THE IDENTIFICATION OF PHYSIOLOGICAL TRAITS IN WHEAT CONFERING PASSIVE RESISTANCE TO FUSARIUM HEAD BLIGHT

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List of Abbreviations

µg - Microgram

13C-NIV - Carbon-13 labelled nivalenol

13C-DON - Carbon-13 labelled deoxynivalenol

13C-T2 - Carbon-13 labelled T2-toxin

13C-ZEAR - Carbon-13 labelled zearalenone

Ac-DON - Acetyl-deoxynivalenol

AL - Awn length

ANOVA - Analysis of variance

APCI - Atmospheric Pressure Chemical Ionisation

BFR - Brown foot rot

CV - Co-efficient of variation

cm - Centimetre

DON - Deoxynivalenol

DON-3-Glc - Deoxynivalenol-3-glucoside

DS - Dead and dying shoots

EA - Extruded anthers

EH - Hair on ear

ESI - Electrospray Ionisation

ESI- - Negative electrospray Ionisation

ESI+ - Positive electrospray Ionisation

EL - Ear length

ELISA - Enzyme linked immuno-sorbant assay

FA - Flag leaf angle

Fa - *Fusarium avenaceum*

Fc - *Fusarium culmorum*

FDG - Fusarium damaged grains

Fg - *Fusarium graminearum*

FHB - Fusarium head blight

FI - Canopy fractional light interception

FL - Flag leaf length

Fl - *Fusarium langsethiae*

FLA - Flag leaf area

Fp - *Fusarium poae*

FR - Rolled flag leaves

FR - Rolled flag leaves

FS - Fertile shoots

FSP - Fertile shoots per plant

FW - Flag leaf width

FWE - Fresh weight of ears

g - Grams

GC-MS - Gas chromatography mass spectrometry

GS 39 - Growth stage 39

GS 65 - Growth stage 65

h - Hours

HI - Harvest index

HPLC - High pressure liquid chromotography

HT2 - HT2 toxin

IS - Potentially infertile shoots

ISP - Potentially infertile shoots per plant

kg - Kilograms

LC - Liquid chromatography

LC-MS - Liquid chromatography mass spectrometry

Log_{10} - Logarithm base 10

LSD - Least significant difference

MC - Moisture content

Min - Minutes

MLR - Multiple linear regression

Mm - *Microdochium majus*

Mn - *Microdochium nivale*

MS - Mass spectrometry

NIV - Nivalenol

PBS - Phosphate buffered saline

PCR - Polymerase chain reaction

pg/ng DNA - Picograms per nanogram of DNA

PH - Plant height

PN - Plant number

PPB - Parts per billion

RA - Retained anthers

RLA - Remaining leaf area

RT-PCR - Real time polymerase chain reaction

SA - Days between sowing and anthesis

SD - Spikelet density

SDM - Stem diameter

SE - Spikelets per ear

SIR - Select ion recording

SLA - Second leaf area

SW - Specific weight

TA - Trapped anthers

TDW - Total plot dry weight

TGW - Thousand grain weight

TFW - Total fresh weight

TLA - Total leaf area

T2 - T2 toxin

YI - Yield

ZEAR - Zearalenone

ZEAR-14-GU - Zearalenone-14-glucoside

Conference presentations

1) Jones, S.P.T. Sparkes, D.L. Foulkes, M.J. Ray, R.V. 2012. Identification of physiological traits in wheat conferring passive resistance to Fusarium Head Blight (FHB). Epidemiology Canopy Architecture International Conference, French National Institute for Agricultural Research (INRA), Rennes : France, 1-5 July 2012.

(Appendix : Conference presentation I)

2) Jones, S.P.T. Sparkes, D.L. Foulkes, M.J. Ray, R.V. 2013. Physiological traits in wheat conferring passive resistance to Fusarium Head Blight. 12th European Fusarium Seminar, French National Institute for Agricultural Research (INRA), Bordeaux : France, 12-16 May 2013.

(Appendix : Conference presentation II)

Abstract

Fusarium head blight (FHB) is a devastating fungal disease of wheat and other small grain cereals worldwide caused by a complex of toxigenic *Fusarium* spp. and non-toxigenic *Microdochium* spp. Infection leads to a reduction of grain yield, loss of grain quality and the production of harmful mycotoxins. Control methods for FHB include both cultural and chemical strategies, however the development of cultivars with improved FHB resistance is considered as the most sustainable method for controlling the impact of this disease.

Resistance to FHB is a polygenic trait and can be improved by stacking multiple resistance genes together, however there are currently no highly FHB resistant genotypes with acceptable agronomic characteristics available, therefore passive disease resistance can potentially make significant contributions to improved FHB resistance. The aim of this study was to identify novel physiological traits in wheat conferring passive resistance to FHB using ground inoculated field experiments to enable disease escape mechanisms to be expressed, and spray inoculated glasshouse experiments to test if the identified traits were associated with genetic resistance.

The most consistent traits relating to FHB were flag leaf length, plant height and awn length. Since these relationships were present in both field and glasshouse experiments, they are likely caused by a genetic linkage or pleiotropy, with genes conferring FHB resistance or susceptibility. Remaining leaf area was less consistently related to FHB, however, since canopy leaf area showed a positive relationship with the development of FHB in ground inoculated field experiments, there is a basis to support the role of reduced canopy leaf area as conferring passive resistance to FHB.

In general, relationships were poor between visual FHB symptoms and both pathogen DNA and grain mycotoxin contamination. This was theorised to be due to the use of a mixed species inoculation which introduced antagonism between FHB species and which included more than one producer of several mycotoxins quantified. Therefore the use of visual FHB assessments under mixed species inoculation is concluded to be a poor indicator of both pathogen infection and mycotoxin contamination.

Chapter 1.

Literature Review

1.1 INTRODUCTION

The UN forecasts that the world population will reach 9.6 billion by 2050, increasing from 7.2 billion in 2013, with the majority of the population increase coming from less developed countries (UN, 2014). The world must therefore develop the capacity to feed another 2.4 billion people within the next 40 years. Wheat is one of the world's oldest cultivated crops, with the first evolutionary steps occuring approximately 10,000 years ago (Evans *et al.* 1975). Common hexaploid wheat was derived from three different diploid donor species, including *Triticum urartu* which contributed with genome A, *Aegilops speltoides* which contributed with genome B and *Triticum tauschii* which contributed with genome D (Gill and Friebe, 2002). Wheat is currently the world's second largest cereal crop, with 713 million tonnes produced in 2013, second only to rice with a total production of 745 million tonnes (FAO STAT, 2014). Average wheat losses per year, attributed to plant pathogens, is estimated at around 10% in western Europe, but far higher in developing countries (Oerke, 2006).

Fusarium FHB Blight (FHB) is a widespread and destructive disease of small-grained cereals that can cause potentially significant crop losses. Up to 17 *Fusarium* spp. and a small number of *Microdochium* spp. have been linked to causing FHB (Parry *et al.* 1995; Pirgozliev *et al.* 2003) of which several F*usarium* spp. are known to be producers of mycotoxins (Bottalico and Perrone, 2002). FHB causing *Fusarium* species in wheat can infect several of the floral parts including the lemma, palea, anther and stigma (Pritsch *et al.* 2001) resulting in loss of grain yield by causing wheat grains to develop improperly (Kimura *et al.* 2006) or by causing sterility of the florets (Goswami & Kistler, 2004). Grain of potential milling or malting quality is damaged via the inclusion of *Fusarium* damaged grains resulting in a substantial reduction in resulting loaf volumes (Nightingale *et al.* 1999) or gushing in beer. The contamination of grain with mycotoxins, produced by

Fusarium species, is a concern for public health. The trichothecene group has been shown to cause depression of the immune responses, nausea and vomiting in humans (Peraica *et al.* 1999). Unfortunately deoxynivalenol (DON) is a very stable contaminant and is not degraded via processing (Wang *et al.* 2005), therefore it is vitally important that cultural and chemical controls are effectively used to prevent losses in both grain yield and quality, as well as to minimise mycotoxin production which results from FHB infections.

1.2 FUSARIUM HEAD BLIGHT IN WHEAT

Fusarium head blight (FHB) is one of the most important diseases of wheat jeopardizing food and feed safety in many regions of the world. FHB was first described in 1884 in England and since then there have been increased FHB incidences across the world (Goswami & Kistler, 2004). FHB pathogens are ubiquitous in nature (Miraglia *et al.* 2009) and cause disease sporadically, being of high incidence in some seasons yet low in others (Jennings & Turner, 1996). There were reports of a large rise in epidemic FHB out breaks in America during the 1990s, and in areas of North America during 1993 it caused one of the greatest economic losses of any plant disease in a single year (McMullen *et al.* 1997). This epidemic was attributed to severely wet weather followed by humid periods and confirms how variable this disease can be between years.

FHB can cause severe losses in grain yield (Saur, 1991), reductions in milling quality (Nightingale *et al.* 1999) and can cause contamination with mycotoxins (Prandini *et al.* 2009). Symptoms of FHB start as brown lesions on the glumes, which may spread between spikelets and eventually cause tissues to senesce (Bushnell *et al.* 2003) As the disease develops, bleaching of the ear may occur above the point of infection (Brown *et al.* 2010). The fungal species which is causing the disease cannot be inferred from visual symptoms alone (Xu *et al.* 2008). Several factors influence the incidence of FHB epidemics each year, including previous cropping and tillage (Schaafsma *et al.* 2005), varietal resistance and climatic conditions during flowering (Xu *et al.* 2008).

1.2.1 Causal organisms and geographical distribution

A range of *Fusarium spp*. have been associated with the development of FHB in small grain cereals in Europe. These predominantly include *F. graminearum, F. avenaceum* and

F. culmorum, but other less frequently encountered species can also be present including, *F. poae, F. cerealis, F. equiseti, F. sporotrichioides, F. tricinctum* and some even more sporadically identified species include *F. acuminatum, F. subglutinans, F. solani, F. oxysporum, F. verticillioides, F. semitectum* and *F. proliferatum* (Bottalico and Perrone, 2002). Other FHB causing species commonly found in small grain cereals include *Microdochium majus* and *M.nivale* (Nielsen *et al.* 2013). Bottalico & Perrone (2002) describe how it is common to isolate from a single fragment of infected tissue, up to nine differing *Fusarium* species, and up to 17 in freshly harvested grain. The species profile of FHB pathogens in different geographic areas depends on multiple factors, but primarily upon climatic conditions, especially temperature (Miraglia *et al.* 2009). *F. graminearum* is associated with warm and humid conditions, *F. avenaceum* and *F. culmorum* with cooler, damper and humid conditions, *F. poae* with drier and warmer climates, and *Microdochium spp.* with mild temperatures and frequent rainfall (Xu *et al.* 2008). The incidence of *Fusarium* species throughout Europe is split between the cooler north and central regions, and the warmer south (Table 1.1). Isolates of *F. graminearum*, originating from both America and China, have also been shown to vary in their cultural characteristics and ability to cause FHB (Bai & Shaner, 1996) showing that that there could be significant differences in an isolates ability to cause disease may vary by geographical location. Significant quantitative variation in the aggressiveness of *F. graminearum* isolates has been observed within populations, this variation however did not differ between the Asian or European populations studied (Gagkaeva and Yli-Mattila, 2004).

Table 1.1. Mycotoxigenic species isolated from FHB of wheat in Europe

	Geographical incidence	
Species	North/Centre	South
F. gramineaum	+ + +	+ + +
F. avenaceum	+ + +	+ +
F. culmorum	+ + +	+ +
F. poae	+ +	+
F. equiseti	+ +	+
F. tricinctum	+	+
F. cerealis	+	±
F. sporotrichioides	+	±
F. acuminatum	±	±
F. subglutinans	±	-
F. solani	±	-
F. oxysporum	±	-

Key: + Low incidence; + + Moderate incidence; + + + High incidence; ± Sporadic between locations.

(Source: Bottalico and Perrone, 2002)

1.2.2 Losses due to Fusarium Head Blight

FHB causes crop losses in a multitude of ways, including reductions in grain yield, reductions in grain quality and contamination with harmful mycotoxins.

1.2.2.1 *Grain yield*

Growers use multiple control measures to protect crops against FHB infections and prevent yield loss. Severe yield losses can occur during epidemic years, which are largely determined by the weather (Parry *et al.* 1995). Yield reductions of between 6-39% have been reported in experiments containing over 500 wheat lines, inoculated with *F. graminearum* and *F. culmorum* spores (Saur, 1991) and Parry *et al.* (1995) report that yields can be reduced by as much as 60% in *F. culmorum* inoculated trials. McMullen *et al.* (1997) reported average losses to FHB of 45% in commercial crops under high disease pressure years. FHB epidemics lead to the increased production of shrivelled grains with a white or pink discolouration. Additionally, FHB epidemics can lead to seed-borne contamination with *Fusarium* and *Microdochium* spores. Infected grain, when used to establish new crops, can lead to poor establishment and reduced grain yield (Jones, 1999).

1.2.2.2 *Grain quality*

FHB epidemics have detrimental effects on the quality of wheat crops making them less suitable for their respective end uses. Epidemics reduce the hectolitre weight of grain (McMullen *et al.*. 1997), reducing quality premiums from the milling sector or leading to the end use of grain being downgraded. Wheat grains, damaged by *F. graminearum* and *F. avenaceum*, have been shown to suffer from significant degradation of endosperm proteins (Nightingale *et al.* 1999) and starch granules (Snijders, 2004). Nightingale *et al.* (1999) showed that the consistency and resistance to extension of dough was decreased when *Fusarium* damaged grains were included in the flour constituents. This resulted in substantial reductions in loaf volume and was attributed to the presence of fungal proteases. Fungal proteases have been shown to lead to weak dough properties and

unsatisfactory bread quality (Wang *et al.* 2005). Additionally, reductions in gluten strength in durum wheat, through the inclusion of *Fusarium* Damaged Grains (FDG) is likely caused by lower proportions of glutenin (Dexter *et al.* 1997) of which there was a liner relationship between FDG and dough quality. The quality of grain, for use as seed to establish new crops, is reduced through FHB epidemics as it can lead to poor establishment of crops where seed-borne Microdochium *spp.* is present (Haigh and Hare, 2012).

1.2.2.3 *Mycotoxins*

The word mycotoxin refers to toxins produced by fungi, and although not all fungi produce toxins, those that do are referred to as being toxigenic (Prandini *et al.* 2009). The trichothecenes are a major group of mycotoxins that are grouped into type A trichothecenes (e.g. T-2 toxin and HT-2 toxin) and type B trichothecenes (e.g. DON and NIV) (Larsen *et al*, 2004) (Fig. 1.1 and 1.2). Other common mycotoxins such as zearanelone belong to separate groups of trichothecenes despite their ability to be produced together.

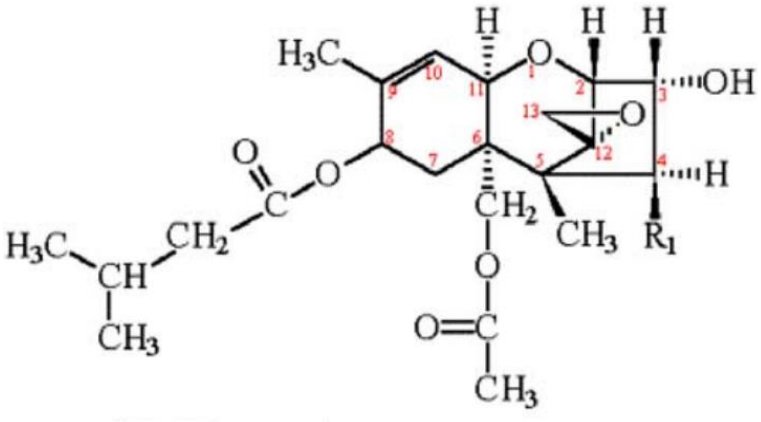

Figure 1.1. Chemical structure of Type A trichothecenes
(Source: Champeil *et al.* 2004)

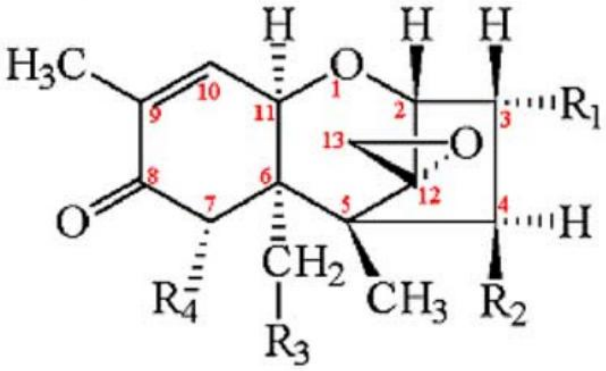

Figure 1.2. Chemical structure of Type B trichothecenes
(Source: Champeil *et al.* 2004)

Mycotoxins occurring in food and feed are important due to their negative impacts on human and animal health; therefore legislative limits exist to ensure that mycotoxin contaminated grains are controlled within the supply chain. Within the European Union (EU), limits of DON and ZEAR within unprocessed cereals are 1250 µg/kg and 100 µg/kg, respectively (European Union, 2006). Surveys of UK wheat have however shown that up to 13.1% of crops produced in 2008 exceeded the 1250 µg/kg limit for DON (HGCA, 2010b). Limits for T-2 toxin and HT-2 toxin contamination in grains have not yet been imposed within the EU, despite being highly toxic.

In UK grown wheat, DON is the most commonly occurring mycotoxin (Edwards, 2009a) with water availability and temperature being the most important environmental factors influencing mycotoxin production (Milani, 2013). The largest DON producing *Fusarium* pathogens are *F. graminearum* and *F. culmorum* as they are more pathogenic than other species (Edwards, 2004) although they are also the predominant producers of ZEAR (Edwards, 2006) (Table 1.2). Acetylated forms of DON known as 3- and 15-acetyl DON,

can also be co-produced with DON dependent upon which chemotype of *F. graminearum* or *F. culmorum* is present (Edwards, 2006).

Table 1.2. The main FHB causing *Fusarium* species in Europe and their associated mycotoxin production.

F. graminearum	DON, NIV, ZEN, AcDON, FUS.
F. culmorum	DON, ZEN, ZOH, NIV.

Key: DON (Deoxynivalenol); NIV (Nivalenol), ZEN (Zearalenone); AcDON (Monoacetyl-deoxynivalenols); FUS (Fusarenone-X); ZOH (Zearalenols - alpha and beta isomers).

(Source: Bottalico and Perrone, 2002).

DON is a very stable contaminant and neither storing, milling, processing, cooking nor processing cause its degradation (Wang *et al.* 2005). This is an issue especially for the ethanol producing industry as processing can concentrate DON within spent grains, creating problems in production of a waste feedstock. Superheated steam treatment of wheat grains between 160-185^{o}C has been shown to cause significant ($P<0.05$) reductions in DON content by up to 52% (Pronyk *et al.* 2006). These reductions were confirmed as being caused by thermal degradation of DON and not just through solubilisation and extraction.

Symptoms of trichothecene poisoning in humans often include depression of the immune system, nausea and sometimes vomiting (Paraica *et al.* 1999). These symptoms are often mediated through damage to all major organs, most frequently including the liver, kidneys, lungs and the nervous, endocrine and immune system (Bhatnager *et al.* 2002). A study of twenty-eight individuals found to be suffering from mycotoxin-induced disease, showed

that patients could be successfully treated using a combination of heat and physical therapy, immune modulators, anti-fungal medication, and oxygen therapy (Rea *et al.* 2009). In farm animals, symptoms of excess mycotoxin consumption include reduced feed intake, loss of weight, reproductive disorders, faintness and uncoordinated movement (Hussain & Brasel, 2001) although these symptoms are not always clear cut and many others may be present depending upon the affected species and the dose ingested. Ruminant animals have been shown to be more resilient to the negative effects of mycotoxins since the microbiota within the rumen is able to degrade such toxins (Hussain & Brasel, 2001). Preventive measures are widely believed to provide greater potential for treatment that remedial procedures, with the selection of resistant cultivars to control mycotoxin contamination in feed being the most effective strategy (D'Mello *et al.* 1999). Within the trichothecene group of mycotoxins, the mechanism for their toxicity is through the inhibition of ribosomal protein synthesis (Desjardins and Proctor, 2007).

Research focusing on the effect of diets containing a mixture of several mycotoxins, in terms of their antagonistic, synergistic or additive effects, have become of increasing importance to public health. Information on this subject is limited, especially with tricothecenes, and Speijers and Speijers (2004) suggest future research should focus on those mycotoxins most likely to have a syngergistic effect with each other and to establish a group Tolerable Daily Intake (TDI) for trichothecenes due to their potential to have an additive effect.

Improved monitoring of mycotoxins has ensured that they have not caused wide scale human death from oral consumption since the episode of alimentary toxic aleukia in the USSR (1932-1947). Research into the production and effects of mycotoxins has increased since the death in the UK of 100,000 young turkeys in 1960 which was due to the inclusion of aflatoxin contaminated groundnut meal in feed (European Mycotoxins Awareness

Network, undated). Studies aiming to anticipate emerging food hazards (Marvin and Kleter, 2009) and improved methods of reporting such hazards (Kleter *et al.* 2009) have allowed for the risks of wide scale mycotoxin contamination to be reduced.

