount of Ceph DNA (pg) per 100ng of sample tested
WH 9	3.48	OSTS9	0.9
WH 21	53.1	OSTS10	0.61
WH 23	4.65	OSTS11	0.61
WH 32	0.56	OSTS12	0.04
WH 33	1.04	OSTS13	0.56
WH 48	3.09	OSTS14	0.24
WH 61	11.38	OSTS15	0.51
OSTS1	3.93	OSTS16	0.73
OSTS2	4.05	OSTS17	0.03
OSTS3	2.32	OSTS18	0.04
OSTS4	3.12	OSTS19	0.01
OSTS5	0.72	OSTS20	0
OSTS6	4.13	OSTS21	0.01
OSTS7	1.7	OSTS22	0
OSTS8	1.02		

Table 20. qPCR values for retested seed samples.

All of the samples from the RL Trial tested positive for *C. gramineum* with the exception of WH21. The lane on the gel appeared smeared indicating DNA degradation may have taken place. All of the official seed testing station (OSTS) samples which had qPCR levels above 0.04pg gave a band on the gel indicating the pathogen is present. The presence of the fungus in the samples confirms the possibility of the pathogen spreading in seed.

4.7 Soil analysis

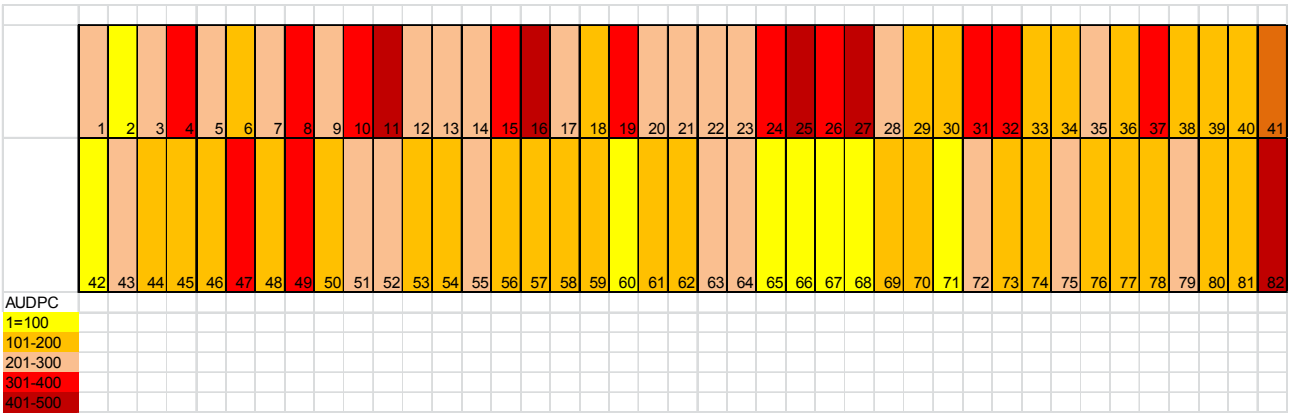


Figure 21. Cephalosporium AUDPC in winter wheat trial, Humbie 2010

Cephalosporium AUDPC levels were much higher in Rep 1 compared to Rep 2. Highest values were in Plot 25 and 27 (Q-Plus and CPBT-W160).