A wide range of environmental factors, mainly temperature, relative humidity, insect attack, drought and plant stress all greatly influence the ability of fungi to produce mycotoxins (Miraglia *et al.* 2009) and therefore climate change presents a changing hazard to the production of mycotoxins within crops. Predicting the influence of climate change on the colonisation of crops and the subsequent mycotoxin production, should be made on a case-by-case basis as each species has its own optimum conditions for fungal growth and mycotoxin production (Miraglia *et al.* 2009). Climate change is predicted to advance the flowering and harvest dates of wheat, and to lead to increased risk of deoxynivalanol contamination in grain, which will be further exacerbated by the increased frequency of wheat and maize cropping (van der Fels-Klerx *et al.* 2012). There is evidence to suggest that there could be a shift in dominant populations from *F. culmorum* to *F. graminearum* (Bateman, 2005; Jennings *et al.* 2000) as global temperatures rise.

The use of FHB visual symptoms is not a reliable predictor of mycotoxin contamination within harvested grain. Several studies have found a correlation between the two variables (He *et al.* 2014), while other studies have failed to identify this (Suzuki *et al.* 2012). Edwards *et al.* (2001) found no correlation between the visual symptoms of FHB and DON content in harvested grain, however there was a good correlation between the amount of trichothecene producing *Fusarium* DNA present and DON concentration. Additionally, strong positive relationships between percentage FDK in wheat samples and levels of DON contamination have been shown (Beyer *et al.* 2007) although this positive correlation did not stretch to NIV, 3AcDON or 15AcDON but did include positive correlations with visual symptoms. The presence of *Microdochium* species on the ear can, in some years, partially

account for the lack of relationship between FHB symptoms and mycotoxin contamination, as *Microdochium* species produce visual symptoms but are non-mycotoxigenic. Further exacerbating the difficulty in predicting mycotoxin production is that the distribution of *Fusarium* pathogens and mycotoxin production is often distributed randomly within a field (Oerke *et al.* 2010) indicting that a comprehensive sampling method is required to adequately assess the presence of *Fusarium* species and mycotoxin production.

Studies between the mycotoxin contamination of conventionally and organically grown crops have found organic farming systems to have lower rates of FHB infection, and subsequent DON contamination, than conventionally farmed wheat crops (Birzele *et al.* 2002). Additional studies in barley have, however, shown there to be no difference in mycotoxin contamination between the two systems (Edwards, 2009b).

The genome of *F. graminearum* has recently been mapped (Chang *et al.* 2007) which should facilitate research into control of mycotoxin production. Several genes are associated with the production of trichothecenes with roughly one half of the required genes for tricothocenes biosynthesis being found on a 25-kb gene cluster. Genes of particular importance include Tri5, which encodes for the enzyme trichodiene synthase and is the first committed step in the biosynthetic pathway of tricothocene (Goswami and Kistler, 2004). The production of trichothecene mycotoxins by *F. graminearum* is believed to be a virulence factor for its pathogenicity to certain host plants, as without it virulence is reduced (Proctor *et al.* 1995). DON production has been shown to be a requirement of *F. graminearum,* to facilitate its spread from its infection point through the ear (Bai *et al.* 2002) and is also used to contribute to bleaching and cellular death in the latter stages of infection, as the pathogen undergoes the saprophytic stage of its lifecycle (Bushnell, 2010).

1.3 FHB EPIDEMIOLOGY

Fusarium and *Microdochium spp*. are the causal pathogens of a complex of diseases in small-grained cereals including Fusarium Seedling Blight (FSB), Brown Foot Rot (BFR) and Fusarium Head Blight (FHB). Each disease poses its own risk during the crops lifecycle; FSB causes a reduction in plant establishment; BFR increases lodging and the occurrence of white ears; FHB reduces grain quality, increases the risk of mycotoxin contamination and reduces yield (Edwards, 2004) and each aforementioned disease can cause significant yield loss. Each disease within the complex is important from a epidemiological perspective as each plays a role in the transmission and survival of the pathogen from one season to the next.

1.3.1 Dispersal and spread

Fusarium species are both saprophytes and facultative parasites that are able to colonise living hosts before establishing themselves in crop stubble through their presence in senescent tissue (Liddell, 2003). This debris is a primary source of FHB inoculum as it allows reserves of *Fusarium* pathogen to survive between seasons. This ensures FHB inoculum to be abundant within the environment (Osborne and Stein, 2007). A general overview of the *Fusarium* pathogen lifecycle is shown in Fig. 1.3. Fusarium seedling blight (FSB) is the first disease of the pathogens life cycle, which additionally allows the pathogen to overwinter within the host. Fusarium seedling blight (FSB) is predominantly caused by the *Fusarium* and *Microdochium* species *F. graminearum, F. culmorum, M. majus* and *M. nivale* (Glynn *et al.* 2006). It leads to the premature death of establishing wheat seedlings both pre- and post-emergence with one of the most common consequences of the disease being poor crop establishment (Humphreys *et al.* 1995). The multiple seedling blight pathogens have differing optimal climatic conditions for infection. *M.*

nivale thrives under cool (10-15^{o}C) and dry conditions, whereas the true *Fusarium* species which are also FSB pathogens are more suited to warm (15^{o}C>) and dry conditions (Doohan *et al.* 2003). Significant negative relationships exist between the percentage of seed infected by *M. nivale* and *F. culmorum,* and plant establishment (Hare *et al.* 1999). Additionally, while the percentage of *M. nivale* infected seed was not correlated with seed weight, the percentage of *F. culmorum* infection was, with infection levels being greater in lighter batches of seed (Hare *et al.* 1999). This shows that selecting seed based on a high TGW is a useful tool in minimising the effects of FSB. The main source of FSB inoculum is infected seed, but soil borne inoculum may also contribute to the disease (Doohan *et al.* 2003). The establishment of FSB in wheat crops allows for the pathogen to develop into Brown Foot Rot (BFR) and to infect additional crop residues ensuring survival until the next season.

Brown Foot Rot (BFR) forms part of the stem base complex in wheat and has the potential to cause significant yield reductions (Nicholson *et al.* 1996). The causal pathogens of BFR include several of the same *Fusarium* species which cause FHB, including: *F. culmorum*, *F. graminearum*, *F. avenaceum*, *F. poae* and *M. nivale* (Nicholson *et al.* 1996) although it is predominantly caused by *M. nivale.* It is worst during cool (10-15^{o}C) and dry spring conditions which favour natural stem-base infection (Doohan *et al.* 2003; Jennings & Turner, 1996). Symptoms include dark brown staining of the stem base and lower nodes before causing them to rot. BFR results in the colonisation of stem base tissue and will therefore later contribute to infected debris in the soil, facilitating the pathogen's spread between seasons.

FHB, while not a direct consequence of FSB or BFR, is certainly influenced by the infected residues that these pathogens colonise. FHB inoculum can come from several sources, including soil and infected seed, as well as crop debris (Edwards, 2006). However,

some crop debris contains more inoculum than others, with residues of grain maize being important sources of *F. graminearum* and *F. culmorum* (Maiorano *et al.* 2008). Both conidia and ascospores are able to cause FHB in cereals; however the routes via which such spores reach the ear of the plant are not clearly understood (Champeil *et al.* 2004), although it is known to be unrelated to systemic growth through the stem (Snijders, 1990). Areas with an abundance of infected residues, such as regions producing maize or cereal crops, contribute to the presence of airborne spores within the local area (Osborne and Stein, 2007). Such airborne spores can travel long distances (Maldonado-Ramirez *et al.* 2005) and should be considered an important dispersal method of FHB inoculum. Sutton (1982) found that disease severity and incidence of FHB had close relations to the quantity of primary inoculum present in the crop, and that since the disease is polycyclic it does not spread between ears.

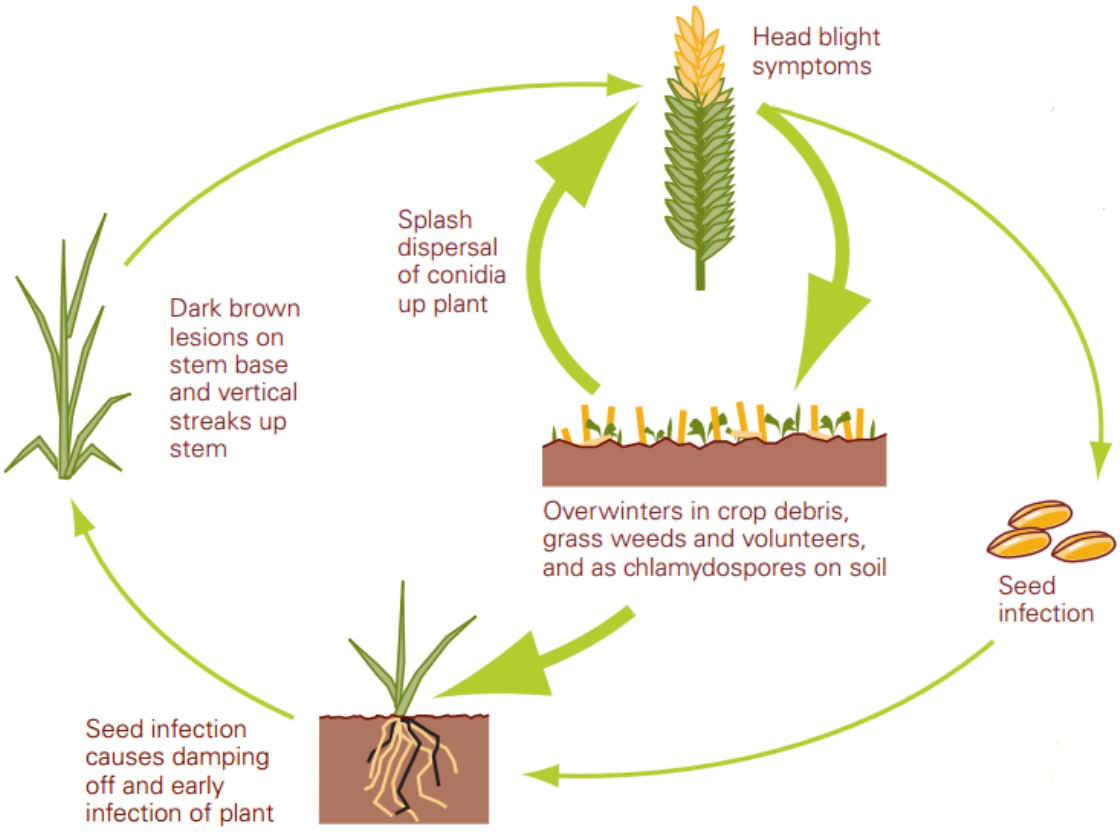

Figure 1.3. Lifecycle of Fusarium species
(Source: HGCA, 2010)

1.3.2 Infection

The successful colonisation of a wheat ear by *Fusarium* and *Microdochium* pathogens causing FHB can be split into three distinct stages. These stages are; spore dispersal onto the wheat ear, entry into floral tissues and colonisation of the floret and spike.

The route of spore dispersal onto wheat ears needs consideration as to how spores achieve movement from the soil where they are harboured, to the site of infection. FHB infections caused by the systemic growth of *Fusarium* fungi was proposed in the 1920s, but Atanasoff (1920) was the first researcher to discredit this as a route of FHB infection. Recent experiments have also shown that systemic growth is an unlikely contributing factor to the development of FHB as it was found that just 3% of plants inoculated at their stem bases were colonised with *Fusarium* above the second node, while no colonisation at all was found at the fifth node or within the ear (Clement and Parry, 1998). Snijders (1990) also had similar findings, concluding that although stem base infections could lead to the systemic growth and colonisation of higher stem internodes, there was no evidence that this systemic growth led to the development of FHB in wheat ears. This explains the lack of relationship between BFR and FHB, and why they are to date, considered as separate to one another (Champeil, *et al.* 2004).

The principal source of FHB inoculum being deposited onto the wheat ear is thought to be through the splashing and wind dispersal of ascospores and conidia, both of which are considered to be forms of aerial contamination (Champeil *et al.* 2004). Splashing is the main route by which macroconidia are spread, since they are too heavy to be transported by wind, whereas ascospores rely more on wind dispersal. The climate, pathogenic complex present and this species complex ascospore production capacity all impact upon the relative importance of each dispersal method (Champeil *et al.* 2004). The spread of

inoculum via leaf-to-leaf contact has been suggested as a route for FHB dispersal to the ear (Champeil et al, 2004) and this possibility has shown how, under field conditions, spores attached to the upper sides of leaves can be further spread to higher leaves towards the wheat ear by continuous splash events (Horberg, 2002). Furthermore to the role that wind dispersal plays in deposition of spores on ears, *Fusarium* spores have been found in the atmosphere at a height of 60m, and consideration must therefore be given to the role that long distance transport of *F. graminearum* inoculum can play in contribution to regional epidemics, as atmospheric transport may move spores in the order of tens to hundreds of kilometres (Maldonade-Ramirex *et al.* 2005; Schmale *et al.* 2012). Significantly more spores were found in the atmosphere during cloudy conditions than during clear conditions (Maldonade-Ramirex *et al.* 2005).

Once *Fusarium* spores have been deposited onto the ears of wheat, spores can exploit multiple pathways through which to gain entry into wheat floral tissues. Several pathways of entry have been shown, including via stomatal openings, direct penetration, hyphal extension into the floret mouth and crevices in between adjacent lemma or palea (Lewandowski *et al.* 2006). There are large differences in the susceptibility of floral cells to infection between the internal and external surfaces. The external surface cells of the palea, lemma and glumes that are in contact with the environment, have extremely thick epidermal and hypodermal cell walls, which prevent direct penetration by the fungus (Shaner, 2003). The surface cells within florets however have thin walls and lack resistance to *Fusarium* invasion (Lewandowski *et al*, 2006) making the internal surfaces of floral parts much more easily invaded than their external surfaces.

Primarily, *Fusarium* spores produce short infection hyphae that can penetrate the epidermal cuticle of the internal surfaces of the palea, lemma and glumes via the use of hydrolyzing enzymes (e.g. cutinases and lipases) (Walter *et al.* 2010). Intercellular fungal

growth then occurs. Pectinases are released which begin to degrade the middle lamella and cell walls, and this, in turn, exposes other polysaccharides in the cell wall to degradation from hemicellulases and cellulases.

1.3.3 Disease development and accumulation

Once the *Fusarium* pathogen has gained initial entry into the floral tissues, the pathogen will continue to develop by colonising the floret and spreading within the ear (Brown *et al.* 2010). Development of the FHB pathogens is subject to an array of both structural and defence mechanisms within the ear. Once the FHB pathogen has achieved penetration of the cuticle layer within the floral tissues, hyphae will continue to grow subcutaneously, before spreading within the cell apoplast leading to cytological modifications and eventually cell death (Walter *et al.* 2010). Growth within these tissues involves the use of extracellular enzymes such as cellulases, xylanases and pectinases to degrade the host cell wall (Kang and Buchenauer, 2002). Trichothecene toxins have been detected in host tissues during early stages of the infection process (Kang and Buchenauer, 2002). The production of trichothecene mycotoxins is thought to enhance pathogen virulence, as the development of ear blight is slowed when the ability to produce such toxins is absent within mutant *F. graminearum* strains (Proctor *et al.* 1995). Once colonisation of floral tissues has been achieved, *F. graminearum* hyphae spread down the rachis node to the central rachis, before eventually spreading up and down (Brown *et al.* 2010). This spread leads to the invasion of further spikelets and the appearance of bleached ears.

1.4 CONTROL OF FHB

1.4.1 Cultural control

The use of cultural control methods are an effective and sustainable way of minimising the risks of *Fusarium* pathogens in cereal crops and growers should utilise cultural control methods prior to chemical controls.

Cultural control methods of FHB include crop rotation, ploughing, nitrogen fertilisation, weed control and varietal resistance, with appropriate crop rotation being considered the most effective of all cultural controls (Pirgozliev *et al*, 2003) as it reduces the build up of the pathogen in the soil to infect new crops. This is the same as with other crop diseases, such as septoria leaf blotch (*Mycosphaerella graminicola*) in which extended rotations can help control build up of the disease and reduce infection in following crops (Pedersen and Hughes, 1992). For example, previous crops of maize can lead to significantly increased levels of grain DON contamination as maize is an important source of *F. gramiearum* and *F. culmorum* inoculum (Maiorano *et al.* 2008). Wheat crops following crops such as potatoes or sugar beet could also lead to elevated grain DON concentrations than in wheat following other small grain cereals (Obst *et al.* 1997). This could be explained in part by several species of *Fusarium* being able to also infect some non-cereal crops (Smith, 1988), for example, *F. graminearum* is known to contribute to dry rot in potatoes tubers (Delagdo *et al.* 2010) and this could act as a source of future FHB inoculum. However, in contrast to the earlier statement by Pirgozliev *et al.* (2003), Smith *et al.* (1988) describe how DON producing *F. culmorum* can survive between crops saphrophytically, meaning that it cannot readily be controlled by rotation alone. This is perhaps reflected in Fig. 1.4 and 1.5 in which a break between wheat crops does not totally eliminate DON and ZEAR production.

Parry *et al.* (1995) mention that infection risk is increased where *Fusarium* infected debris is present after direct drilling or reduced cultivations. Inversion cultivations, such as ploughing, are better at reducing FHB infections as they reduce the pathogen's ability to survive and reproduce (Pereyra *et al.* 2004). With the loss in the ability to stubble burn crop residues, a very effective means to control FHB has been lost and ploughing remains the only viable option for disposal of crop debris, which is believed to provide a less effective control of FHB (Parry *et al.* 1995). Evidence of the relationship between surface crop residues and FHB has been shown by Maiorano *et al.* (2008) who found a strong positive correlation between the quantity of surface residues and grain DON contamination. Explanations for this increase in DON due to increased surface residues were was given as, a more favourable environment for fungal over bacterial development, increased surface moisture favouring *Fusarium* spore development and increased splash dispersal from crop residues at the soil surface. An investigation into the effects on FHB from ground level *F. culmorum* inoculum found that such inoculum was capable of increasing FHB, but its more severe development was dictated by the presence of significant rainfall during anthesis (Bateman, 2005), showing that while to cultural controls to minimise FHB infections are a good choice to base a control programme, the weather is usually the deciding factor in the severity of FHB epidemics. This statement is supported by Lori *et al.* (2009), who undertook a study examining the impact of tillage and other agronomic factors played upon natural *Fusarium* infections in wheat. Favourable weather conditions were found to be more important than tillage or nitrogen fertiliser treatments on FHB infection. This is surprising as Dill-Macky and Jones (2000) attributed the move to reduced tillage in the upper Midwest of the US, to be the causative factor of recent FHB epidemics. Fernandez *et al.* (2009) found that *F. avenaceum* was more prevalent under

reduced tillage than conventional tillage, while *F. graminerum* was lowest under zero-tillage, showing that each FHB pathogen may respond differently to methods of tillage.

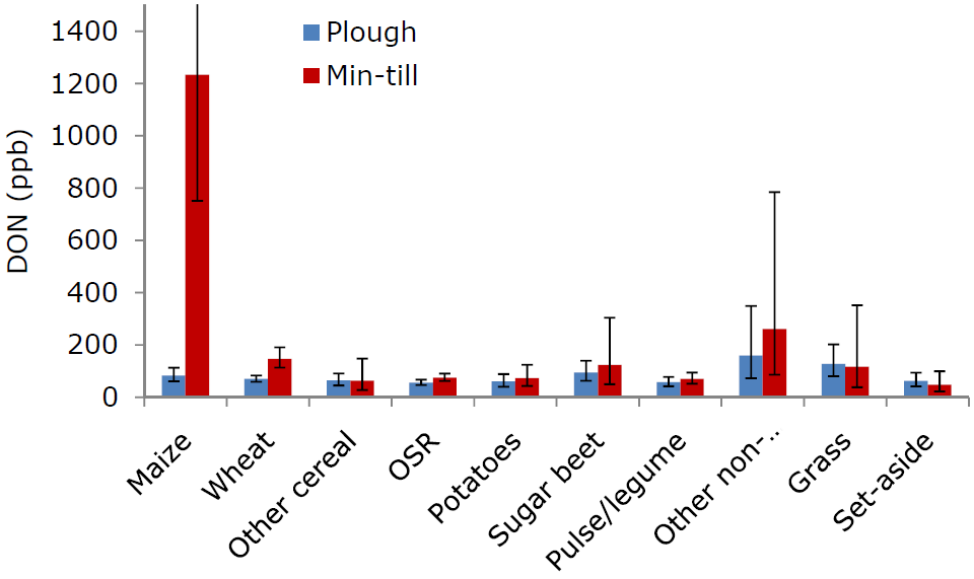

Figure 1.4. Effect of cultivation and previous crop on DON contamination of wheat grain (Source: Edwards, 2011).

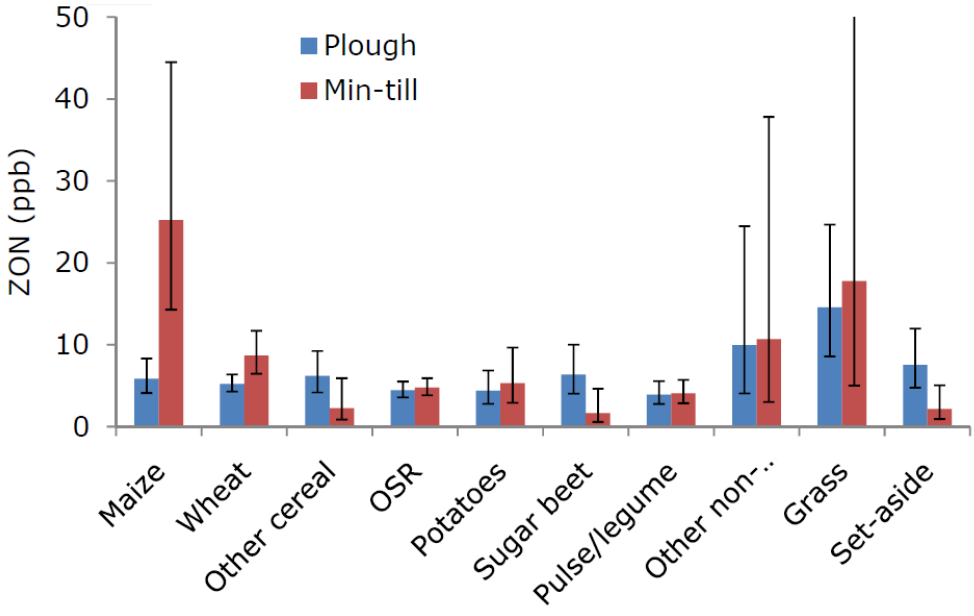

Figure 1.5. Effect of cultivation and previous crop on ZEAR contamination of wheat grain (Source: Edwards, 2011).

1.4.2 Varietal resistance

Varietal resistance to FHB is considered the most sustainable method to control future FHB epidemics. UK wheat varieties however, do not have high levels of genetic resistance with most varieties having a resistance rating of between 5-7 (HGCA, 2014).

Wheat has five types of resistance to FHB (Mesterhazy, 2003) which are described as Type I resistance (Resistance against initial infection), Type II resistance (resistance to pathogen spreading in infected tissue), Type III resistance (resistance to kernel infection), Type IV resistance (tolerance to *Fusarium* head blight) and Type V resistance (resistance to toxins). Type V resistance is a fairly broad factor and can be used to refer to the plant's ability to decompose the toxin, be tolerant to the toxin or to limit its accumulation in the ear. The terms Type III, IV and V resistance are however, used tentatively by researchers, given the problems with appropriate methodologies to study and measure each type of resistance on its own merits (Shaner, 2003). Type I and II are the most studied types of resistance although Bai and Shaner (2004) report that Type II resistance is more genetically stable than Type I resistance. The use of a combination of FHB resistance components (i.e. Type I + Type II + Type V) has been shown by Burlakoti *et al.* (2010), in an experiment involving the use of 113 F9 recombinant inbred spring wheat lines, to offer improved FHB resistance over their already resistant parentage.

Wheat spikes infected with FHB pathogens respond by inducing a set of defence response genes within the infected spikelets (Pritsch *et al.* 2001). The production of the defence response transcripts are produced even in uncolonised parts of the spikelets showing that direct contact with the *Fusarium* pathogen is not required to induce the defence response genes (Pritsch *et al.* 2001). Host plants can identify the invading FHB pathogens by the presence of chitin and glucans in the pathogen's cell walls, and the plant responds by

producing fungal degradative enzymes and by reinforcing its own structural barriers (Walter *et al.* 2010). QTL analysis experiments on wheat germplasm have shown that a vast array of genes are able to provide resistance to FHB infections, with resistance traits being found on all chromosomes within the wheat genome, except that of chromosome 7D (Buerstmayr *et al.* 2009). A small selection of the 52 QTL studies referenced by Buerstmayr *et al* (2009) is shown in Table 1.3. Several additional references on evaluating germplasm for FHB resistance via marker-assisted selection (nine studies) and marker-assisted germplasm evaluation (seven studies) are also given by Buerstmayr *et al.* (2009).

Table 1.3. A small selection of studies showing chromosomes which have been identified as containing traits for FHB resistance.

Plant material	Chromosomes identified with resistance QTL	FHB trait	Reference
Sumai-3 x Stoa	3BS, 6BS, 2AL, 4B	FHB spread	Waldron *et al.* (1999)
Huapei 57-2 x Patterson	3BS, 3BL, 3AS, 5BL	FHB spread	Bourdoncle and Ohm (2003)
CM-82036 x Remus	3BS, 5A	FHB severity	Buerstmayr *et al.* (2003a,b)
Wangshuibai x Alondra	2D, 3BS, 4B, 5B, 7A	FHB severity	Jia *et al.* (2005)
Patterson x Goldfield	2BS, 2B, 7B	FHB incidence	Gilsinger *et al.* (2005)

(Source: Buerstmayr *et al.* 2009).

The most likely QTL sources for incorporation into a wheat breeding programme for FHB resistance is likely to come from chromosome 3BS, 5AS and 6BS (Buerstmayr *et al*, 2009), since studies have shown these to be the most repeatable QTLs. Additionally, numerous genes from monocots and dicots have been found, upon their transgenic over expression, to confer enhanced resistance to FHB in wheat (Walter *et al.* 2010) as shown in Table 1.4.

Table 1.4. Transgenic overexpression of genes associated with FHB resistance in wheat or barley.

Gene/protein name	Pathogen species	Phenotype	Reference
Maize *b-32* gene (ribosome Inactivating protein)	*F. culmorum*	Reduced FHB severity	Balconi *et al.* (2007)
Barley chitinase II	*F. graminearum*	Enhanced Type II resistance/ reduced FHB severity	Shin *et al.* (2008)
Wheat chitinase + b-1,3-glucanase	*F. graminearum*	Slower disease spread, but no improved resistance	Anand *et al.* (2003)
Barley b-1,3-glucanase	*F. graminearum*	Reduced FHB severity	Mackintosh *et al.* (2007)
Arabidopsis npr1 (non-expressor of PR proteins)	*F. graminearum*	Enhanced Type II resistance	Makandar *et al.* (2006)
Wheat α-1-purothionin	*F. graminearum*	Reduced FHB severity	Mackintosh *et al.* (2007)
Barley *tlp-1 (*thaumatin like protein)	*F. graminearum*	Reduced FHB severity	Mackintosh *et al.* (2007)
Rice *tlp (*thaumatin like protein)	*F. graminearum*	Slower disease development	Chen *et al.* (1999)

(Source: Walter *et al.* 2010)

1.4.3 Biological Control

The use of biological methods for controlling FHB in wheat have been suggested. Kohl & Fokkema (1998) describe how, by applying antagonistic fungi to crop debris in the field, the survival and multiplication of necrotrophic pathogens may be reduced. Luongo *et al.* (2005) investigated the potential use of fungal antagonists in the control of *Fusarium spp*. It was found that the use of *Clonostachys rosea* was able to consistently suppress the sporulation of *F. culmorum* and *F. graminearum* on wheat straw.

The use of *Alternaria alternata*, *Botrytis cineria* and *Cladosporium herbarum* as fungal competitors with *F. culmorum* each showed a decrease in spikelets infected with FHB and this was attributed to their production of volatile and non-volatile anti-biotics (Liggitt *et al.* 1997). The use of various fungicides on these fungal competitors was shown to reduce their mycelial growth and it was concluded that the use of fungicides to control FHB may have detrimental effects on saprophytic microflora whose presence normally help suppress FHB occurrence.

1.4.4 Chemical control

Applications of fungicides are made to ears around GS60-GS65 to help control pathogens of the ear disease complex. This has the potential to reduce DON concentration in wheat by targeting FHB pathogens.

Spray timing during flowering is key to the targeting of FHB with fungicides. Timing is based on crop growth stages, and not due to visual symptoms, as these underestimate the extent of fungal infection (Brown *et al.* 2010). When visual symptoms are present on a few

spikelets, it is possible for up to a third of the ear to be fully infected (Hammond-Kosack, 2010). Pirgozliev *et al.* (2008) showed that tebuconazole applied at GS65 gave the greatest reduction in FHB when applied two days prior or two days post artificial inoculation as GS65. This contrasts with Brown *et al.* (2010) who showed that natural infection occurs earlier than previously thought and that applying fungicides mid-anthesis may be too late since symptomless infection may have already occurred, and that for fungicides to be fully effective, they need to be applied immediately after infective spores are detected within the crop. For maximum fungicide efficacy, the application should cover as much of the ear as possible (Liggitt *et al.* 1997). Research has shown that earlier fungicide applications at GS31 and GS39, in addition to ear sprays provides better FHB control and DON reduction than an ear spray alone (Edwards & Godley, 2010). It was found that applications of prothioconazole at GS31, GS39 and GS65 gave an additive level of control by reducing FHB infections by 97% compared to 83% reduction achieved by treating at GS65 alone, and by reducing DON concentration by 83% compared to 57% reduction achieved at GS65 alone.

The effects of fungicide treatment on various FHB pathogens has been investigated (Jennings *et al.* 2000; Paul *et al.* 2007) showing that the triazole fungicides tebuconazole and metconazole are able to give effective control against mycotoxin producing *Fusarium* species and hence give a reduced DON content in grain. Beyer *et al.* (2006) showed that full dose applications of both tebuconazole and metconazole gave a 58% and 60% reduction in DON respectively. It has been hypothesised by Edwards *et al.* (2001) that tebuconazole and metconazole successfully reduce DON contents in harvested winter wheat grain by reducing the presence of trichothecene-producing *Fusarium* in the ear and not by changing the rate at which the DON mycotoxin is produced. Kang *et al.* (2001) however contradict this hypothesis by indicating that tebuconazole reduced the ability of *F.*

culmorum to produce DON. Paul *et al.* (2007) found a large variation in DON control which could not be explained, showing that the use of tebuconazole can give variable reductions in DON and it is not a complete solution. In addition to reductions in FHB and DON, the use of tebuconazole in artificially infected wheat has been found to achieve an increased yield, Thousand Grain Weight (TGW) and kernel numbers per ear (Homdock *et al.* 2000; Jones, 2000). The most recently developed FHB active fungicide is prothioconazole. Studies by Lechoczki-krstak *et al.* (2008) found that the greatest reduction in DON of 81% was achieved using prothioconazole whereas epoxiconazole + krezoxim-methyl and tetraconazole achieved only a 45% DON reduction. In comparison to triazole fungicides, the strobilurin fungicide azoxystrobin gave effective control against the non-mycotoxigenic *M.nivale,* but did not prove effective against *Fusarium* species (Jennings *et al.* 2000). In these experiments, where both *Fusarium* and *Microdochium spp.* were present on ears, the use of azoxystrobin removed competition for the mycotoxigenic *Fusarium* species resulting in a rise in grain DON content. Pirgozliev *et al.* (2008) found that tebuconazole worked most consistently at reducing DON when applied alone, although research by Haidukowski *et al.* (2005) showed that tebuconazole mixed with azoxystrobin, in a studies using *F. graminearum* and *F. culmorum*, managed to achieve a significant DON reduction compared to the inoculated control.

Selection of *F. graminearum* for tebuconazole resistance in vitro has shown that the continual use of tebuconazole in the field *could* potentially lead to fungicide resistance (Becher *et al.* 2010) and this may need to be a factor considered in future years. First signs of *Microdochium* resistance to strobilurins have been found (Walker *et al.* 2009) and attributed to several resistance mechanisms which have evolved independently in populations.

1.5 PASSIVE RESISTANCE MECHANISMS

Resistance types are usually classed as being either morphological or physiological (Rudd *et al.* 2001), of which the term 'morphological' is synonymous with 'passive' in that it is independent of the physiological status of the plant (Mesterhazy, 2003). This means that resistance is not due to an active response from the plant to resist the pathogen, but due to the plant's intrinsic morphology, which results in a reduced likelihood of contact occurring between the plant and the pathogen (Arquaah, 2007). In terms of passive resistance to FHB, it is generally agreed that these sources of resistance are of minor significance when compared to active resistance (Rudd *et al.* 2001).

Several interchangeable, and perhaps poorly defined, terms are used within the subject of passive disease resistance in plants. Commonly used terminology includes disease escape, disease avoidance and apparent resistance. Burdon (1987) describes how disease 'escape' and 'avoidance' relate to very different aspects of passive resistance and therefore further discussion of each term is required.

1.5.1 Disease escape

Disease escape, as defined by Burton (1987), is the result of chance circumstances in which host and pathogen fail to come into contact, despite both being present in the same environment at the same time. Examples can be given in which plant populations are described as able to '*escape*' from the development of biotic disease in both a natural and cultivated environment.

Within the natural environment, lower infection rates of anther smut fungus (*Microbotrytum violaceum)* were found on smaller patches of the perennial plant *Viscaria*

vulgaris, with no infection being found at all on patches smaller than 35 host plants (Jennersten *et al.* 1983). This was attributed to the pollinating insect vector of the fungus visiting each individual *Viscaria vulgaris* plant at random, irrelevant of patch size, therefore the probability of an insect-carried spore reaching a given host population is greater the larger the population size. Frantzen (2000) extrapolates in relation to Jennersten *et al.*'s (1983) work that smaller plant populations are a method that wild plants use to help escape being 'detected' by pathogens. This contrasts cultivated crops, which are planted over large areas, and hence can be easily 'detected' by pathogens. Furthermore, seed dispersal can also be a mechanism via which wild plant species may escape pathogens by causing a distance between the infected mother plant and the resulting offspring, which the pathogen may not be able to bridge (Augspurger, 1983).

Within the farmed environment, strategies can also be applied for assisting disease escape within cultivated crops. The production of crops utilising a combination of both a susceptible and a resistant variety can hinder the spread of a pathogen within the susceptible variety (Wolf, 1985). This leads to the overall crop's resistance to the pathogen being similar to that of the resistant variety alone. The use of varietal mixtures with differing levels of resistance can slow a pathogen's development by both increasing the distance between susceptible plants and by using resistant varieties as a physical barrier between susceptible plants, known as the 'barrier effect' (Frantzen, 2000). The net result of these two aforementioned factors is that plants susceptible to the invading pathogen may escape from the disease. However, as disease escape results from a fortuitous set of circumstances, Burdon (1985) states that it should not be seen as a plant resistance mechanism. Several cultural control practices may, in themselves, be regarded as a form of disease escape for crops: for example ploughing aids disease escape by fully inverting

infected crop residues under the soil, therefore assisting the crop to escape from pathogenic inoculum.

1.5.2 Disease avoidance

Disease avoidance, as defined by Burton (1987), has a genetic basis that may be the result of selection pressures on the host plant population from past pathogen encounters. Several examples can be given in which plant populations are able to '*avoid*' the development of biotic disease. Since disease avoidance is defined as having a genetic origin, Burton classifies it as being an actual plant resistance mechanism, albeit a passive one. The concept of disease avoidance is based upon multiple factors, two of which are described as being the physical protection of susceptible tissue parts against the pathogen's infection, or differences in the rate of development (i.e. flowering dates) (Frantzen, 2000).

Closed-flowering varieties of wheat and barley display disease avoidance traits as they can avoid spike infections with ergot (*Claviceps purpurea*) and loose smut (*Ustilago nuda*), since the pathogen's route of infection via the stigma is blocked until after pollination has occurred, resulting in significantly reduced infections. From a breeding perspective, Arraiano *et al.* (2009) showed that several morphological traits in wheat (plant height, leaf morphology and heading date) could confer an element of disease avoidance to infection of aerial plant parts from *Mycosphaerella graminicola*. It is interesting to note that in Arraiano *et al's* study, they refer to this as a disease escape mechanism whereas, since it has a genetic basis and is not a result of chance circumstance, it would actually be classified as disease avoidance according to Burden's (1987) definition.

1.6 PASSIVE RESISTANCE TRAITS

Several morphological and physiological traits in cereals have been proposed to influence Type I resistance to FHB infections (Walter *et al,* 2010; Graham and Browne, 2009; Schmolke *et al,* 2005; Gautam *et al,* 2012). Such proposed traits include plant height, heading date and flowering duration, anther extrusion, presence or absence of awns, ear density, flower opening width, waxy surfaces on ear tissue, tiller number and ear length. Additionally, several other traits in cereals have been associated with influencing Type II resistance to FHB (Walter *et al,* 2010), including smaller diameter vessels, denser vascular bundles in the rachis, stronger thickened cortical sclerenchyma and cell walls and shorter internodes in the upper part of rachis joints.

1.6.1 Plant height

Early research into the relationships between plant height and FHB resistance found that shorter plants, in naturally infected field trials, tended to have increased levels of FHB severity. This relationship was however not identified in artificially inoculated glasshouse experiments (Mesterhazy, 1995). This led to the assumption that taller varieties exhibited a disease escape mechanism through the wheat ear being further away from the primary source of inoculum on the soil surface. Work by Hilton *et al.* (1999) however showed that this negative relationship still existed when spore suspensions were applied directly to the ear in the field, negating the role of disease escape. The authors reasoned that the negative relationship between FHB severity and plant height had an underlying genetic basis in which gene linkage, pleiotropy, or a combination of the two, were likely to be involved. Additional studies (Miedaner, 1997; Steiner *et al.* 2004; Yan *et al.* 2011) have suggested

that taller plants have reduced FHB symptoms due to differences in microclimate effects at ear height. Yan *et al.* (2011) showed that at their natural height, tall wheat isolines displayed better Type I FHB resistance than short isolines, but this difference largely disappeared when the short isolines were physically raised up to ensure their ears were at the same height as the taller isolines. This difference was put down to either direct or indirect effects of height differences *per se*, including microclimate. However, in contrast Hilton *et al.* (1999) showed that differences in relative humidity during flowering at differing ear heights could not explain the differences in severity between wheat lines.

It is now widely accepted that there is a clear negative relationship between FHB susceptibility and plant height that is due to deleterious gene linkages rather than effects of plant height *per se* (Draeger *et al.* 2007). Multiple studies including Hilton *et al.* (1999); Buerstmayr *et al.* (2000); Gervais *et al.* (2003); Somers *et al.* (2003), Schmolke *et al.* (2005); Klahr *et al.* (2007); Srinivasachary *et al.* (2008); Srinivasachary *et al.* (2009); Tamburic-Ilincic (2012); Yan *et al.* (2011); Chu *et al.* (2011); Buerstmayr *et al.* (2012); Nemati and Hokmalipor (2012); Suzuki *et al.* (2012); Lu *et al.* (2013); Liu *et al.* (2013) and He *et al.* (2014) have identified correlations between plant height and FHB susceptibility ranging from r=-0.22 to r=-0.70 and several of these studies have successfully identified the genes underlying this relationship. A relationship between plant height and FHB has not however been found in every study. For example, Chu *et al.* (2011) reported that, while their study found strong negative relationships between plant height, FHB severity and FDK, the relationship between plant height and DON production was much more variable. Additionally, Suzuki *et al.* (2012) found no relationship between plant height and measurements of FHB within the same year. This suggests that a reduction in plant height is not always followed by increases in FHB infection or mycotoxin production and this is of relevance to wheat breeders and producers alike.

Studies have shown that several plant height QTLs overlap with QTLs relating to FHB resistance. Such overlapping QTLs have been found on chromosomes 1A (Schmolke *et al.* 2008), 2B (Gervais *et al.* 2003), 3BS (McCartney *et al.* 2007), 4B (Buerstmayr *et al.* 2012; McCartney *et al.* 2007), 5A (Gervais *et al.* 2003; Paillard *et al.* 2004), 6A (Schmolke *et al.* 2005; Haberle *et al.* 2007), 7A (Klahr *et al.* 2007) and 7BS (Haberle *et al.* 2007). Several semi-dwarfing genes have been identified, including *Rht-B*1b and *Rht-D*1b, as having pleiotropic effects on increased FHB susceptibility (Lu *et al.* 2013). Despite this, not every dwarfing QTL leads to increases in FHB susceptibility, such as that found on chromosome 4A (Gervais *et al.* 2003).

The development of new FHB resistant wheat varieties has become an increasing priority for wheat breeders as has the mitigation of the negative effects of semi-dwarfing genes on FHB resistance. This can be achieved through careful selection of semi-dwarfing genes. For example, *Rht-B*1b and *Rht-D*1b semi-dwarfing genes both give similar height reductions to each other yet *Rht-B*1b can provide the desired reduction in crop height, without compromising FHB resistance as much as *Rht-D*1b (Miedaner and Voss, 2008; Srinivasachary *et al.* 2009). Currently, most varieties of UK winter wheat are highly susceptible to FHB as they are carriers of the *Rht-D*1b gene (Srinivasachary *et al.* 2009). Lui *et al.* (2013) suggested that the use of dwarfing genes from group 8 (e.g. Rht8c) could be of use in breeding programmes where resistance to FHB is a priority due to its reduced deleterious effects on FHB resistance when compared to semi-dwarfing of the *Rht-B*1b and *Rht-D*1b genes. However, not all sources of FHB resistance can however be easily incorporated into a breeding programme. He *et al.* (2014) excluded several wheat lines that were taller than 120cm from further study within their experiment, stating that, while new resistance genes or alleles may be present in these tall lines, experience in China has shown such lines to be low yielding and of little use in breeding programmes. A key development

in the future breeding of FHB resistant lines is the creation of recombinant lines in which the genetic linkage between plant height and FHB resistance is broken (Suzuki *et al.* 2012).