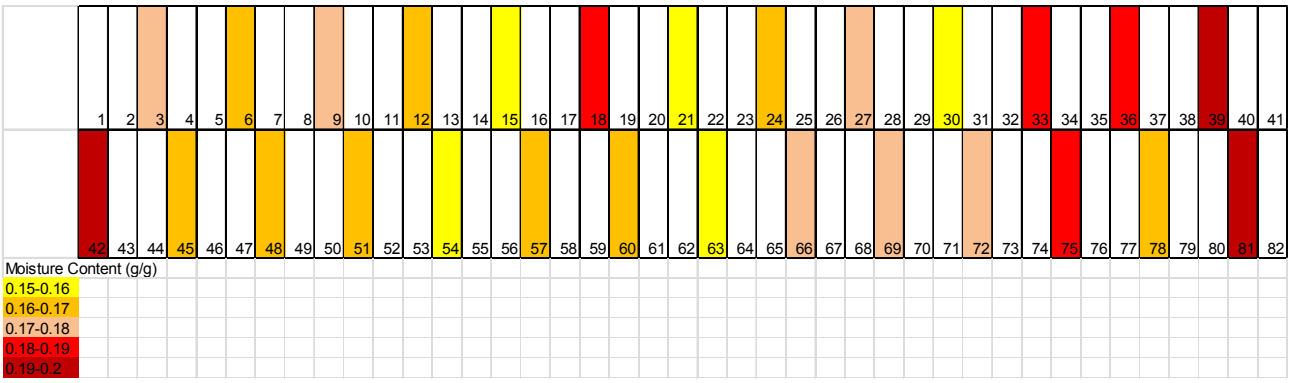


Figure 22. Soil moisture content in winter wheat trial, Humbie 2010

The highest moisture content was recorded in plots 39, 42 and 81 (JB-Diego, Kingdom and Duxford).

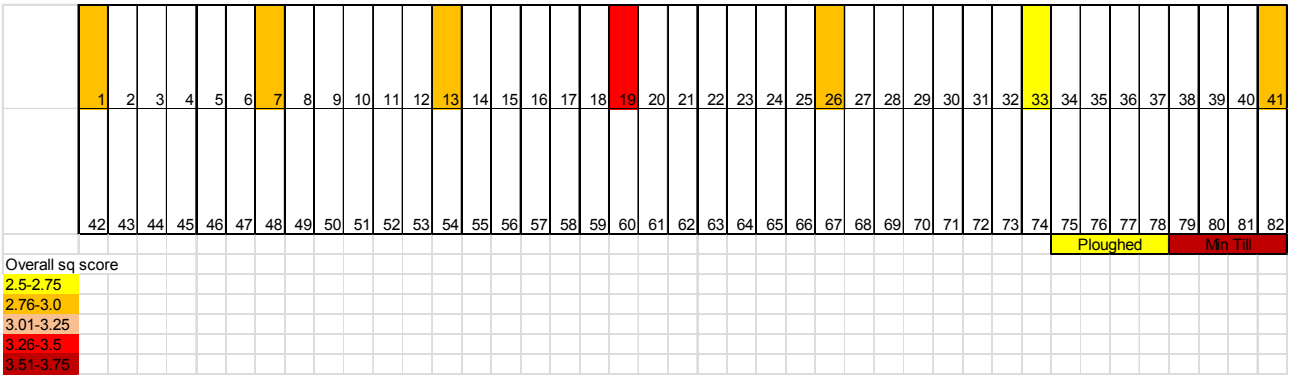


Figure 23. Soil Quality Assessment in winter wheat trial, Humbie 2010

The lowest soil structure quality scores were recorded in Plot 19 and in the minimum tillage plots.

Structure quality	Size and appearance of aggregates	Visible porosity and Roots	Appearance after break-up: various soils	Appearance after break-up: same soil different tillage	Distinguishing feature	Appearance and description of natural or reduced fragment of ~ 1.5 cm diameter
Sq1 Friable Aggregates readily crumble with fingers	Mostly < 6 mm after crumbling	Highly porous Roots throughout the soil			Fine aggregates	The action of breaking the block is enough to reveal them. Large aggregates are composed of smaller ones, held by roots.
Sq2 Intact Aggregates easy to break with one hand	A mixture of porous, rounded aggregates from 2mm - 7 cm. No clods present	Most aggregates are porous Roots throughout the soil			High aggregate porosity	Aggregates when obtained are rounded, very fragile, crumble very easily and are highly porous.
Sq3 Firm Most aggregates break with one hand	A mixture of porous aggregates from 2mm -10 cm; less than 30% are <1 cm. Some angular, non-porous aggregates (clods) may be present	Macropores and cracks present. Porosity and roots both within aggregates.			Low aggregate porosity	Aggregate fragments are fairly easy to obtain. They have few visible pores and are rounded. Roots usually grow through the aggregates.
Sq4 Compact Requires considerable effort to break aggregates with one hand	Mostly large > 10 cm and sub-angular non-porous; horizontal/platy also possible; less than 30% are <7 cm	Few macropores and cracks All roots are clustered in macropores and around aggregates			Distinct macropores	Aggregate fragments are easy to obtain when soil is wet, in cube shapes which are very sharp-edged and show cracks internally.
Sq5 Very compact Difficult to break up	Mostly large > 10 cm, very few < 7 cm, angular and non-porous	Very low porosity. Macropores may be present. May contain anaerobic zones. Few roots, if any, and restricted to cracks			Grey-blue colour	Aggregate fragments are easy to obtain when soil is wet, although considerable force may be needed. No pores or cracks are visible usually.

Figure 24. Visual assessment scheme for soil (Ball *et al*., 2007)

Mapping of the winter wheat RL plots in 2010 was aimed at establishing a pattern across the field. AUDPC figures indicated a higher range of values in the upper bank of varieties (Rep 1). However, soil moisture figures indicated higher moisture in the upper plots in Rep 1. The visual assessment of soil structure indicated variability across the plots. Soil structure was poorest in Plot 19 (Rep 1), which also had a high cephalosporium AUDPC value.

5. Discussion

The results from three years of field trials with the winter wheat RL varieties has indicated that quantifiable differences exist between varieties, in terms of resistance to cephalosporium disease symptoms. Differences in susceptibility to cephalosporium leaf stripe in winter wheat have been known since the 1950s (Breuhl, 1957). Resistance has been attributed to genotypic variation within the wheat germplasm in slowing the movement of the fungus within the plant and in reducing the entry via roots (Mathre and Morton, 1990). There is no information available on the mechanisms by which the resistance in UK wheat varieties is operating and this is worthy of further investigation. Recent work in the USA and South America has looked at the impact of the *Compactum* gene and other associated QTLs in conferring resistance to cephalosporium (Quinkce, 2009). No such study has been undertaken on UK wheat varieties. Nevertheless the production of resistance ratings from field data will allow growers using continuous or short rotations to select a variety with increased resistance to cephalosporium leaf stripe. With an effective range of 8 to 4 (HGCA, 2013), the current recommended list does offer a degree of choice to growers.