1.6.2 Tiller number

The tiller production process commences upon the initiation of tiller bud primordia at the shoot apex after germination, after which tillers are produced from these buds in the leaf axils on the main stem (Kirby and Appleyard, 1984). Tiller number often differs between wheat cultivars due to differences in tiller initiation and survival, with many tillers usually aborting before producing fertile ears, usually between the onset of stem extension and anthesis (Sharma, 1995). Tiller mortality occurs during this period due to the increased competition for assimilates from the stem (Slafer and Rawson, 1994).

Few studies have examined the relationship between tiller number per unit area and FHB severity. Gautam *et al.* (2012) hypothesised that tillers may act either as a barrier to the movement of Fusarium spores up the plant to the ear, or that tillers could contribute to the late increases of grain DON content due to the later tillers' delayed physiological maturity. The study found levels of DON in grain to be statistically higher in the main stem ears of barley and spring wheat, yet higher in the grain of secondary tillers in winter wheat, although this was not statistically significant. It was hypothesised that levels of DON in main stem ears may be due to the main stem ear reaching anthesis earlier and allowing the invading pathogen more time to invade the wheat tissues leading to higher DON content. The study concluded that breeding work to create cultivars with an increased tiller number could be used to dilute the presence of DON in the harvested crop, and that altering planting densities to increase the proportion of tillers in a crop may have the same effect of

reducing DON content. Wheat has been shown to compensate for low plant populations up to 60 plants m^{-2} with increased tillering (Whaley *et al.* 2000), and ear fertility to maintain grain yield, but a strategy of breeding for reduced ear population density would need to ensure that significant reductions in yield did not occur.

1.6.3 Heading date

The earliest reports of a relationship between heading date and FHB severity stated that earlier maturing cultivars had improved FHB resistance (Arthur, 1891; Found in Steiner *et al.* 2004). Studies have since found correlations between heading date and measurements of FHB severity that are positive (Hori *et al.* 2005; Steiner *et al.* 2004; Tamburic-Ilincic 2012), negative (Schmolke *et al.* 2005; Gervais *et al.* 2003; Paillard *et al.* 2004; Buerstmayr *et al.* 2012; He *et al.* 2014), positive and negative, (Klahr *et al.* 2007; Chu *et al.* 2011; Suzuki *et al.* 2012) and some found no correlation at all (Liu *et al.* 2007; Buerstmayr *et al.* 2000). This shows that there is no fixed consensus as to the direction of this relationship.

Several of the above studies have attempted to rationalise their results. He *et al.* (2014) found negative correlations between heading date, DON and FDK, reasoning that early flowering lines had been exposed to the epidemic environment for a longer period of time than late flowering lines. The authors continue, however, to warn that selecting for late flowering lines with low infection levels was not advisable as the negative association between days to heading and DON/FDK was caused by developmental or epidemiological conditions, showing that He *et al.* (2014) believed heading date could be a passive resistance trait. Lui *et al.* (2007) propose that FHB associations with heading date are artifacts caused by field inoculation protocols, rather than real effects. They state that in

their glasshouse studies in which inoculation can be made at exact developmental stages, more often than not, resistances to FHB were independent of heading date.

Multiple studies have found overlapping QTL for FHB resistance and heading date. Schmolke *et al.* (2005) identified an overlapping QTL for FHB resistance and heading date on chromosome 7BS. Paillard *et al.* (2004) identified six QTLs related to heading date of which two, QEet.fal-6DL and QEat.fal-5BL, overlapped with a FHB resistance QTL. A subsample of lines were used to confirm that overlapping QTLs were not solely a result of inoculation or scoring methods, concluding that correlations between FHB and heading date was not due to escape mechanisms of later flowering genotypes. Klahr *et al.* (2007) also found multiple overlapping QTLs for FHB severity and heading date, of which QFhs.whs-5B remained significant after adjusting the Area Under Disease Progress Curve (AUDPC) to avoid confounding effects of plant height and heading date. The authors therefore concluded, in agreement with Paillard *et al.* (2004), that heading date is not a true disease escape mechanism. In contrast, Salameh *et al.* (2011) found no overlapping QTL between FHB resistance and heading date.

Within RILs and DH populations, heading dates have been found to vary widely (Gilsinger *et al.* 2005; Klahr *et al.* 2007; Chu *et al.* 2011; Suzuki *et al.* 2012; Liu *et al.* 2007; Buerstmayr *et al.* 2000) yet variation in heading dates between years appears to be fairly constant (r= 0.853) (Suzuki *et al.* 2012). Broad-sense heritability for heading dates of between 0.76 and 0.95 have been found (Paillard *et al.* 2004; Klahr *et al.* 2007; Schmolke *et al.* 2005 and Buerstmayr *et al.* 2000) showing that selection of specific heading dates in a breeding programme is possible and will be a stable trait in the field. The use of variation in heading date by breeders to create lines with improved FHB resistance has been discussed. Buerstmayer *et al.* (2000) concluded from their experiments that it is possible to select for FHB resistant genotypes that are largely independent of flowering time.

Buerstmays *et al*'s conclusion is however contradicted by Emrich *et al.* (2008) who proposed a statistical approach to breeding to allow for the selection of FHB resistant lines while avoiding indirectly selecting for later maturing lines, which as Emrich *et al.* (2008) state, is required due to negative correlations between heading date and FHB severity. Additionally, in relation to genes on chromosome 7BS controlling both heading date and FHB resistance (Schmolke *et al.* 2005), Emrich *et al.* (2008) discuss how their statistical approach to breeding would block such pleotrophic genes from selection, exerting selection pressure instead on genes determining FHB resistance and not for heading date.

1.6.4 Ear length and density

Variation in the shape, size and density of wheat ears can differ noticeably between lines (Waines and Hegde, 2003) although these traits are fairly uniform within a single variety. Each ear is made up of between 10-30 spikelets, which appear in an alternating pattern along the length of the central rachis.

Hexaploid wheat populations derived from wide genetic crosses involving *Agropyron elongatum* L., *Triticum polonicum* L. and *Triticum aestivum* L. var. Morocco wheat (Rajaram and Reynolds, 2001) have been developed at CIMMYT to exploit heterosis, which, when grown as spaced plants, have intermediate tillering capacity (up to 10 tillers), long spikes (30 cm) and high spike fertility (up to 200 grains per spike). CIMMYT has developed new advanced lines derived from crosses with these novel wheats which have more grains per spike compared to modern CIMMYT releases when grown as spaced plants. Gaju *et al.* (2009) showed for a CIMMYT large spike phenotype that spikelets per spike (+4%), grains per spike (+5%) and individual grain weight (+10%) were increased compared to a check line Bacanora, but grain yield was reduced (-8%) due to fewer spikes

per square metre (-26%). Large-spike "Gigas" phenotypes, having up to 30 spikelets, 9 grains per spikelet and individual grain weight of 63 mg, were characterised by Atsmon and Jacobs (1977), exhibiting tiller inhibition attributed to a single recessive gene *Tin1A* on chromosome 1AS (Richards, 1988; Spielmeyer and Richards, 2004).

Ear traits have been studied as a potential source of FHB resistance. Weak, but significant, correlations between FHB and ear density have been found, with both positive (Buerstmayr *et al.* 2012) and negative (Steiner *et al.* 2004) relationships reported. Negative relationships between FHB and ear length (Suzuki *et al.* 2012) have been found. However, other studies have failed to identify any significant relationship between FHB and ear traits in wheat (Somers *et al.* 2003; Schmolke *et al.* 2005; Liu *et al.* 2007; Nemati and Hokmalipour, 2012) or barley (Hori *et al.* 2005; Dahleen *et al.* 2012).

Despite the lack of reported relationships between FHB and ear traits, overlapping QTLs controlling both have been identified. The 'Sumai-3' alleles at the 4BS and 5AS QTLs which reduce FHB severity have been identified as increasing both stem and ear length in wheat, with a significant positive correlation between ear and stem length of r=0.55 (Suzuki *et al.* 2012). QTL on the 6AL chromosome controlling ear density have also been found to overlap with QTL for FHB resistance and plant height, despite no significant correlation between ear density and FHB severity being observed within the study (Schmolke *et al.* 2005). Studies have found individual QTL on chromosomes for both ear density (5A and 7A) and FHB severity (3B, 4B, 6A, 6B and 7B) which did not overlap for the two traits (Buerstmayr *et al.* 2012) while other studies failed to identify any QTL at all relating to ear traits, despite finding multiple QTL associated with FHB severity on chromosomes 2B, 3B 4BL and 5A (Liu *et al.* 2007).

Theoretical passive resistance mechanisms related to low ear density include the ability for an ear to dry quickly after a rain shower, creating a less favourable environment for FHB infection. Alternatively, less dense ears may have increased Type II resistance (spread within the ear) by the invading fungal pathogen having to travel a greater distance between spikelets within the ear.

1.6.5 Awn presence and length

The presence and length of awns has long been discussed as a potential route of FHB resistance or susceptibility. As a potential passive resistance trait, awns may theoretically reduce FHB infections by acting as a barrier to spores being deposited on the ear, or from the opposite perspective, awns may increase FHB infections by increasing the overall area of the ear through which spores can infect.

Mesterhazy (1995) first reported that lines of wheat with longer awns were of increased susceptibility to FHB under natural conditions. Since then, several studies have shown that longer awns can increase FHB infection (Somers *et al.* 2003; Lui *et al.* 2013), some that longer awns can decrease FHB infection (Buerstmayr *et al.* 2000; Ban and Suenaga. 2000; Buerstmayr *et al.* 2012), and some that there is no relationship between the two (Buerstmayr *et al.* 2000; Buerstmayr *et al.* 2002; Hori *et al.* 2005; Liu *et al.* 2007).

Several studies have found QTLs that overlap for the presence of awns and FHB resistance, of which the first such report of a genetic linkage existing was identified on chromosome 4B (Snijders, 1990). QTLs on chromosome 5A have since been identified which overlap both FHB resistance and the phenotypic marker of awnedness, B1 (Gervais *et al.* 2003; Lui *et al.* 2013). Ban and Suenga (2000) concluded too that the awn suppressor

gene on chromosome 5A (either B1 or B2) was responsible for controlling awnedness in their population whereas, although Buerstmayr *et al.* (2002) found significant effects on FHB from chromosome 5A, this QTL was not associated with the presence or absence of awns in their study. An entirely different major gene for awn length has been identified on chromosome 6BS, although it was found not to be coincident with any FHB resistance QTL within the study (Somers *et al.* 2003). Despite the reported genetic linkage between FHB and the presence of awns, it has been shown that the linkage between the two can be easily broken (Gervais *et al.* 2003) and the development of awnless cultivars with improved levels of FHB resistance being easy to produce.

1.6.6 Anther extrusion

Wheat is a kleistogamus plant, meaning that it is self-pollinating and that pollination and fertilization occur prior to the anthers being extruded from the florets (Rawson & Evans, 1970). Anthesis does not occur simultaneously within each ear, but spreads both up and down from an initial flowering point in the middle, taking around 2-3 days to be complete within an individual ear.

It was previously thought that anthers extruded from the wheat ear were the initial route of *Fusarium* infection (Dickson *et al.* 1921). It was however, later observed that anthers retained within the floret provided this initial route instead (Pugh, 1933). Three distinct anther characteristics exist in wheat, including ‘retained anthers’ (anthers not extruded from the ear), ‘trapped anthers’ (partially extruded and trapped between the lemma and palea of the ear) and ‘fully extruded anthers’ (Graham and Browne, 2009) and these three scenarios can occur in continuously variable ratios within each ear.

The first reports of associations between anther extrusion and FHB were made in Chinese germplasm in the 1980s (He *et al.* 2014). The majority of studies since have shown that there is a negative correlation between the degree of anthers extruded from the floret, and the amount of FHB infection and DON contamination (Skinnes *et al.* 2005; Skinnes *et al.* 2008; Graham and Brown. 2009; Skinnes *et al.* 2010; Buerstmayr *et al.* 2012; Lu *et al.* 2013; He *et al.* 2014). In contrast, other studies have found that closed flowering lines have less FHB than lines that extrude their anthers (Kubo *et al.* 2010; Kubo *et al.* 2013; Gilsinger *et al.* (2005). In Kubo et al's (2010) study, 110 open flowering lines and only 16 closed flowering lines were used. This probably occurred because anther extrusion is a continuously distributed trait (Skinnes *et al.* 2008; Lu *et al.* 2013) and classing lines into open or closed flowering may be an over simplification of the situation.

It has been suggested (Skinnes *et al.* 2010) that retained anthers provide a good growth medium for *Fusarium* to infect and colonise the ear as anthers are the best growth substrate for *F. graminearum* (Pierce, 1976). Additionally, anther extracts have been shown to enhance fungal growth more than that of either palea or lemma extracts (Engle *et al.* 2004) with the study suggesting that this was due to higher sugar concentrations in the anthers than that of other floral parts. Anthers containing increased levels of choline and betaine have been shown to act as a stimulant to *Fusarium* growth (Strange *et al.* 1974). However, further research appears to refute this by showing that anther extracts of a resistant variety increased mycelial growth whereas anther extracts from a susceptible cultivar reduced it (Nkongolo *et al.* 1993). Early research showed that the emasculation of anthers after point inoculation successfully reduced FHB infection (Liang *et al.* 1981). Research by Engle *et al.* (2004) and Liang *et al.* (1981) both help to explain why negative correlations exist between the degree of anther extrusion and FHB, in that, having anthers extruded from the floret removes both a route of infection and source of energy from the pathogen.

Skinnes *et al.* (2008) explain how low anther extrusion lines enable inoculum to penetrate the floret and activate active resistance mechanisms, whereas for high anther extrusion lines, active resistance mechanisms contribute much less to overall resistance due to the reduced initial inoculum load entering the floret. This shows that anther extrusion, as a disease escape mechanism (as proposed by Skinnes *et al.* 2008; Skinnes *et al.* 2010 and He *et al.* 2014), may act as the first barrier to FHB (Type I resistance) in the early stages of infection (Skinnes *et al.* 2010; Graham and Brown. 2009; Lu *et al.* 2013), followed by active resistance mechanisms as the second barrier once passive resistance mechanisms have been overcome. The Type I resistance that anther extrusion could offer may provide sufficient protection in situations where FHB epidemics are sporadic (He *et al.* 2014) and this could be an important consideration for breeding programmes where FHB resistance is a priority. High levels of anther extrusion are however not the pinnacle of *Fusarium* resistance as hyphae have been shown to be able to grow around the external ear tissue reaching the inner lemma and palea surfaces (Kang and Buchenauer, 2000).

Across several years of trials, correlations between anther extrusion and FHB severity have been shown to be relatively stable (Lu *et al.* 2013), with the authors proposing that this indicated a genetic basis. The study identified that all QTLs for anther extrusion overlapped with QTLs for FHB severity. However, most of the QTLs detected for anther extrusion by Lu *et al.* (2013), except that on chromosome 7AL, differed from those found by Skinnes *et al.* (2010) who found 3 major anther extrusion QTL on chromosomes 1AL, 4DL and 6AS, suggesting that anther extrusion is controlled by several genes, each individually having small to moderate effects, of which some of the QTL may overlap with FHB resistance. A significant positive correlation between anther extrusion and plant height ($r = 0.43$) has been identified (Lu *et al.* 2013), of which plant height is a well documented trait that is negatively correlated with FHB severity (Hilton *et al.* 1999; Chu *et*

al. 2011; Lu *et al,* 2013; He *et al,* 2014). Two common QTL were found between anther extrusion and plant height, in which low anther extrusion and reduced plant height led to increased FHB susceptibility (Lu *et al.* 2013). The authors concluded that, despite some common genetic control between the traits, completely independent genes mostly controlled their variability and that developing FHB resistant lines with high anther extrusion and short plant height is entirely possible.

Early screening for high anther extrusion may be a useful first step in reducing the contamination of mycotoxins in hexaploid wheat (Skinnes *et al.* 2010). The distribution of anther extrusion versus FHB is fan shaped, with high anther extrusion lines having consistently low FHB severity, and low anther extrusion lines having a much wider range of severities (He *et al.* 2014; Skinnes *et al.* 2010) indicating that high anther extrusion could be a reliable trait in breeding programmes by offering a consistent reduction in FHB infection. Low interactions between anther extrusion and year (Skinnes *et al.* 2005) and high broad-sense heritability for anther extrusion (0.90 - 0.91) (Skinnes *et al.* 2005; Skinnes *et al.* 2010) make this trait suitable for use in a breeding programme. Studies have shown the degree of flower opening to be related to FHB severity (Gilsinger *et al.* 2005) however Skinnes *et al.* (2010) comment that anther extrusion is significantly easier to monitor in a breeding programme than flower opening and that anther extrusion itself may well encompass a degree of flower opening.

1.7 OBJECTIVES AND HYPOTHESES

The objectives of this study were:

1) To quantify a range of physiological traits in field and glasshouse experiments using a selection of double haploid, UK elite genotypes and highly FHB resistant wheat genotypes.

2) Assess FHB resistance of the selected wheat genotypes in ground inoculated field experiments and spray inoculated glasshouse experiments using a combination of visual disease assessments, real-time PCR and mycotoxin quantification.

3) Identify significant relationships between physiological traits and FHB in ground inoculated field experiments.

4) Identify significant relationships between physiological traits and FHB in spray inoculated glasshouse experiments.

5) Elucidate which physiological traits significantly related to FHB provide a true passive resistance mechanism, by drawing comparisons between field and glasshouse experiments.

The following hypotheses were developed within the study:

i) Strong correlation between visual FHB symptoms, pathogen DNA and grain mycotoxin contamination will be present.

ii) Each grain mycotoxin contaminant will be related to a specific *Fusarium* producer species.

iii) A number of physiological traits in wheat, including tiller number, will be positively related to visual FHB symptoms and mycotoxin contamination in ground inoculated field experiments.

iv) A number of physiological traits in wheat, including plant height and anther extrusion, will be negatively related to visual FHB symptoms and mycotoxin contamination in ground inoculated field experiments.

v) A number of physiological traits in wheat will be significantly positively related to visual FHB symptoms in spray inoculated glasshouse experiments.

vi) A number of physiological traits in wheat will be significantly negatively related to visual FHB symptoms in spray inoculated glasshouse experiments.

vii) The physiological traits related to FHB in ground inoculated field experiments will be different under high and low disease pressures created by misting during anthesis.

viii) A number of physiological traits related to FHB in ground inoculated field experiments, but not spray inoculated glasshouse experiments, will confer true passive FHB disease escape mechanisms.

Chapter 2.

General materials and methods

2.1 PLANT MATERIAL

All field and glasshouse studies were conducted using a combination of the UK elite winter wheat varieties Claire, Rialto, Solstice, Ambrosia and Grafton and a set of doubled-haploid (DH) lines jointly developed by CIMMYT, Mexico and the University of Nottingham. The DH lines were derived from a cross between a large-spike phenotype CIMMYT spring wheat advanced line L8 and the UK winter wheat Rialto. The CIMMYT advanced line L8 is the result of a wide-crossing programme performed by CIMMYT in the 1990s involving *Agropyron elongatum*, *Triticum polonicum* and *Triticum aestivum* (var. Morocco) to create restructured hexaploid wheat plant types exploiting heterosis (Rajaram and Reynolds, 2001). The UK winter wheat cultivar Rialto was crossed with the CIMMYT advanced line L8 (CMH8OA.763-1B-1Y-2B-3Y-OY) to generate a DH population of 88 lines using the maize pollination technique (Laurie and Bennett, 1986). A selection of 10-16 lines from this L8 x Rialto DH population were chosen for use in each experiment based on their wide expression of traits such as plant height, canopy density, ear length and spikelet number, using a previous field data set (Foulkes. pers. comm.). Seed used in the experiments was provided by CIMMYT, via John Foulkes (The University of Nottingham), and was grown each year in the field at the University of Nottingham to maintain stocks of each line.

2.2 GROUND INOCULUM PRODUCTION

All 20 isolates (Appendix 2A) were stored on Potato Dextrose Agar (PDA) slopes at 4^{o}C before being subcultured onto PDA plates (Sigma-Aldrich, UK). After 7 days each isolate had 5 plugs removed to inoculate a conical flask containing 100 ml of sterile potato dextrose broth (Sigma-Aldrich, UK). Flasks were sealed and placed on an orbital shaker for 5 days at 20^{o}C while being mixed at 90 rpm. Oats (650 g) and 65 ml of deionized water

were each added to 40 autoclave bags before being autoclaved at 121^{o}C for 1 hour, cooled overnight and the autoclave process repeated the following day. Inoculated PDB (50 ml) was added to each bag of sterile oats, giving two bags of each isolate. Bags were incubated at room temperature for 14 days before being thoroughly mixed together to create a compound inoculum and evenly applied at GS 31 to plots at a rate of 37.5g m^{-2}.

2.3 FIELD TRAIT ASSESSMENTS

When each plot reached GS39 (Zadoks *et al.* 1974) and GS65, a 0.25m^{2} quadrat (0.5m x 0.5m) of plants was sampled avoiding the outer row of the plot. The above-ground plant tissues underwent a detailed laboratory growth analysis to determine crop canopy architecture traits, as described in Sections 4.3 and 5.3. Regular assessments of developmental stage were made every 2-3 days to ensure that each genotype was sampled at the correct growth stage.