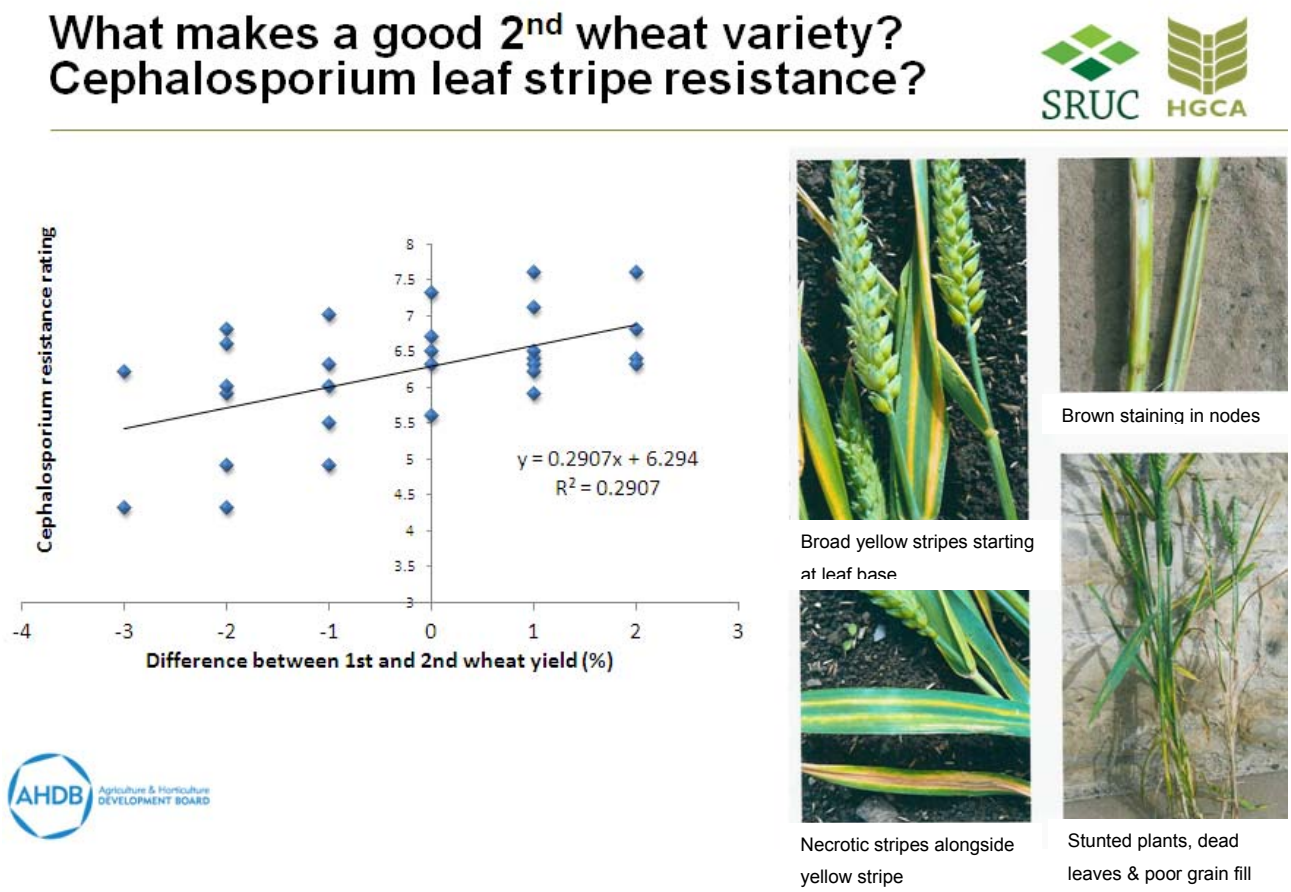


Figure 25. Relationship between leaf stripe resistance and yield difference (first and second wheats).

Information from the trials was also used to assess the value of second wheats in rotation. This slide was produced by Dr Simon Oxley and plots yield differences between first and second wheats and cephalosporium resistance ratings. The data appears to indicate that a relationship does exist between the two factors and that despite cephalosporium not being reported in trials, an increased susceptibility to cephalosporium in second wheat can lead to a yield loss. Other factors, such as take all resistance, will also impact on second wheat yield. A comparative study on the effect to soil health of continuous and short rotations would be useful for growers.

Analysis of the 2009 winter wheat trial results shows a non-surprising strong positive interaction between cephalosporium levels and the AUDPC figures. There were strong negative interactions between late season cephalosporium and yield and also between cephalosporium and mid-season septoria. The effect of cephalosporium on yield has been studied in a number of countries with yield losses quoted as high as 80% in extreme cases (Quincke *et al.,* 2012). In inoculated trials, using a range of susceptible and resistant varieties, the fungus reduced yield by 41% and also affected grain weight, grain diameter and grain protein (Quincke *et al.,* 2012). In these trials resistant and susceptible varieties performed similarly in respect to yield at two sites, while

intermediate varieties were more inconsistent. In the 2009 trial at Humbie yield loss due to cephalosporium was strongly correlated to disease severity in the plots.

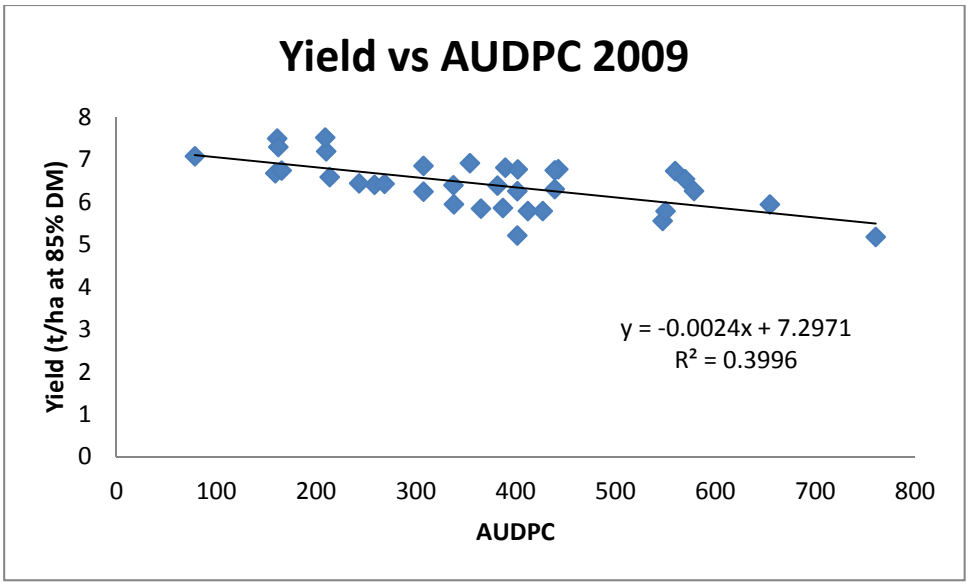


Figure 26. Relationship between yield and Cephalosporium DNA, Humbie 2009

This correlation was not as strong in following years. Yield was correlated to late season green leaf area in 2010. Previous research in cephalosporium has shown that infection of the crop does not lead to a reduced number of heads but rather a reduced number of grains/ear and grain size (Johnston and Mathre, 1972). In the 2009 trial there was a significant interaction between small tillers and final yield. The interactions in the first year of wheat trials were not repeated in later years when disease levels were much lower.

One year of yielded trials with winter barley was not sufficient to establish a relationship between disease symptoms and yield loss. However, there was a negative interaction between mid-season disease levels and yield (Table 18). Interactions between cephalosporium and the other major barley diseases were seen in 2011 but disease levels in both years were low. In 2009, the winter barley crop surrounding the winter wheat plots was badly affected by cephalosporium. The high disease levels observed may just reflect the high disease levels seen in that cropping season. Cephalosporium leaf stripe has been observed in barley fields grown with no rotation in Scotland (F Burnett, pers. comm.). Cephalosporium levels were even lower in oats than barley, with no variety giving a score above 2%. In conclusion, the fungus is much less of a problem in barley and oats than wheat. This confirms findings from Europe that indicated a variation in susceptibility to cephalosporium in different graminaceous species e.g. triticale is more susceptible than wheat and rye (Martyniuk *et al.,* 1995).

The results from the minimum tillage trials in this project are inconclusive. Evidence from previous work suggests that minimum tillage plots would have greater trash levels than ploughed plots and

that this should in turn lead to higher disease levels (Christian *et al*,. 1983). However, only in 2011 did the minimum tillage plots have more disease. The reasons for this are not known. Inoculum can survive in undisturbed trash for 3 years or on buried crop residue in soil for 0.5 to 2.5 weeks at 23 °C and a few months if the soil is dry at 7 °C (Weise and Ravenscroft, 1975). Little is known about fungal survival at cooler temperatures and much wetter conditions. This area requires much further investigation. Previous work has shown that deep ploughing for 3 years was as effective as straw burning in reducing cephalosporium leaf stripe (Bockus *et al.,* 1983).

The fungus is not considered to be controlled by fungicides and control relies on cultural methods. However the sensitivity assays carried out on pure fungal colonies indicates that the major fungicides used in seed treatments do have an inhibitory effect on fungal growth (Table 19). Mean EC 50 values were in the range of 0.83 to 9.9 ppm. Graphs for each fungicide indicate a range in susceptibility. Early work on infection of wheat by isolates of *C. gramineum* indicated that race structure may exist for *C. gramineum* (Van Wert *at al.,* 1984). More recently Baaj *et al.* (2011) studied variation in isolates from Japan, USA and Europe. They discovered at least four genotypes within the isolates tested and were able to develop genotype specific markers. The fungicide tests carried out in this project also appear to suggest variation within UK population. However, further testing of isolates used in this report would be required. The effect of seed treatment fungicides on *C. gramineum* levels in emerging plants were not conclusive (Figure 13). Testing of whole plants and roots rather than leaf layers may have given different results. This is an area which would benefit from further investigation.

Leaf samples collected from infected trial sites were visually scored and then *C. gramineum* DNA quantified. No clear relationship was established between fungal DNA and leaf infection levels (data not shown). The position of the fungus in plant xylem tissue in the stem and leaves may have accounted for this result. A clearer interaction may have been observed between the levels of *C. graminuem* in whole plants and disease symptoms.