At each growth stage, the plants in the sample were separated from one another and counted to determine plant number per m^{-2}. Then five plants were blindly selected for assessments of tiller number and plant height, flag leaf traits and ear traits on the main stem. Tiller number was recorded as the total number of fertile shoots per plant minus the main stem, and plant height was measured from the base of the stem to the tip of the ear. Flag leaf length was measured from the ligule to the leaf tip, flag leaf width was measured across the leaf at half its length, and flag leaf height was measured from the base of the stem to the flag leaf ligule. Ear traits included the ear length, measured from the ear base to the tip, spikelet number counted the total number of fertile spikelets per ear, spikelet density was calculated as the spikelet number divided by the ear length, awn length measured awns on two randomly selected spikelets on opposing sides of the ear to

calculate the mean, and hair on ears was recorded as either present or absent. The roots of all the plants from the plot were then removed and the above-ground fresh weight of the whole sample measured. Further growth analysis was carried out on a 25% subsample (by fresh weight) in which the plant material was separated into plant components. These plant components were primarily split into fertile shoots, potentially infertile shoots and dead/dying shoots, which were counted and the fresh and dry weights recorded. The flag leaf, second leaf and all remaining leaves were then individually separated from the fertile shoots and their leaf area, fresh weight and dry weight recorded. Different traits were measured between experiments as described in the methodology section of each results chapter. Sampled plots which were unable to have their growth analysis completed on the day of sampling were stored in a cold room (4^{o}C) for up to three days prior to analysis.

2.4 FIELD VISUAL DISEASE ASSESSMENT

Visual disease progression on each genotype was carried out from mid-anthesis (GS65) onwards with 4 day intervals. Twenty ears per plot were assessed in which the total number of spikelets and the number of infected spikelets per ear were recorded. FHB incidence was calculated as the average percentage of spikelets within an ear showing infection symptoms and this was used to create an Area Under Disease Progress Curve (AUDPC) over a 33 day period post each genotype's mid-anthesis date. Symptoms of FHB included both lesions and bleaching, with lesions classified as characteristic round brown areas developing on the glumes or lemma, and bleaching classified as individual spikelets or whole proportions of an ear with a light bleached appearance. The criteria for an infected spikelet were a clearly defined lesion or bleaching symptom.

2.4.1 AUDPC calculation

Area Under Disease Progress Curve (AUDPC) is a mathematical technique used to measure the rate of disease development over time and was used to standardise against the differing flowering dates of the assessed genotypes. A mathematical formula originally devised by Shaner and Finney (1977) and adapted by Buerstmayr *et al.* (2000) was utilised for the AUDPC calculations as shown in Equation 1, where y_i is the score of visually infected spikelets on the *i*th day and x_i is the day of the *i*th observation and *n* is the total number of observations.

$$\text{AUDPC} = \sum_{i=1}^{n} \{[\frac{y_i + y_{i-1}}{2}](x_i - x_{i-1})\} \qquad \text{(Equation 1)}$$

2.5 FIELD GRAIN HARVEST

Due to the wide range of maturity dates between genotypes, each genotype was harvested individually as its grain ripened (GS93) so that mycotoxin and *Fusarium* infection did not continue to increase in the field once physiological maturity had been reached. As each line reached GS93, grab samples of around fifty fertile shoots were blindly selected from the plot and placed into paper bags. Ears were removed from straw and threshed using a stationary thresher and the grain was milled to a fine flour using a Krups F203 grinder (Krups, Windsor). Grain yield was measured by harvesting the remaining 2x6m plot with a Sampo plot combine and recording the harvested grain weight, before converting to tonnes per hectare.

2.6 PATHOGEN DNA QUANTIFICATION

2.6.1 DNA extraction

A 4g sample of milled flour was placed into a 50 ml centrifuge tube along with 30ml of CTAB buffer (Appendix 2B). Tubes were mixed and placed for two hours in a water bath at 65^{o}C before being rapidly cooled on ice and 10ml of potassium acetate solution added (Appendix 2B). Tubes were gently inverted for two minutes and frozen overnight. Samples were defrosted and centrifuged at 3,000 RPM. A 1200µl aliquot was removed from the middle layer, avoiding contamination from both the heavier and lighter fractions, and placed into a 2ml tube along with 600 µl of 100% chloroform, inverting gently for two minutes. Tubes were centrifuged at 10,000RPM for 15 minutes, had a 1000µl aliquot of the top layer removed to a fresh 2ml tube, 800µl of 100% isopropanol added and gently inverted to mix. Tubes were frozen overnight to precipitate DNA before being centrifuged at 10,000RPM for 15 minutes and having the isopropanol poured off from the pellet. 1000µl of 44% isopropanol was added to the pellet and the process of centrifugation and pouring off repeated a further two times. Tubes were blotted to remove excess 44% isopropanol and then left open overnight to allow the pellet to dry off. 200µl of Tris-EDTA (TE) buffer was added to the pellet before being placed in a hot block at 65^{o}C for two hours after which the tube was vortexed and stored at 4^{o}C.

2.6.2 DNA quantification

Extracted DNA samples were vortexed then centrifuged for 5 minutes at 12,000RPM. A 20 µl aliquot of sample was added to 180 µl of TE buffer in a 1.5 ml eppendorf tube to create a 10 fold dilution before being vortexed and centrifuged for 5 minutes at 12,000 RPM. A spectrophotometer (Varian Cary 50 UV-Vis) was set-up to read 50 µl of each diluted DNA

sample, measuring absorption at the 260 nm, 280 nm, 328 nm and 360 nm wavelengths having been calibrated to zero using fresh TE buffer.

2.6.3 DNA dilution

Prior to real-time PCR, 20 ng/µl dilutions of all DNA samples were created by adding a quantity of original DNA stock to TE buffer. Once diluted, 50µl of each sample was vortexed, centrifuged for 5 minutes at 12,000 RPM and then read in the spectrophotometer to measure absorption at the 260 nm, 280 nm, 328 nm and 360 nm wavelengths to assess purity and concentration of the sample.

2.6.4 Real-time PCR

Real-time PCR was used to quantify the amount of *Fusarium* and *Microdochium* species DNA in the extracted flour samples. Amplification and quantification of the relevant species DNA was performed using real-time PCR thermal cycler CFX96 (Bio-Rad, UK). DNA standard curves were created from pure isolates of each Fusarium and *Microdochium* species and ranged from 10 to 10^{-6} ng/ul. 2x iQ SYBR Green Supermix (Bio-Rad, UK) was used as per the manufacturer's instructions along with 250 nM of both forward and reverse primers (Appendix 2C) to create the amplification master mix for each species. 2.5 µl of DNA template was used in a total reaction volume of 12.5 µl per well. The negative control used the same PCR water as used to create the master mix. Once each plate had been set up, they were run using different protocols that had been optimised for the species specific primers that were being used. The real-time PCR cycles for each *Fusarium* and *Microdochium* spp. are shown in Appendix 2D. Data was analysed using CFX Manager

3.0 of which the well name, content and starting quantity were exported to calculate the starting concentration of each sample.

2.7 INTERNAL TRANSCRIBED SPACER (ITS) PCR

Prior to real-time PCR, extracted DNA samples underwent ITS-PCR using ITS4 and ITS5 primers, to confirm the presence of DNA and to ensure it was amplifiable. A method published by Edwards *et al.* (2012) was utilised for this work.

Each ITS-PCR well consisted of a 25µl reaction, containing 5µl of 20ng/µl DNA template and 20µl PCR master mix. Each 20µl PCR master mix contained 1µl dNTP, 3.5µl PCR mix, 14.4µl PCR water, 0.5µl of each primer, 0.1µl Taq polymerase. Positive controls consisted of 5µl of a 1ng/ul *F.graminearum* DNA stock and negative control consisted of 5µl PCR grade water.

ITS4 and ITS5 primers (5'-TCCTCCGCTTATTGATATGC and 5'-GGAAGTAAAAGTCGTAACAAGG respectively) were used for the amplification (White *et al.* 1990). Amplification was performed on a GeneAmp 9700 (Applied Biosystems, UK), with an initial denaturation of 94^{o}C for 75s, followed by 35 cycles of 15s at 94^{o}C, 15s at 50^{o}C and 45s at 72^{o}C, with a final extension step at 75^{o}C for 5 minutes (Edwards *et al.* 2012). Samples underwent gel electrophoresis using a 2% agarose gel stained with 0.05% ethidium bromide. A BioRad Gel Doc 2000 with UV transilluminator (BioRad, UK) was used to view the PCR products (c. 600-700bp amplicons in size).

Chapter 3.

Mycotoxin quantification

3.1 INTRODUCTION

This chapter presents the development of a methodology for the accurate quantification of a range of mycotoxins in harvested grain samples, developed for the use on samples generated within the wider scope of this study.

Mycotoxins are harmful secondary fungal metabolites produced by a wide range of naturally occurring mycotoxigenic fungi. Examples of such fungal species include *Aspergillus* spp., *Claviceps purpurea* and *Fusarium* spp. which each have the ability respectively to produce aflatoxins, ergotamine and trichothcene mycotoxins. In UK cereal production, the most prevalent group of mycotoxin producing fungi are the *Fusarium* spp. which produce a group of mycotoxins known as the trichothecenes. The most prevalent UK *Fusarium* spp. and their respective trichothecene mycotoxins are shown in Table 3.1.

Table 3.1. Predominant UK Fusarium pathogens and the mycotoxins they produce

F.graminearum	Nivalanol, deoxynivalanol, acetyl-deoxynivalanol, zearalenone
F.culmorum	Nivalanol, deoxynivalanol, zearalenone
F.avenaceum	Moniliformin, enniatins, beauvericin
F.poae	Nivalanol
F.langsethiae	HT-2, T-2

(Source: Bottalico and Perrone, 2002; Edwards *et al.* 2009)

Dexoynivalanol (DON) is often both the most concentrated and commonly occurring trichothecene mycotoxin in UK wheat, being above the limit of detection in 86% of UK samples collected between in 2001 to 2005 (Edwards, 2008). HT-2 and T-2 toxins are most commonly found in oat grains (Edwards, 2009a) and, although they are produced in low concentrations in wheat (Edwards, 2009b), their toxicity is significantly greater than that of other trichothecene mycotoxins (Edwards, 2009a). Legal EU limits have been set for the

contamination of grain with specific mycotoxins, to minimise grain contaminated with high levels of mycotoxins from being introduced into the food chain, therefore protecting the health of the consumer. Within humans, symptoms of excess consumption of trichothecene mycotoxins often include depression of the immune system, nausea and sometimes vomiting (Paraica *et al.* 1999) whereas these symptoms in farm animals include a reduction of feed intake, loss of weight, reproductive disorders, faintness and uncoordinated movement (Hussain & Brasel, 2001).

Quantification of mycotoxins within harvested cereals can be made using a range of quantitative methods, including Enzyme-Linked ImmunoSorbant Assay (ELISA), Gas Chromatography Mass Spectrometry (GC-MS) and Liquid Chromatography Mass Spectrometry (LC-MS). ELISA assays are regarded as a rapid quantification technique; however they are known to lack sensitivity for quantifying low concentrations and suffer from cross reactivity between structurally related toxins (Pereira *et al.* 2014), therefore limiting their use within research. GS-MS and LC-MS techniques in comparison are both able to achieve very low limits of detection in the region of 10µg/kg, and are able to simultaneously quantify multiple mycotoxins (Pereira *et al.* 2014). However, the use of GS-MS and LC-MS methods require significantly more operator training and sample preparation work than ELISA techniques. LC-MS is now the method of choice for mycotoxin quantification over GC-MS methods, with over 150 published articles using LC-MS between 2008 and 2013, in comparison to only 15 published articles using GC-MS (Pereira *et al.* 2014). Published LC-MS methods for mycotoxin quantification are however not always directly applicable to every LC-MS system, due to variation in equipment and the range of mycotoxins for analysis. Therefore considerable method development is needed for each new LC-MS system, of which previously published material is a useful guide.

Liquid Chromatography (LC) works on the principle that molecules of differing polarities are separated on a column through which the mobile phase and analyte of interest is flowing though under high pressure. This chromatographic process can consist of an isocratic mobile phase, or it can be further refined by using a gradient of differing solvents to improve peak separation.

Mass Spectroscopy (MS) is a powerful tool for the quantification of molecules and works by ionizing chemical compounds to produce charged molecules or fragments that can then be detected and quantified. In scan mode, MS equipment produces a mass spectrum of all ions present within a sample and enables the user to select and monitor specific ions of interest as they are eluted from off the LC column, or the sensitivity of the equipment can be increased using Select Ion Recording (SIR) mode which monitors a much smaller number of molecules of interest. There are two different interfaces most commonly used with MS for the ionization and vaporisation of samples, these being Atmospheric Pressure Chemical Ionization (APCI) and Electrospray Ionization (ESI) interfaces. APCI vaporises the mobile phase plus analyte into the gas phase using a heated gas stream after which chemical ionization occurs as a separate step in the process, whereas with ESI the mobile phase and analyte are ionized during the liquid phase within electrically charged droplets, prior to vaporisation into the gas phase (King et al, 2000). Successful ionisation with either interface relies upon the chemical nature of the molecule being investigated as well as the matrix effects from the sample. Both positive and negative ionisation polarities can be used with APCI and ESI interfaces, with some compounds having a preference for ionisation in one polarity over the other.

Sample preparation is an important aspect of an LC-MS methodology as it reduces matrix effects and increases sensitivity, such as reducing the limits of detection, however these techniques can lead to sample losses for which recovery values must be calculated. The use

of internally labelled (13C) standards is of growing popularity as they are able to compensate for losses during sample clean-up and for matrix effects during ionisation. Internal standards are often exactly the same compound as that being analysed within a sample, except their carbon-12 atoms have been replaced with the much less commonly occurring carbon-13 atoms, allowing for discrimination within the MS based on molecular weight, while behaving exactly the same as the target analyte during all other steps including clean-up and chromatographic separation.

Within the wider context of this study, a method for the quantification of low levels of trichothecene mycotoxins within harvested grain samples was required, to compare between the mycotoxin content of multiple wheat genotypes. An LC-MS method for the simultaneous quantification of multiple mycotoxins was therefore developed so that mycotoxin quantification within harvested grain samples could be undertaken.

3.2 AIMS AND OBJECTIVES

The aim of this work was to develop a validated LC-MS assay to quantify accurately and reliably a range of commonly occurring trichothecene mycotoxins from within samples of wheat, barley and oat grains. The specific objectives included: i) the development of an optimised LC-MS assay for the quantification of mycotoxins, ii) a comparison between sample clean-up columns using a spiked flour experiment, iii) validation of the system and chosen cleanup method using a range of naturally contaminated reference flours.

3.3 METHOD DEVELOPMENT

In brief, method development firstly consisted of detecting a range of mycotoxin standards, to determine their retention times and optimum detection parameters, prior to the limit of

detection and linearity of each individual mycotoxin being assessed. Secondly, comparisons between cleanup columns used during sample preparation were made. Finally, variation during sample extraction and intra-day quantification was assessed.

LC-MS equipment was chosen for use within this method development due to the availability of such equipment from within the University of Nottingham. Relevant published methodologies (Dall'Asta *et al,* 2004; Klotzel *et al,* 2005; Biselli *et al,* 2005; Tanaka *et al,* 2010; Elbert *et al,* 2008) were used to guide the development of this assay, in which both Liquid Chromatography (LC) and Mass Spectrometry (MS) aspects of the equipment had to be optimised. Practical assistance and guidance with the method development was received from Dr Rob Linforth, which proved invaluable. Methodology development took a methodical approach first to optimise the equipment for mycotoxin detection followed by LOD experiments, comparisons between clean-up cartridges and finally method validation.

3.3.1 Equipment and consumables

An Agilent 1100 HPLC system (Stockport, UK) fitted with a reverse phase Luna® 5µm C18(2) 250 x 3mm column (Phenomenex, Macclefield, UK) coupled to a MicroMass® Platform LCZ (Micromass, Manchester, UK) MS was used for the development of this assay. The LC equipment was controlled using Agilent ChemStation software, the MS was controlled using MassLynx® software (version 3.2) and a contact closure was set up between the two systems so that new LC runs were recorded automatically by the MS. The LC-MS equipment utilised LC-MS grade acetonitrile, gradient grade methanol, TraceSelect® grade sodium chloride and HLPC grade ammonium acetate purchased from Sigma-Aldrich. HPLC grade water was produced via a Purite Select (Purite, Oxon, UK),

producing water to a quality of around 16.7MΩ. Additional consumables for standard preparation and sample clean up included 100µg/ml standards of nivalenol (NIV), deoxynivalenol (DON), 3-acetyl-DON (3-Ac-DON), HT-2 toxin (HT-2), T-2 toxin (T-2) and zearalenone (ZEAR) and 25µg/ml internal standards of 13C-NIV, 13C-DON, 13C-T-2 and 13C-ZEAR purchased from Biopure™ (Romer Labs, Runcorn, UK). DZT MS-PREP and Mycosep 226 columns were purchased from R-biopharm (Glasgow, UK) and Romer Labs (Runcorn, UK), respectively. Whatman No. 1 filter paper and phosphate buffered saline (PBS) tablets were purchased from Sigma-Aldrich. Glass microfibre filter paper was purchased from Fisher Scientific. Technical nitrogen gas was supplied by BOC (Derby, UK). Blank (mycotoxin free) wheat flour was produced by growing the highly FHB resistant wheat variety Petrus at the University of Nottingham glass houses and milling the grain to a fine flour using a Krups F203 grinder (Krups, Windsor, UK).

3.3.2 Detection of mycotoxin standards

Assay development began by injecting each mycotoxin standard into the LC system and monitoring ion production on the MS. To achieve this, the LC column was by-passed to speed up the process and ensure mycotoxins were not retained on the column, prior to the development of a suitable mobile phase gradient run. A mobile phase consisting of 50% bottle A (100% acetonitrile) and 50% bottle B (10% acetonitrile) was used at a flow rate of 0.5 ml/min, to which 50 µl injections of each 10,000 ppb standard were made into the mobile phase stream. Standards were evaporated to dryness and redissolved in 1000µl of 10% acetonitrile. The MS was set up in scan mode scanning between 150-600 m/z, with a source temperature of 100°C; desolvation temperature, 450°C; gas flow rate, 646 l h^{-1}; capillary voltage, 3.5 kV and cone voltage, 21 V.

Initially, the MS was set up with an Atmospheric Pressure Chemical Ionization (APCI) interface, as recommended by Razzazi-Fazeli *et al* (1999) and Razzazi-Fazeli *et al.* (2002), however no ions were detected and it was instead replaced with an ElectroSpray Ionization (ESI) interface as recommended by Ren *et al.* (2007). Several mycotoxins were successfully detected in scan mode using the ESI interface, with NIV, DON, Ac-DON and ZEAR being detected in negative mode, and T-2-toxin in positive mode, however HT-2 was not detectable.

Alternative mobile phases were tested to improve HT-2 ionisation, including acetonitrile plus 10mM ammonium acetate (Klotzel *et al,* 2005), methanol plus 10mM ammonium acetate and methanol plus 0.1mM NaCl (Dall'asta *et al*, 2004), in which methanol was used in this case instead of acetonitrile due to its ability to dissolve NaCl. The use of methanol plus 0.1mM NaCl allowed for the successful ionisation of HT-2 in ESI+ mode, but had however caused the masses of several other previously detectable mycotoxins to shift. New spectra for each mycotoxin standard were therefore produced using the new mobile phase (Fig. 3.1 - 3.6). Mobile phases consisting of 10% methanol (without NaCl) in bottle A and 100% methanol + 0.1mM NaCl in bottle B were selected as this would minimise NaCl from entering the MS when the LC was re-equilibrating back to 100% bottle A at the start of the mobile phase gradient run. Standards were redissolved in 10% MeOH, instead of 10% acetonitrile, to reflect the new starting mobile phase conditions. The masses of various carbon-13 (13C) labelled standards were also determined by monitoring the ion spectra produced upon injection of 1,000ppb standards (Fig. 3.7 - 3.10).

Once the key masses had been identified for each mycotoxin standard, of which most mycotoxins produced one primary mass and several other minor masses, the column was reattached to the LC and each standard individually injected to determine retention times and optimum masses. Various mobile phase gradients were tested to ensure sufficient time

for each mycotoxin to be fully eluted from the column, with the selected optimum gradient being shown in Table 3.2. The MS was used in Single Ion Recording (SIR) mode and set to detect multiple masses from the spectra of each mycotoxin. This allowed for the mass producing the strongest signal to be selected for further use, as well as to determine the retention time of each mycotoxin. A selection of cone voltages ranging from 18, 21, 25 and 30 V were trialled to allow selection of the optimum cone voltage for each mycotoxin. The cone voltages for each mycotoxin were optimal at 21 V, except for ZEAR in which 30 V producing the strongest signal (Table 3.3). A run programme was set up on the MS so that the correct polarity, optimum cone voltages and optimum SIR masses were used within each phase of the LC run (Table 3.3). Once the optimisation was complete, a mixed standard containing all relevant mycotoxins was injected into the system to confirm that the developed method was able to correctly detect all mycotoxins within one simultaneous run (Fig. 3.11).

Table 3.2. Optimised mobile phase gradient run for the chromatographic separation of multiple mycotoxins. Bottle B contained 100% methanol plus 0.1mM NaCl, while the remaining mobile phase consisted of methanol : water (10:90 v/v).

Time (min)	% bottle B
0	0
15	100
22	100
22.2	0
27	0

Table 3.3. Optimized ionisation mode, retention time, ion mass, cone volts and dwell time for multiple mycotoxins.

	NIV	13C-NIV	DON	13C-DON	Ac-DON	HT-2	T-2	13C-T2	ZEAR	13C-ZEAR
Ionisation mode	ESI-	ESI-	ESI-	ESI-	ESI-	ESI+	ESI+	ESI+	ESI-	ESI-
Retention time (s)	8.23	8.23	10.07	10.07	13.06	16.13	16.80	16.80	17.65	17.65
Ion mass	347	362	331	346	373	447	489	513	317	335
Cone volts	21	21	21	21	21	21	21	21	30	30
Dwell time (s)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

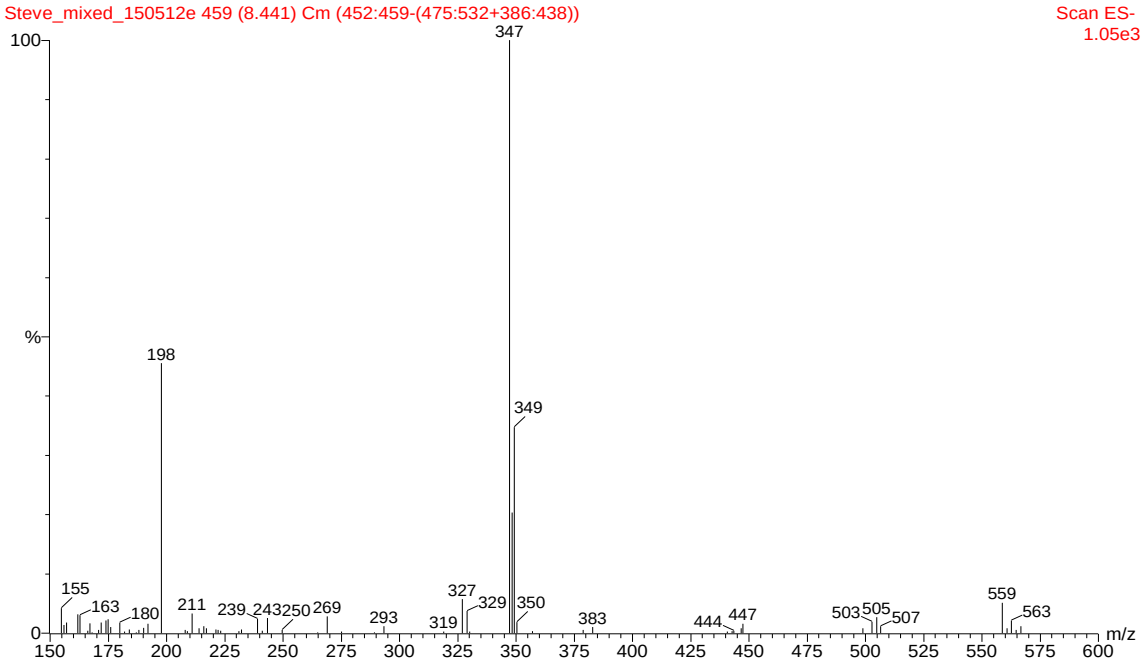

Figure 3.1. Nivalanol ion spectra

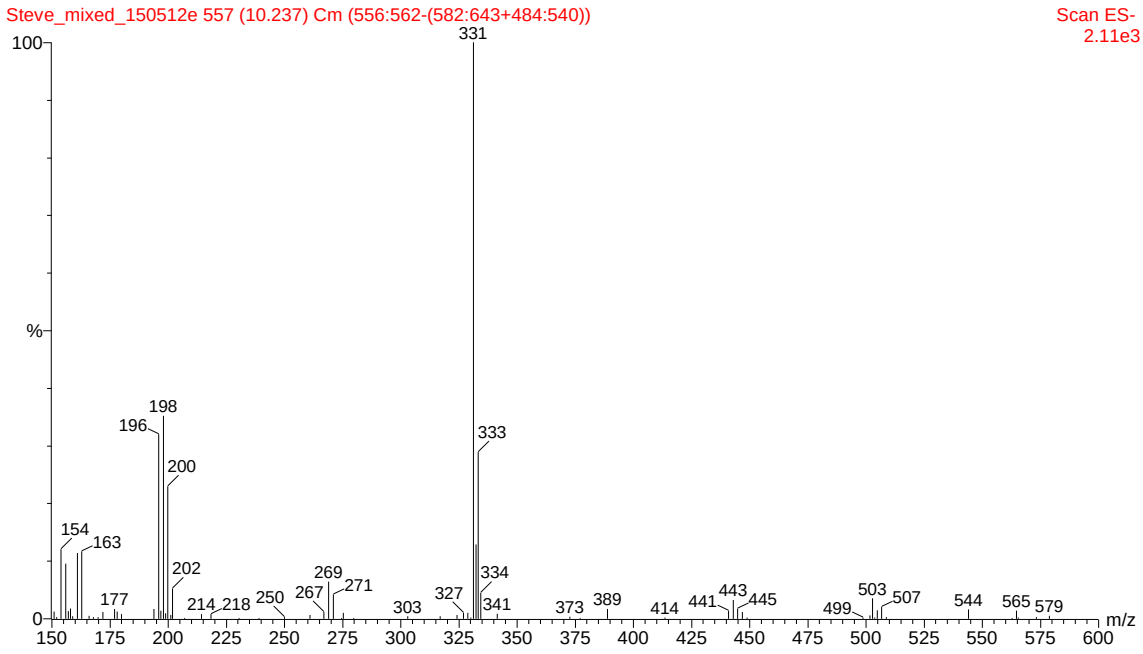

Figure 3.2. Deoxynivalanol ion spectra

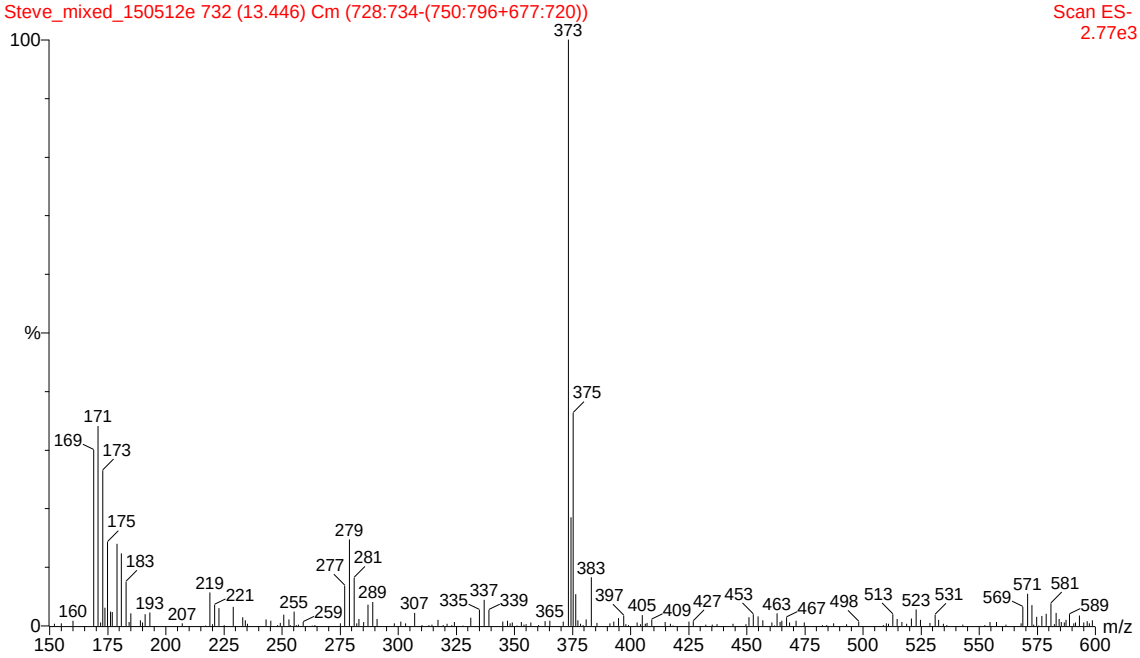

Figure 3.3. Ac-DON ion spectra

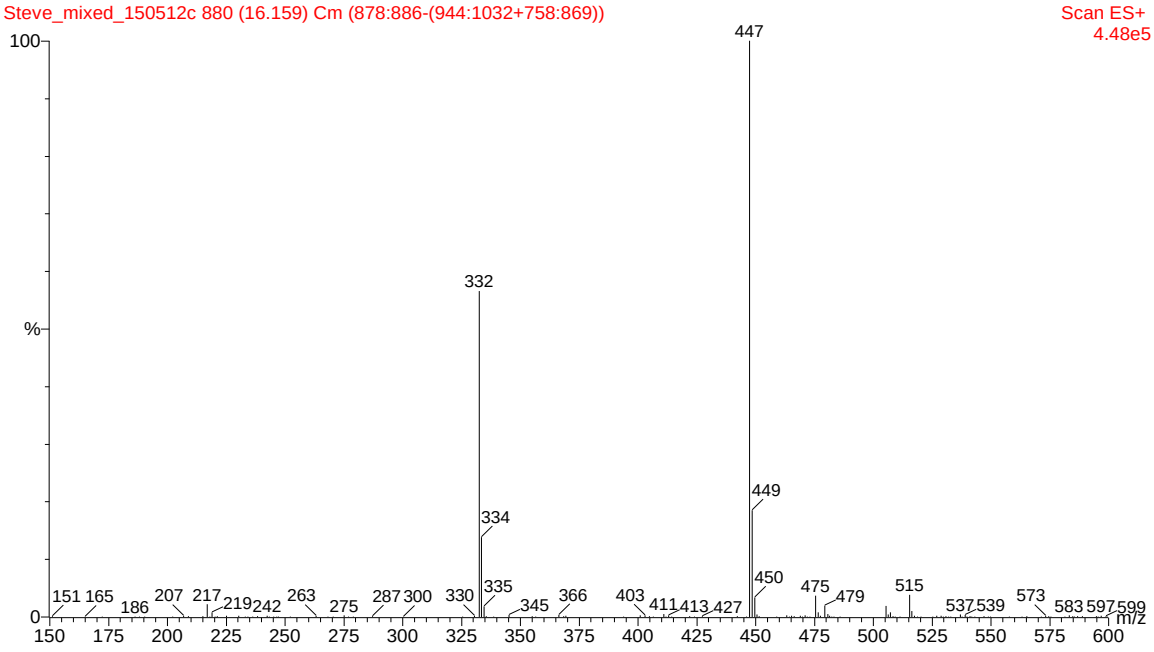

Figure 3.4. HT-2 ion spectra

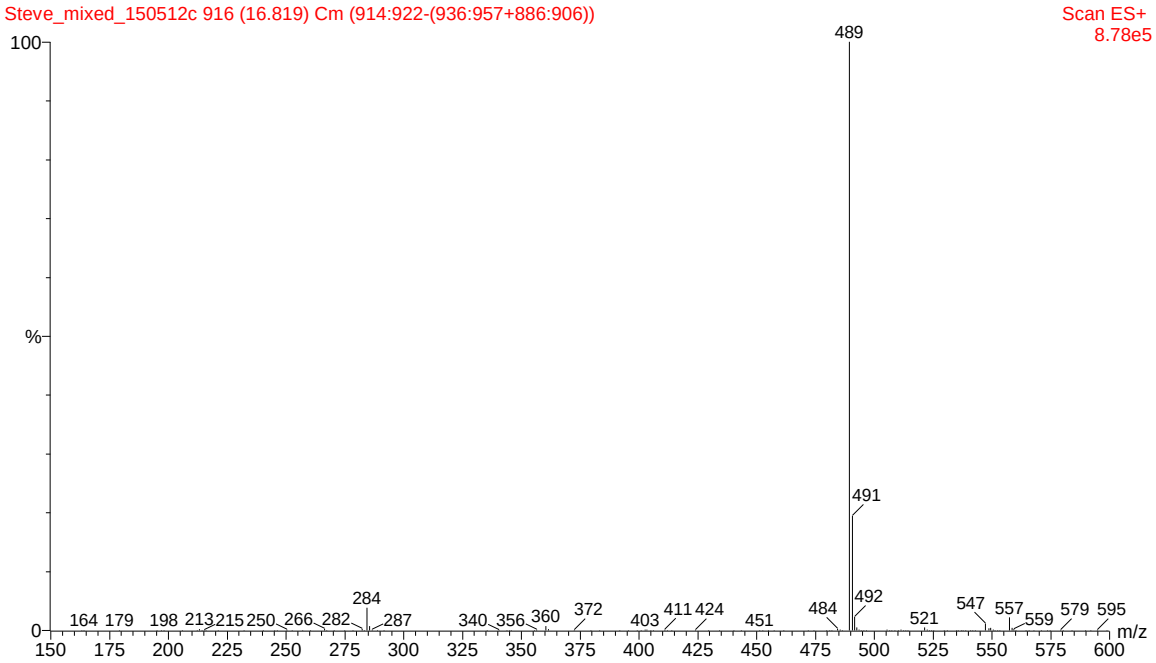

Figure 3.5. T-2 ion spectra

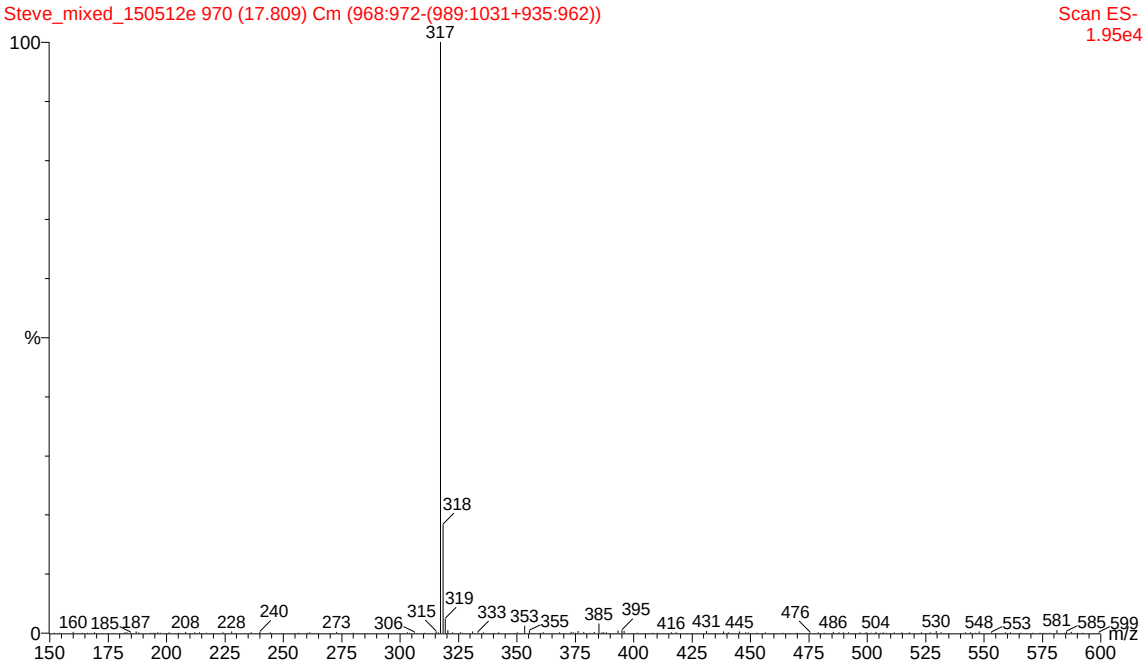

Figure 3.6. ZEAR ion spectra

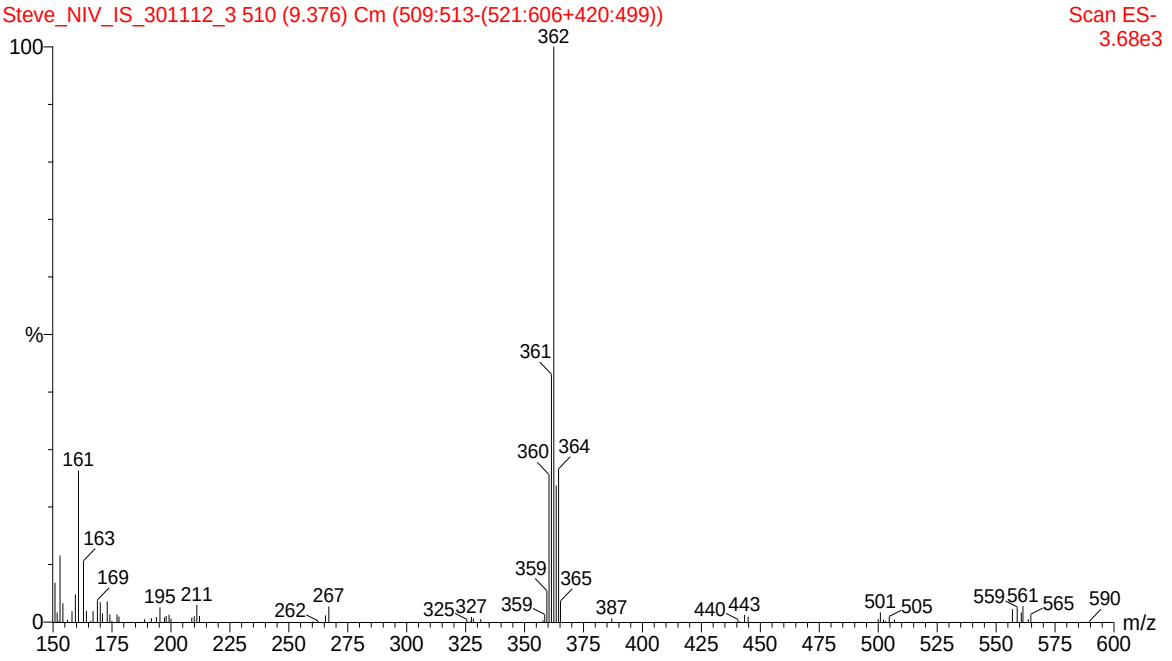

Figure 3.7. 13C-NIV ion spectra

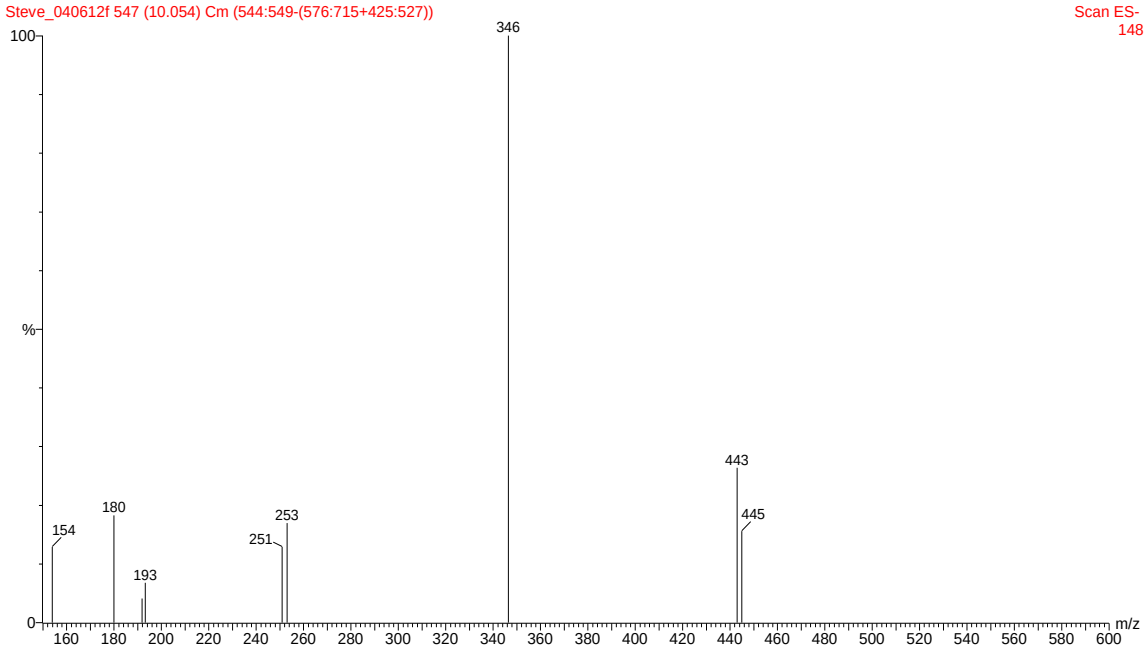

Figure 3.8. 13C-DON ion spectra

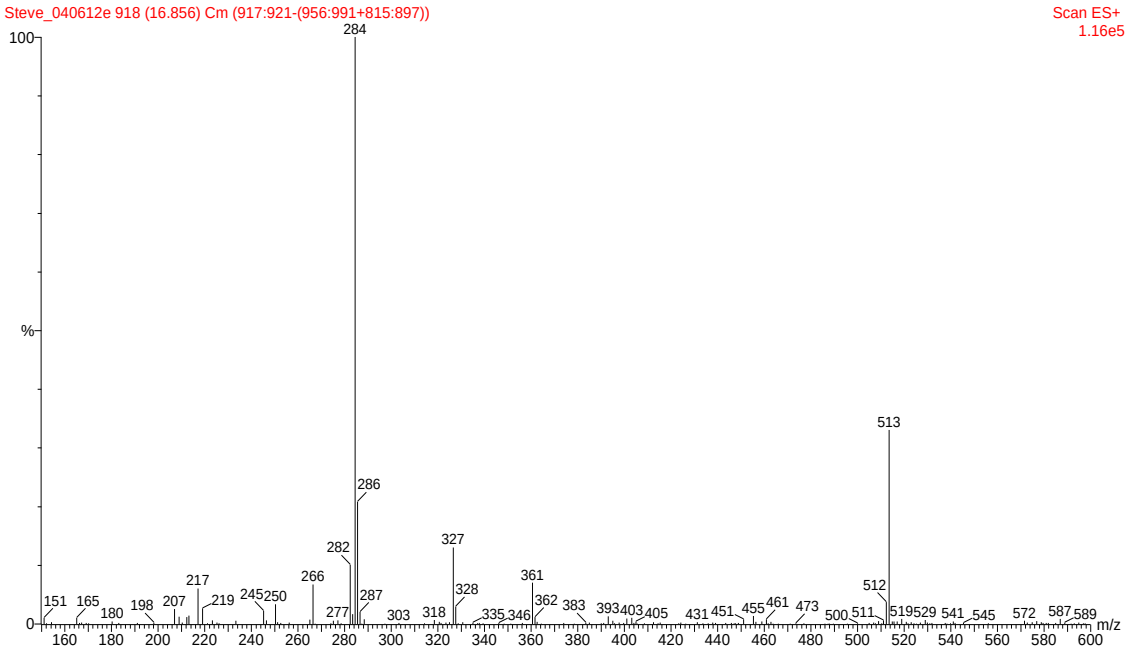

Figure 3.9. 13C-T2 ion spectra

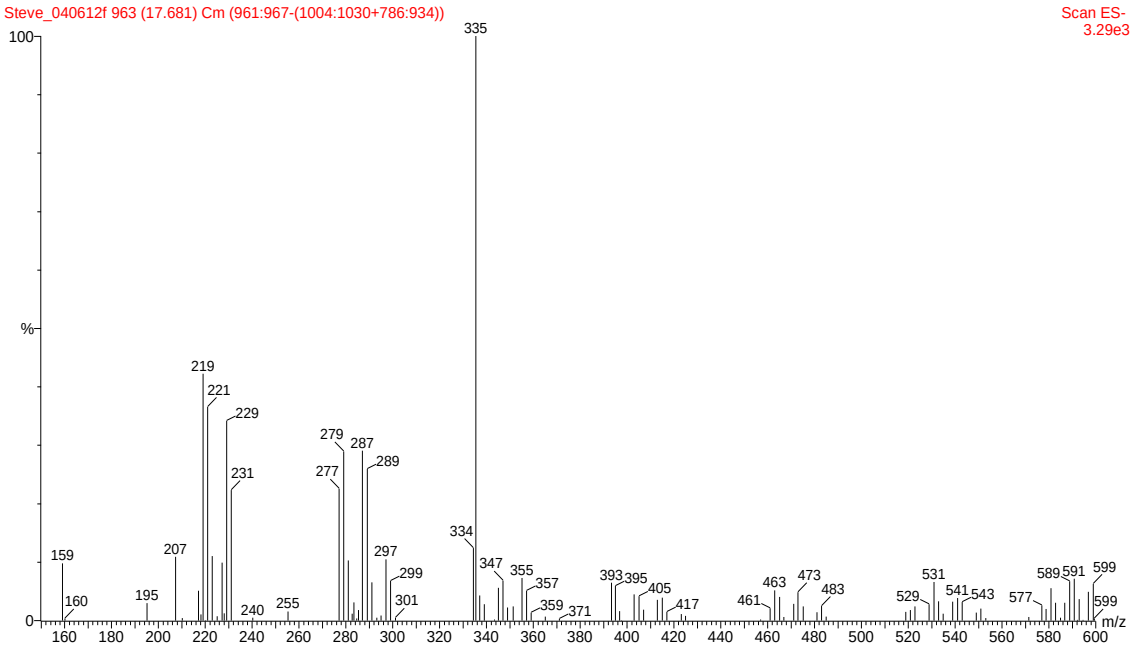

Figure 3.10. 13C-ZEAR ion spectra

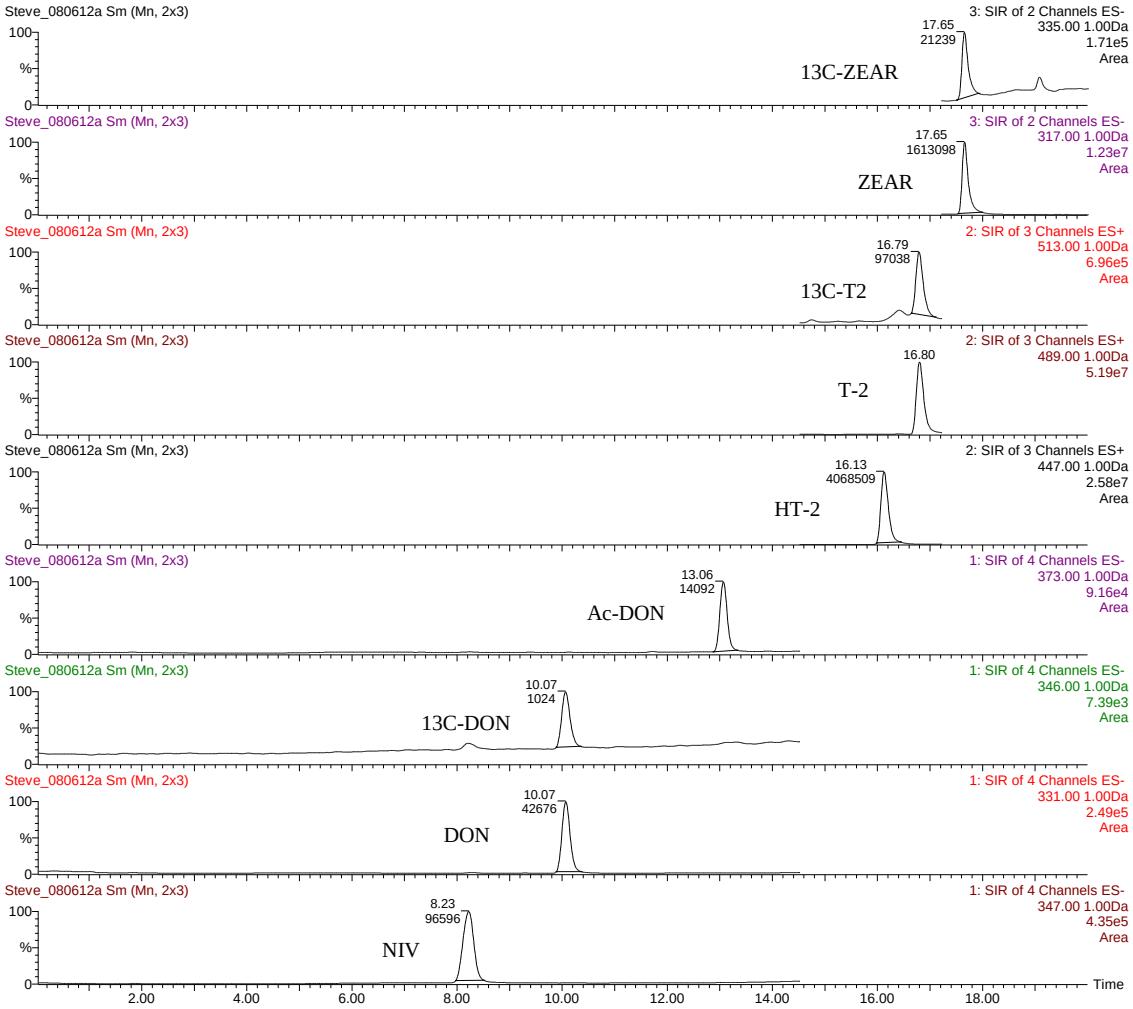

Figure 3.11. A select ion (SIR) chromatogram of a 10,000ppb mixed mycotoxin standard plus 10ppb internal standards. The x-axis shows chromatographic time (min). The ion mass monitored for each of the 9 mycotoxins is shown in the upper right of each trace along with maximum signal height. Each peak is annotated with the name, retention time and peak area of the compound.

3.3.3 Limit of detection and linearity

Mixed mycotoxin standards ranging from 0 to 200 ppb were created to determine the Limit of Detection (LOD) and linearity of the developed LC-MS method shown in section 3.3.2. Standards were injected into the LC-MS system in a random sequence ensuring that carry over between standards could not confound results. LOD was determined as the lowest mycotoxin concentration that could be successfully integrated by the Masslynx software, while linearity of the analysis was determined as the R^2 value between the peak areas of each standard.

The LOD of all mycotoxins was 10ppb, except for DON which had an LOD of 20ppb (Table 3.4). The peak area of differing standard concentrations was found to be highly linear.

Table 3.4. Limit of detection and linearity of peak areas for mixed mycotoxin standards using optimised LC-MS settings

	NIV	DON	Ac-DON	HT-2	T-2	ZEAR
200ppb	3203	2357	899	140903	237484	44585
100ppb	1481	1004	373	63722	122,874	18512
50ppb	704	456	154	31790	62418	7813
20ppb	246	131	57	11700	23031	3033
10ppb	94	-	34	6287	8070	1057
0ppb	-	-	-	-	-	-
LOD	10ppb	20ppb	10ppb	10ppb	10ppb	10ppb
R^2	0.9984	0.9941	0.9899	0.9976	0.9991	0.9911

3.3.4 MycoSep® 226 clean-up cartridge method validation

An experiment was designed to test and validate the use of MycoSep® 226 columns to clean-up extracted flour samples prior to injection with the developed LC-MS method.

Reference flours of known mycotoxin content were supplied by Simon Edwards (Harper Adams University College) and included two wheat flours (samples WC125 and WC133) and two oat flours (samples 05/06/090 and 05/06/094). MycoSep® 226 clean-up columns were purchased from Romer Labs (Runcorn, UK). Mycotoxin extraction and cleanup consisted of weighing 10g of each flour sample into a conical flask, adding 40ml of 84% acetonitrile and then 20ppb aliquots of 13C-DON, 13C-T2 and 13C-ZEAR internal standards. Samples were extracted for 2h on a rotary shaker, followed by adding 40ml of 100% acetonitrile. Extracts were filtered through Whatman No. 1 filter paper and 8ml of the filtrate passed through a MycoSep® 226 column. 5ml of the extract was collected, evaporated to dryness under a gentle stream of nitrogen gas, and re-dissolved in 500µl of 10% methanol.

Results showed large discrepancies between known reference flour mycotoxin concentrations and the values obtained from the LC-MS analysis (Tables 3.5 and 3.6). A comparable experiment was therefore developed to test if improved results could be achieved by using DZT MS-PREP® columns for clean up, instead of MycoSep® 226 columns (section 3.3.5).

Table 3.5. The quantified and reference flour mycotoxin values (ppb) for wheat flour samples WC125 and WC133. Quantified values were calculated using the developed validation methodology, while reference values were the mycotoxin quantities reported in the previous laboratory analysis.

	WC125		WC133	
	Quantified value	Reference value	Quantified value	Reference value
NIV	7ppb	14ppb	160ppb	199ppb
DON	826ppb	269ppb	8,639ppb	3130ppb
Ac-DON	-	-	579ppb	Unknown
ZON	19ppb	13ppb	2,416ppb	1,987ppb

Table 3.6. The quantified and reference flour mycotoxin values (ppb) for oat flour samples 05/06/090 and 05/06/094. Quantified values were calculated using the developed validation methodology, while reference values were the mycotoxin quantities reported in the previous laboratory analysis.

	05/06/090		05/06/094	
	Quantified value	Reference value	Quantified value	Reference value
HT-2	109ppb	226ppb	1499ppb	2321ppb
T-2	71ppb	84ppb	3690ppb	3940ppb

3.3.5 DZT MS-PREP® clean-up column method validation

An experiment was developed to validate the use of DZT MS-PREP® columns to clean-up extracted flour samples prior to injection into the LC-MS system. Reference flours supplied by Simon Edwards (Harper Adams University, Edgmond, UK) were used, including three wheat flours (samples WC128, WC133 and WC139), three barley flours (SM10 021, SM10 039 and SM10 070) and three oat flours (OS/06/092, OS/06/098 and OS/06/099). Several flour samples previously used in the MycoSep® 226 validation experiment were unable to be used within this validation as insufficient sample remained. DZT MS-PREP® clean-up cartridges were purchased from R- Biopharm (Glasgow, UK). For the extraction and clean up of mycotoxins, 12.5g of flour was weighed into a blender jar along with 50ml of methanol/deionised water (70:30 v/v). Samples were spiked with 10µl of C13-DON standard to create a 20ppb internal standard, prior to being blended at high speed for 3 min. The supernatant was passed through a Whatman No.1 filter, before having 2ml of the filtrate added to 48ml of PBS solution and this mixture passed through a glass microfibre filter. 20ml of the filtered solution was passed through a DZT MS-PREP® under gravity followed by washing of the column with 20ml deionised water. Mycotoxins

were eluted from the DZT MS-PREP® using 2ml of 100% methanol before being evaporated to dryness under a gentle stream of nitrogen gas and redissolved in 500µl of 10% methanol. Mycotoxin content within each sample was quantified from three separate injections so that instrumental variation could be estimated (Section 3.3.8). Additionally, four separate DZT MS-PREP® clean-up extractions of samples WC139 and OS/06/098 were undertaken so that within sample extraction variation could be estimated (Section 3.3.8).