Soil samples were taken during the course of the project to attempt to quantify the effect of rotation on *C. gramineum* levels in the soil. The soils were stored at 4 °C as the diagnostic assay was developed and validated. However subsequent testing of the soil samples indicated no *C. gramineum* DNA in the soil. The half-life of the fungus in the soil in Scottish conditions appears to be relatively short. This experiment should have been repeated with trash samples collected as well as soil and DNA extraction from soil should have been carried out immediately. DNA could then have been stored at -80 °C for later analysis. Survival limits for *C. gramineum* are described above. Survivability is also related to soil pH and matric potential (Specht and Murray, 1989). The effect of previous crops, buried trash, rainfall and drainage on these two factors will have been

considerable. No liming was carried out during the course of this project. The fungus is known to sporulate and survive better at soil pH 4.7 (Specht and Murray, 1989).

The fungus is known to be seed borne in wheat (Murray, 2006). Although isolation levels remain low and symptom appearance in sown wheat is less than 1%, this stage could still provide a method for the fungus to survive adverse climatic conditions and slowly build up levels (Murray, 2006). The results from the testing of seed samples from the wheat trials in this project indicate a wide range of *C. gramineum* DNA levels in harvested seed, 29 to 0.86 pgrams (Figure 9). There was only a minor correlation between DNA levels and resistance ratings (r^2 = 0.27, Figure 10). DNA levels were similar in the Scottish Agronomy trial (Figure 11) but here there was less of a correlation with resistance ratings (Figure 12). The mechanisms involved in resistance to *C. gramineum* in wheat are only slowly being elucidated. Research has indicated that two types of resistance are present (Mathre and Morton, 1980). The first type involves pathogen exclusion and is measured in a reduction of infected plants. The second type is restricted pathogen movement after initial colonization and is measured by a reduction in rate and severity of disease symptoms. The production of a reliable qPCR assay for *C. gramineum* offers the opportunity to study the resistance mechanisms in UK winter wheat varieties in more detail.

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7. Appendices

Appendix One

Example of trial management

Agchem details for trial site 2009–10

Application date	Product name	Type (e.g. fungicide)	Rate	Crop growth stage
06.oct.09	PK	fertiliser	70 kg	4
14.0ct.09	Baccara	herbicide	1.0l/ha	8
15.mar.10	Nitram	fertiliser	70kg	22
19.mar.10	Nitram	fertiliser	120 kg	25
22.apr.10	5c cycocel	growth regulator	1.25 l/ha	30
22.apr.10	Talius	fungicide	0.15l/ha	30
22.apr.10	Opus	fungicide	0.5 l/ha	30
11.may.10	Tracker	fungicide	1.5 l/ha	32
11.may.10	BRAVO	fungicide	1 l/ha	32
11.may.10	Flexity	fungicide	0.5 l/ha	32
31.05.10	BRAVO	fungicide	1.0l/ha	39
31.05.10	Talius	fungicide	0.15l/ha	39
31.05.10	Opus	fungicide	0.5l/ha	39
31.05.10	Comet	fungicide	0.75l/ha	39
21.06.10	Comet	fungicide	0.75l/ha	59
21.06.10	Proline	fungicide	0.8l/ha	59

Winter Barley Trial 2010

Plot	Variety	Block	% ceph 16 June	% ceph7 July	Ceph AUDPC	max AUDPC	Ceph AUDPC as % of max
1	Malabar	1	0	0	0	2300	0
2	Sequel	1	2	0	23	2300	1
3	Element	1	2	0	23	2300	1
4	Pelican	1	0	5	57.5	2300	2.5
5	MH02ES58	1	4	0	46	2300	2
6	3429 GH1	1	0	0	0	2300	0
7	Volume	1	5	0	57.5	2300	2.5
8	Boost	1	2	0	23	2300	1
9	SE BC705	1	1	0	11.5	2300	0.5