Regression analysis between the known mycotoxin contents of reference flours and the quantified results using the developed LC-MS assay showed highly linear relationships for DON ($R^2 = 0.99$, $P<0.001$), HT-2 ($R^2 = 0.84$, $P<0.001$), T-2 ($R^2 = 0.90$, $P<0.001$) and ZEAR ($R^2 = 0.99$, $P<0.001$) (Table 3.7). Quantification of mycotoxins underestimated mean reference flour DON values with a percentage difference of 43%, underestimated HT-2 values by 17%, underestimated T-2 values by 15% and overestimated ZEAR values by 30%.

3.3.6 Nivalanol clean-up procedure method validation

A clean-up procedure for the detection of NIV in samples was developed for use with the developed LC-MS methodology. This was required as DZT MS-PREP® columns are unable to clean up NIV within samples, therefore a cost effective alternative method had to be developed.

The developed clean-up procedure utilised 80ml of 70% methanol solution and 10g of ground sample, blended together at high speed for 3 min. The blended mixture was filtered through a Whatman No.1 filter, before being passed through a fine glass microfibre filter.

A 5ml aliquot of filtrate was placed in a 50ml tube with 2.5µl of 13C-NIV standard to spike the sample to the equivalent of 100ppb 13C-NIV in the original flour. The sample was evaporated to dryness under a gentle stream of nitrogen before being redissolved in 1000µl of 10% methanol. The redissolved sample was placed into a 1.5ml tube, and centrifuged at 11,000rpm for five min. The clear top layer of liquid was carefully removed, avoiding the waste pellet, and placed into a 2ml vial. Samples were stored at -20^{o}C until 50 µl injection into the LC-MS system.

NIV content was quantified in triplicate so that machine variation could be calculated (section 3.3.8). Additionally, four separate NIV clean up procedures were carried out on samples WC139 and OS/06/08 so that within sample extraction variation could be calculated (section 3.3.8). Regression analysis between known mycotoxins content of reference flours and NIV quantification using the NIV clean-up procedure showed a highly linear significant relationship (R^2=95.6; P<0.001) (Table 3.7). Mycotoxin quantification overestimated mean reference flour NIV values with a percentage difference of 2.97%.

Table 3.7. Mycotoxin concentration (ppb) validation using reference flour samples with DZT MS-PREP® clean up cartridges and crude NIV clean up method. Quantified values were calculated using the developed validation methodology, while reference values were the mycotoxin values reported by the laboratory which previously quantified the samples.

	Quantified NIV	Reference NIV	Quantified DON	Reference DON	Quantified HT-2	Reference HT-2	Quantified T-2	Reference T-2	Quantified ZEAR	Reference ZEAR
WC128	24	17	469	790	-	-	-	-	71	39
WC133	152	199	2002	3130	-	-	-	-	2699	1987
WC139 (1)	14	31	277	406	-	-	-	-	104	112
WC139 (2)	19	31	306	406	-	-	-	-	151	112
WC139 (3)	20	31	261	406	-	-	-	-	143	112
WC139 (4)	10	31	294	406	-	-	-	-	173	112
SM10 021	14	15	63	56	94.1	87	31.2	27	-	-
SM10 039	43	52	67	51	24.8	29	7.6	16	-	-
SM10 070	206	209	18	26	90.5	67	23.8	52	-	-
OS/06/092	69	70	30	39	499.1	557	239.8	248	-	-
OS/06/098 (1)	399	427	0	17	203.7	269	110.4	135	-	-
OS/06/098 (2)	463	427	0	17	219	269	142.8	135	-	-
OS/06/098 (3)	454	427	0	17	211.6	269	99.9	135	-	-
OS/06/098 (4)	547	427	0	17	246.3	269	117	135	-	-
OS/06/099	342	301	0	62	161.1	263	53.3	79	-	-
P-value	<0.001		<0.001		<0.001		<0.001		<0.001	
LSD	47.76		66.27		86.81		32.68		233.5	
CV (%)	15.4		15.6		25.8		20.6		22.3	
Regression	R2 = 95.6, P<0.001		R2 = 99.3, P<0.001		R2 = 84.8, P<0.001		R2=90.4, P<0.001		R2=98.8, P<0.001	

3.3.7 Comparison between DZT MS-PREP® and MycoSep® 226

An experiment was developed to test between MycoSep® 226 and DZT MS-PREP® clean up cartridges for giving the best mycotoxin quantification results. The experiment used blank flour samples, spiked with 0ppb, 20ppb, 100ppb and 500ppb of each DON, HT-2, T-2 and ZEAR as well as 20ppb of 13C-DON, 13C-T2 and 13C-ZEAR internal standards. Spiked samples were each extracted and cleaned up using both MycoSep® 226 and DZT MS-PREP® as per the protocols described in sections 3.3.4 and 3.3.5 respectively, prior to analysis using LC-MS. While the MycoSep® 226 is able to clean up nivalanol from extracted samples, the DZT MS-PREP® columns does not, therefore this mycotoxin was excluded from the experiments.

Results showed significant ($P<0.001$) differences between the concentration of spiking treatments for all mycotoxins and significant differences in quantified values for Ac-DON, HT-2 and T-2 between MycoSep® 226 and DZT MS-PREP® (Table 3.8). For Ac-DON, HT-2 and T-2, the quantified values were closer to the spiked concentrations when cleaned-up using a DZT MS-PREP® cartridge rather than a MycoSep® 226 column (Table 3.8). The DZT MS-PREP® was used for all analyses of mycotoxin content thereafter. Further data analysis showed that DZT MS-PREP® quantification of mycotoxin concentrations yielded similar results when using only the 13C-DON internal standard, thereby offering significant cost savings within the method.

Table 3.8. Blank flour spiking experiments and comparison between Mycosep 226 and DZT MS-PREP clean up columns. Blank flour samples spiked with either 0, 20, 100 and 500ppb of mycotoxin prior to clean up. ANOVA used to detect significant differences between the spiking concentration (spiking) and significant differences in sample concentrations between Mycosep 226 and DZT MS-PREP clean up columns (columns).

Toxin	Clean-up	0ppb mean	20ppb mean	100ppb mean	500ppb mean	P-value (spiking)	P-value (columns)	LSD (spiking)	LSD (columns)	CV (%)
NIV	MycoSep	0	7.9	34.6	154.5	<0.001	-	25.91	-	19.0
	DZT	-	-	-	-	-	-	-	-	-
DON	MycoSep	0	20.2	99	505.2	<0.001	0.627	30.06	21.25	11.3
	DZT	0	21.1	109.1	512.5					
AC-DON	MycoSep	0	46.8	175.3	756.1	<0.001	<0.001	43.17	30.53	12.80
	DZT	0	19.8	97.7	516.3					
HT-2	MycoSep	0	13.7	63.9	315.1	<0.001	<0.003	44.09	31.18	20.5
	DZT	0	19	100.3	514.8					
T-2	MycoSep	0	22.1	119.0	591.8	<0.001	<0.001	8.06	5.70	2.8
	DZT	0	19.9	103.2	515.8					
ZEAR	MycoSep	0	19.8	100.2	488.7	<0.001	0.159	29.35	20.76	11.7
	DZT	0	20.9	84.4	448.8					

3.3.8 Intra-day and extraction variation

Data from both the DZT MS-PREP® and NIV clean-up validation experiments was used to calculate the intra-day variation between samples. Samples used within the validation were each run in triplicates, so that calculations of the machines Co-efficient of Variation (CV) (%) could be made. Results showed an intra-day variation of between 10.7 - 24.9% across the range of mycotoxins quantified (Table 3.9).

Within the DZT MS-PREP® and NIV clean-up validation experiments, one wheat flour (WC139) and one oat flour (OS/06/098) were each separately extracted and cleaned up four times via each clean-up procedure so that variation within the extraction procedure could be quantified. Extraction variation within samples was calculated using analysis of variance (Genstat v14.1) and ranged from 7.0 – 19.2% for DON, HT-2, T-2 and ZEAR, cleaned up via the DZT MS-PREP®, and 19.8% for nivalanol, as cleaned-up via the NIV clean-up procedure (Table 3.10).