10	Purdey	1	0	0	0	2300	0
11	Vanquish	1	0	0	0	2300	0
12	Cassata	1	0	5	57.5	2300	2.5
13	KWS-Cassia	1	1	0	11.5	2300	0.5
14	Flagon	1	3	0	34.5	2300	1.5
15	Suzuka	1	4	0	46	2300	2
16	Blazing	1	0	0	0	2300	0
17	Saffron	1	3	0	34.5	2300	1.5
18	Trick	1	2	0	23	2300	1
19	Retriever	1	0	0	0	2300	0
20	Winsome	1	3	0	34.5	2300	1.5
21	Pearl	1	0	5	57.5	2300	2.5
22	Vanquish	2	0	0	0	2300	0
23	Flagon	2	0	0	0	2300	0
24	Pearl	2	0	0	0	2300	0
25	Purdey	2	2	0	23	2300	1
26	Cassata	2	2	0	23	2300	1
27	Saffron	2	0	0	0	2300	0
28	SE BC705	2	2	0	23	2300	1
29	Suzuka	2	0	0	0	2300	0
30	Winsome	2	1	0	11.5	2300	0.5
31	Trick	2	0	0	0	2300	0
32	Retriever	2	1	0	11.5	2300	0.5
33	Blazing	2	0	0	0	2300	0
34	KWS-Cassia	2	0	0	0	2300	0
35	Malabar	2	3	0	34.5	2300	1.5
36	Boost	2	0	0	0	2300	0
37	Volume	2	2	0	23	2300	1
38	Pelican	2	0	0	0	2300	0
39	MH02ES58	2	1	0	11.5	2300	0.5
40	Element	2	3	0	34.5	2300	1.5
41	Sequel	2	2	0	23	2300	1
42	3429 GH1	2	0	0	0	2300	0

Maximum AUDPC is calculated as 100% infection in the crop through the season

Winter Barley Trial 2011

		19 May	16 Jun			
plot	Variety	% ceph infection	% ceph infection	ceph AUDPC	max AUDPC	Ceph AUDPC as % of max
1	Escadre	0	1	14	2800	0.5
2	Volume	0	1	14	2800	0.5
3	Pelican	1	1	28	2800	1
4	Element	0	0	0	2800	0
5	Sequel	1	2	42	2800	1.5
6	LP-6-728	0	0	0	2800	0

7	SYN-208-52	0	1	14	2800	0.5
8	Archer	0	4	56	2800	2
9	Florentine	0	0	0	2800	0
10	Pearl	0	2	28	2800	1
11	Sinatra	1	1	28	2800	1
12	SYN-208-57	0	0	0	2800	0
13	KWS-Cassia	0	0	0	2800	0
14	Suzuka	0	0	0	2800	0
15	Winsome	0	0	0	2800	0
16	Canyon	0	0	0	2800	0
17	Retriever	0	0	0	2800	0
18	Cassata	0	0	0	2800	0
19	Matros	0	1	14	2800	0.5
20	Flagon	0	0	0	2800	0
21	Purdey	0	0	0	2800	0
22	Saffron	1	0	14	2800	0.5
23	LP-6-728	0	0	0	2800	0
24	Volume	0	0	0	2800	0
25	Escadre	1	1	28	2800	1
26	Pelican	0	1	14	2800	0.5
27	Element	0	0	0	2800	0
28	Sequel	0	0	0	2800	0
29	SYN-208-52	0	1	14	2800	0.5
30	Retriever	0	0	0	2800	0
31	Sinatra	0	0	0	2800	0
32	Matros	0	1	14	2800	0.5
33	Flagon	0	0	0	2800	0
34	Archer	0	3	42	2800	1.5
35	Cassata	1	1	28	2800	1
36	Saffron	1	1	28	2800	1
37	Suzuka	0	0	0	2800	0
38	Florentine	0	1	14	2800	0.5
39	Canyon	0	1	14	2800	0.5
40	KWS-Cassia	0	0	0	2800	0
41	SYN-208-57	0	1	14	2800	0.5
42	Purdey	0	1	14	2800	0.5
43	Pearl	1	1	28	2800	1
44	Winsome	0	0	0	2800	0

Maximum AUDPC is calculated as 100% infection in the crop through the season

Appendix Two

Resistance Ratings

HGCA Recommended Lists® Trials 2012 Harvest

Winter wheat RL disease ratings

				Cephalosporium			
				Rating (1–9)			
				NEW old			%
	Variety ID	dif			log	count	retns
1	Solstice	WW1282	6	6.4	1.58	3	5.75
2	Oakley	WW1658	5	5.2	1.98	3	9.17
3	JB-Diego	WW1737	6	6.3	1.61	3	5.79
4	Gallant	WW1766	6	6	1.71	3	6.18
5	Scout	WW1787	6	6.4	1.59	3	5.38
6	Invicta	WW1853	6	5.9	1.74	3	5.76
10	Claire	WW1070	7	6.6	1.51	3	5.67
11	Einstein	WW1376	6	6.3	1.77	3	6.72
12	Cordiale	WW1388	6	5.9	1.75	3	5.61
14	Alchemy	WW1564	6	5.5	1.89	3	7.39
15	Timber	WW1644	4	4.1	2.37	1	17.96
16	Duxford	WW1725	7	6.8	1.44	3	4.89
17	Panorama	WW1801	8	7.6	1.15	3	2.82
18	Grafton	WW1811	8	7.6	1.18	3	3.08
19	Viscount	WW1812	7	7.1	1.35	3	3.74
20	Conqueror	WW1813	6	6.3	1.6	3	5.83
22	Warrior	WW1865	6	5.6	1.86	3	8
23	KWS-Sterling	WW1880	7	7.3	1.27	3	3.26
24	Beluga	WW1885	6	6.3	1.61	3	6.04
25	Denman	WW1895	4	4.3	2.3	2	10.52
26	KWS-Podium	WW1907	7	7	1.36	2	5.39
27	KWS-Target	WW1911	6	6.2	1.64	2	6.27
28	KWS-Santiago	WW1916	7	6.7	1.47	2	4.96
29	Cocoon	WW1922	6	6.2	1.65	2	5.39
30	Gravitas	WW1940	7	6.8	1.45	2	4.64
31	Stigg	WW1941	6	6	1.71	2	5.96
32	Tuxedo	WW1954	7	6.5	1.55	2	5.39
33	KWS-Solo	WW1975	6	5.8	1.8	1	6.11
34	KWS-Gator	WW1977	7	6.9	1.4	1	4.61
35	Chilton	WW1980	8	7.5	1.2	1	4.11
36	Torch	WW1986	4	4.3	2.3	1	9.11
37	Relay	WW1988	7	6.5	1.55	1	5.11
38	SY-Epson	WW2001	7	6.9	1.4	1	4.61
39	Crusoe	WW2009	5	4.9	2.1	1	7.61
40	Horatio	WW2018	5	4.9	2.1	1	7.61
41	Monterey	WW2022	6	6.1	1.69	1	5.61
42	Delphi	WW2023	7	6.5	1.55	1	5.11

Appendix Three

qPCR Primers designed in project

Hymenula cerealis sequence KF053537. Primers are highlighted in green and probe in red.

CGCTACTACCGATTGAATGGCTCAGTGAGGCTTTCGGACCGGCCCAGAGAGAGTGGCAACAC
TCCCTCAGGGCTGGAAAGTTGTACAAACTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGG
TTTCCGTAGGTGAACCTGCGGAAGGATCATTAATAAACTAGTCGTTCGTGTAGGGGCAACCTG
ATCATCACGATGTAAGCTCACTAGTCACCCTTGAATATCATACCTTTGTTGCTTTGGCGGGCC
GCCCTCGTGGCCAGCGGNTCCGGCTGCTGCGTGCCCGCCAGAGGACCCCAACTCTTGTTTT
TAGTGATGTCTGAGTACTATATAATAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCAT
CGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA
TCTTTGAACGCACATTGCGCCCTTTGGTATTCCGAAGGGCATGCCTGTTCGAGCGTCATTATA
ACCACTCAAGCCTTCGCTTGGTATTGGGGTTCGCGGTCTCGCGGCCCCTAAAATCAGTGGCG
GTGCCTGTCGGCTCTACGCGTAGTAATACTCCTCGCGATTGAGTCCGGTGGGTAGCTTGCCA
ACAACCCCCAAATTTTTACAGGTTGACCTCGGATCAGGTAGGGAT

TABLE 1. Oligonucleotides used for cloning and real-time PCR

Oligonucleotide name Oligonucleotide type Sequences (5′–3′) and labeling
1406f forward primer TGYACACACCGCCCGT
3126r reverse primer ATATGCTTAAGTTCAGCGGGT
CephF1 forward primer TGATGTCTGAGTACTATATAATAG
CephR1 reverse primer GTTATAATGACGCTCGAA
CephP1 TaqMan®probe FAM-ATCTCTTGGTTCTGGCATCG-BHQ1[a]

[a] Probe labelled with 6-carboxy fluorescein (FAM) and Black Hole Quencher 1 (BHQ-1).