Table 3.9. Average intra-day variation using DZT MS-PREP® for DON, HT2, T2 and ZEAR clean-up and the nivalanol clean-up procedure for NIV.

	NIV		DON		HT-2		T-2		ZEAR	
	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)
WC128	24	2.6	469	17.7	-	-	-	-	71	48.6
WC133	152	26.1	2002	5.3	-	-	-	-	2699	10.6
WC139 (1)	14	52.1	277	8.9	-	-	-	-	147	34.2
WC139 (2)	19	59.5	306	10.9	-	-	-	-	195	12
WC139 (3)	20	53.1	261	10.2	-	-	-	-	143	26.1
WC139 (4)	14	37.2	294	1.9	-	-	-	-	173	18.1
SM10 021	14	22.1	63	15.1	94	31.7	31	38.4	-	-
SM10 039	39	7.8	67	9.3	25	26.6	8	21.7	-	-
SM10 070	206	11.5	19	18.5	91	25.2	24	43.8	-	-
OS/06/092	68	13.9	32	8.6	499	25.1	240	18.1	-	-
OS/06/098 (1)	399	7.2	-	-	204	22.9	110	16.5	-	-
OS/06/098 (2)	463	13.2	-	-	219	8.8	143	15.1	-	-
OS/06/098 (3)	454	6.7	-	-	212	24.9	100	11.2	-	-
OS/06/098 (4)	547	5.7	-	-	246	11.8	117	18.2	-	-
OS/06/099	342	14.5	-	-	161	24.8	53	16	-	-
Average CV (%)		22.2		10.7		22.4		22.1		24.9

Table 3.10. Extraction variation using DZT MS-PREP® for DON, HT2, T2 and ZEAR clean-up and the nivalanol clean-up procedure for NIV.

	NIV	NIV	DON	HT-2	T-2	ZEAR
WC139 (1)	14	-	277	-	-	147
WC139 (2)	19	-	306	-	-	192
WC139 (3)	20	-	261	-	-	128
WC139 (4)	14	-	294	-	-	189
OS/06/098 (1)	-	399	-	204	110	-
OS/06/098 (2)	-	463	-	219	143	-
OS/06/098 (3)	-	454	-	212	100	-
OS/06/098 (4)	-	547	-	246	117	-
P-value	0.776	0.013	0.201	0.595	0.101	0.053
LSD	18.15	75.8	46.72	73.9	34.93	50.58
CV (%)	19.8	13.1	7.0	8.3	15.5	19.2

3.4 DISCUSSION

Literature shows the use of both APCI and ESI interfaces for the ionization of mycotoxins. Dall'Atra *et al.* (2004) reported that APCI was the most frequently used ionisation technique for detecting trichothecenes at trace levels and that ESI is seldom used due to poor response of molecules to the technique, difficulty in simultaneously detecting type A and type B trichothecenes and due to increased matrix effects within ESI. Several methodologies have however shown successful use of ESI to detect mycotoxins, including Elbert *et al.* (2008), Tanaka *et al.* (2010), Biselli *et al.* (2005), Klotzel *et al.* (2005), Senyuva *et al.* (2012), Romagnoli *et al.* (2010) and even Dall'Asta *et al.* (2004). Research by Biselli *et al.* (2005) and Klotzel *et al.* (2005) compared sensitivities for mycotoxin ionisation using ESI and APCI interfaces, finding that ESI consistently produced much stronger peak signals which is consistent with the findings of this study. ESI is however known to be more liable to increased suppression or enhancement by different matrix effects (Cunha & Fernandes, 2012), although within this method development this factor was compensated for through the use of internal standards. In contrast to the earlier statement by Dall'Atra *et al.* (2004), only a small group of studies have shown the successful use of APCI for mycotoxin ionisation (Razzazi-Fazeli *et al.* 2002) on a very limited range of mycotoxins.

Ionisation polarity was also found within this study to have a large effect on the successful ionisation of different mycotoxin types, in which Type A mycotoxins required positive ionisation and Type B mycotoxins required negative ionisation, thereby requiring multiple switches in ionization polarity within a single gradient run. The need for different ionization polarities between Type A (positive ionization) and B (negative ionization) mycotoxins has also been consistently shown within other published methods (Biselli *et al.* 2005; Klotzel *et al.* 2005; Dall'Asta et *al.* 2004; Senyuva *et al.* 2012; Romagnoli *et al.*

2010 and Elbert *et al.* 2008). The only exception to this was Tanaka *et al.* (2010) who used positive ionisation mode for both Type A and B mycotoxins, a technique which Dall'Asta *et al.* (2004) state would not work for Type B mycotoxins.

Acetonitrile/water was the initial mobile phase of choice, as recommended by Ren *et al.* (2007) stating improved mycotoxin ionisation using acetonitrile rather than methanol, although this was contrasted by Ran *et al.* (2013) who stated that methanol is usually used over acetonitrile, as it gives DON a higher response value. Initial experiments using an acetonitrile and water mobile phase however failed to successfully ionize and detect HT-2, therefore alternative mobile phases containing additional ionization agents were tested. Results showed the successful ionisation of HT-2 using methanol + 0.1mM NaCl, in which Type A trichothecenes formed sodium adducts, in agreement with the findings of Dall'Asta *et al.* (2004) and Biselli *et al.* (2005). The majority of methods utilise an ionisation agent dissolved within the mobile phase, the most common being ammonium acetate (Tanaka *et al.* 2010; Elbert *et al.* 2008; Romagnoli *et al.* 2010 and Klotzel *et al.* 2005) followed by ammonium bicarbonate (Senyuva *et al.* 2012) and sodium chloride (Dall'Asta *et al.* 2004), although Biselli *et al.* (2005) succeeded without using an ionisation agent.

Mobile phase gradient used within the method validation (Table 3.2) consisted of a linear gradient from 10% methanol in water, to 100% methanol containing 0.1mM NaCl over 15 min, held for 7 min to ensure full elution of all mycotoxins and potential contaminants before column re-equilibration over 5 min. This reflects the general mobile phase gradients used within similar studies in which high concentrations of eluting solvent were reached over 5.5 to 25 min via a linear gradient (Biselli *et al,* 2005; Klotzel *et al,* 2005; Elbert *et al.* 2008). The order in which each mycotoxin was eluted from the column (Fig. 3.11) was the same as in other studies (Dall'Asta *et al,* 2004; Klotzel *et al,* 2005; Biselli *et al,* 2005;

Elbert *et al,* 2008; Tanaka *et al,* 2010). This would be expected as the elution order is dependent on the polarity of the compound.

The detection of a range of mycotoxin concentrations was shown to be highly linear (Table 3.4) in agreement with other studies (Dall'Asta *et al.* 2004; Biselli *et al.* 2005; Klotzel *et al.* 2005 and Tanaka *et al.* 2010) which reported linearity values of R^2>0.98 for the same mycotoxins. Elbert *et al.* (2008) however, despite finding highly linear relationships between mycotoxin concentrations, showed that these highly linear ranges were for solutions of a lower mycotoxin concentration than the other studies. The results of the LOD analysis (Table 3.4) were broadly similar to that shown by Ibanez-Vea *et al.* (2011) in which NIV and Ac-DON had an LOQ of 10 ppb and DON, HT-2 and T-2 had an LOQ of 20 ppb.

It is generally accepted that prior to detection and quantification via LC-MS, clean up of the extracted sample is necessary to reduce matrix effects and to reduce the LOD. Senyuva *et al.* (2012) found that LC-MS without sample clean-up had background interference, ion detection against a noisy background, raised LODs and reduced repeatability, concluding that while the absence of a clean-up step was adequate for mycotoxin screening, clean-up remains an essential tool for definitive quantitative measurements. Biselli *et al.* (2005) also found that mycotoxin quantification of crude extracts resulted in unacceptable high matrix effects, a situation that was improved significantly through sample clean-up. Senyuva *et al.* (2012) observed no evidence of adverse effects on MS performance from continued injections of crude extracts, however within this study a reduced detection sensitivity was observed over repeated injections of crude extracts, causing for the LC-MS cone to become encrusted in waste matter that had to be frequently removed, proving the necessity of sample clean-up.

Two common methods of sample clean-up utilise either a MycoSep® or DZT MS-PREP® column (Pereira *et al.* 2014), and these were therefore the two clean-up methods tested within this validation. MycoSep® columns use a non-specific extraction method in which adsorbants such as charcoal, celite, ion-exchange resins and polymers are used to remove interfering substances such as proteins, fats and pigments from the sample (Pereira *et al.* 2014). Immunoaffinity columns (IAC), such as the DZT MS-PREP® column, comprise mycotoxin specific anti-bodies bound to an activated solid phase support, so that as the extract passes over the anti-bodies the mycotoxins are bound to the column, the impurities removed by a washing phase and the mycotoxins eluted from the column using a solvent to denature the antibody (Pereira *et al.* 2014).

Carbon-13 (13C) labelled internal standards (13C-NIV, 13C-DON, 13C-T2 and 13C-ZEAR) were used during sample clean-up to compensate for problems such as losses, ionization and matrix effects (Pereira *et al.* 2014). Mycotoxin recoveries without internal standards are between 54 - 93% using the MycoSep® 226 column (Biselli *et al.* 2005) and 60 - 100% using the DZT MS-PREP® (Romagnoli *et al*, 2010). The use of 13C internal standards during sample clean-up is able to compensate for losses and matrix interference. Table 3.11 shows that the use of 13C internal standards are an excellent choice for compensating for clean-up losses, making it unnecessary to compensate for differences in the efficiency of recovery.

Table 3.11. Sample recovery between internal and external standards

	External standards (CV %)	Internal standards (CV %)
DON	49 (6)	96 (5)
HT-2	148 (7)	98 (7)
T-2	127 (5)	99 (6)
NIV	89 (10)	103 (11)

(Source: Varga *et al.* 2012)

The initial method validation using MycoSep® 226 columns was unsuccessful, with quantified mycotoxin values being significantly different from reference flour values (Table 3.5 & 3.6) and providing inaccurate quantification of NIV, Ac-DON, HT-2 and T-2 within the spiking experiment (Table 3.8) despite the use of internal standards. This is in disagreement with multiple other studies (Dall'Asta *et al.* 2004; Biselli *et al.* 2005) which successfully validated an LC-MS method using MycoSep® 226 cartridges. Biselli *et al.* (2005) and Klotzel *et al.* (2005) however reported strong signal suppression and matrix effects when using MycoSep® clean-up cartridges. These matrix effects, if present within this validation, could have led to inaccurate mycotoxin quantification, despite the use of internal standards, if baseline interference had led to misleading peak areas being integrated. Low levels of internal standards (20ppb) were used to spike flour within the MycoSep® validation experiment, primarily due to the expensive nature of the product, therefore it is possible that the occurrence of signal suppression or matrix effects as described by Biselli *et al.* (2005) and Klotzel *et al.* (2005), prevented accurate integration of internal standard peak areas. Evidence of this is seen in Fig. 3.12 and 3.13, comparing MycoSep® and DZT MS-PREP® chromatograms from the flour spiking experiments (Table 3.8), which show that while MycoSep® columns generally produced larger peak areas than DZT MS-PREP® columns, the baseline of the MycoSep® had significantly more interference, leading to a source of error during peak integration and quantification.

Method validation using DZT MS-PREP® cartridges was shown to be successful, finding strong relationships with reference flour samples (Table 3.7) plus accurate mycotoxin quantification in spiked experiments (Table 3.8), with quantification being significantly different to MycoSep® columns for Ac-DON, HT-2 and T-2. The crude extract method developed for NIV quantification additionally proved successful, accurately quantifying a range of NIV concentrations.

Despite finding strong linear relationships with the reference flour samples, the DZT MS-PREP® columns underestimated the quantity of DON, HT-2 and T-2, and overestimated the concentration of ZEAR. The original mycotoxin quantification of the reference flours used within the validation was reportedly achieved using LC-UV, however the method development was undertaken using more sensitive LC-MS equipment, plus internal standards. It is likely, therefore, that the highly linear relationships between references flour values and the method validation may show that the LC-MS method developed within this validation is more accurate than that used to originally quantify the reference flours. Differences, therefore, in mycotoxin quantification may have reported the true values of the reference samples. This theory was confirmed using the blank flour spiking experiment (Table 3.8) which showed accurate quantification of spiked flour samples using DZT MS-PREP® columns for clean-up prior to quantification, thereby validating the developed method.

Variation between sample clean-up using the DZT MS-PREP® was generally within parameters reported by Senyuva *et al.* (2012) and Romagnoli *et al.* (2010), but generally higher than that reported by Tanaka *et al.* (2010) (Table 3.12). The injection of crude extracts for NIV quantification were found to have sample extraction variations of 19.8% and although there is no published variation figures to directly compare this to, this figure was only slightly higher than the clean-up variability for HT-2 reported by Senyuva *et al.* (2012) when extracted without a clean-up phase. These results are in general agreement with Senyuva *et al.* (2012) in that the variation between sample extractions is increased when clean-up columns are not used.

Table 3.12. Comparison between the validated method and published CV (%) values for sample clean-up using DZT MS-PREP® columns.

	Validated method	Senyuva *et al*. (2012)	Romagnoli *et al*. (2010)	Tanaka et al. (2010)
DON	7.0	4.3 - 8.5	2 - 5	4.4 - 5.4
HT-2	8.3	2.6 - 6.8	15 - 32	1.6 - 5.1
T-2	15.5	3.8 - 6.0	13 - 15	0.8 - 3.2
ZEAR	19.2	3.6 - 3.7	9 - 24	2.4 - 4.8

These results show that DZT MS-PREP® columns are a reliable cleanup method for mycotoxin quantification, partially explaining why IAC are the most commonly used clean-up technique in cereals, being used in almost 40% of the published methods (Pereira *et al*. 2014).

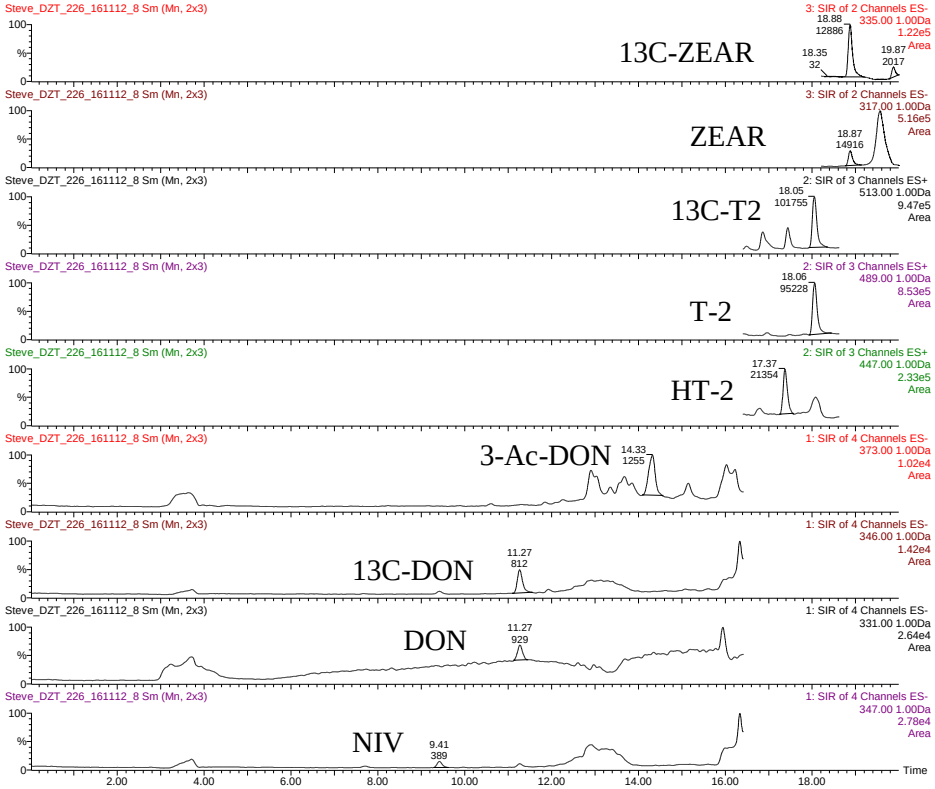

Figure 3.12. Mycosep® 226 chromatogram for 20ppb spiked flour sample with 20ppb of 13C-DON, 13C-T2 and 13C-ZEAR.

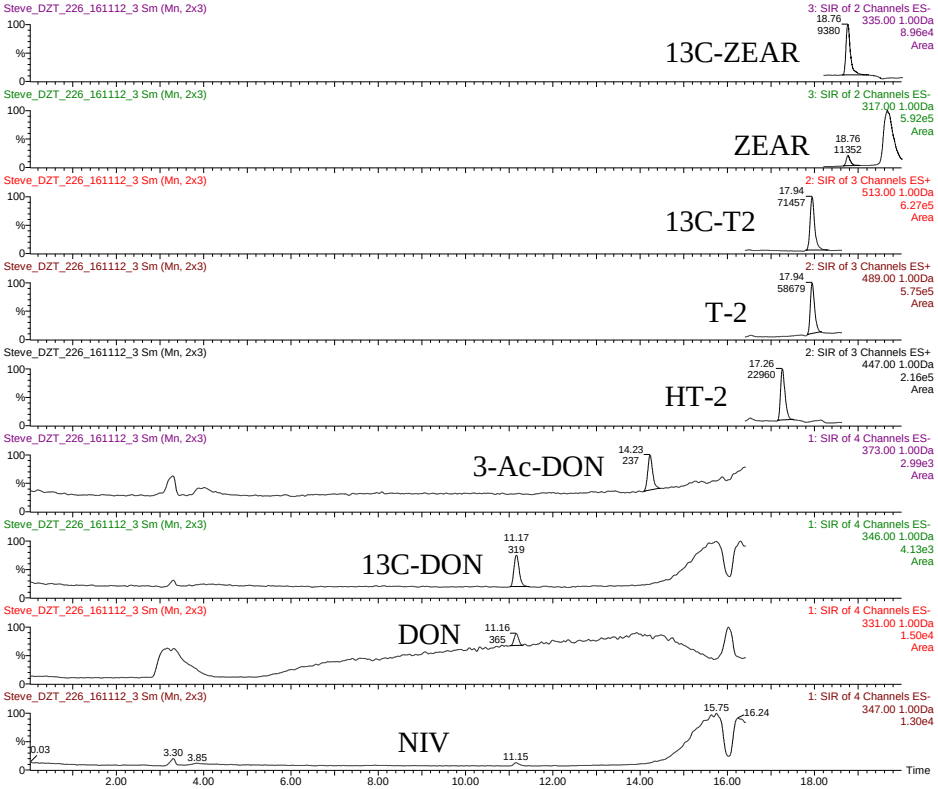

Figure 3.13. DZT MS-PREP chromatogram for 20ppb spiked flour sample with 20ppb of 13C-DON, 13C-T2 and 13C-ZEAR.

Intra-day precision of mycotoxin quantification during method development was compared to similar validation studies. Variation in DON and HT-2 quantification were within the range, and NIV, T-2 and ZEAR variation above the range shown by Di Mavunga *et al.* (2009) (Table 3.13). The intra-day variation shown by NIV, T-2 and ZEAR was slightly higher than the optimum, however this did not seem to effect the strong relationships found between reference flours and quantified values (Table 3.7) and the accurate mycotoxin quantification from the spiked flour experiments (Table 3.8) when using DZT MS-PREP® columns.

Table 3.13. Intra-day CV (%) of extracted samples run in triplicate

	Validated method	Di Mavunga *et al*. (2009)
NIV	13.1 - 19.8	8 - 14
DON	7.0	7 - 8
HT-2	8.3	8 - 11
T-2	15.5	5 - 8
ZEAR	19.2	5 - 9

A recent development in the analysis and quantification of mycotoxins has come in the increased requirement to detect conjugated, or 'masked', mycotoxins. These mycotoxin derivatives are named as such as they are undetected by conventional analytical methods due to alterations in the toxins original chemical structure (Berthiller *et al.* 2013). This includes Deoxynivalanol-3-Glucoside (DON-3-Glc) and Zearanenone-14-glucuronide (ZEAR-14-GU) as examples of a wide range of conjugated mycotoxins, which emerge after metabolism by plants, animals and even fungi (Versilovskis *et al.* 2011). The development of new methods for the clean-up, ionisation and detection of these masked mycotoxins have shown that significant alterations to standard mycotoxin quantification

methodologies are required to successfully quantify this new mycotoxin group (Kostelanska *et al.* 2009; Versilovskis *et al.* 2011).

In conclusion, the LC-MS methodology developed within this study for the simultaneous quantification of multiple mycotoxins has been successfully validated, using DZT MS-PREP® columns for accurate and repeatable clean-up of DON, HT-2, T-2 and ZEAR, and a NIV clean-up procedure for the accurate quantification of NIV.

The completed LC-MS methodology details are presented in Appendix 3A.

Chapter 4.

Field experiment 2010 & 2011

4.1 INTRODUCTION

The 2010 and 2011 field experiments described within this chapter were designed to complete knowledge gaps within the subject of passive resistance to FHB, and represent the first study to rigorously compare the quantitative contribution of a wide range of physiological traits to FHB resistance. The field experiments were designed to allow disease escape mechanisms to be expressed within each genotype, by utilising inoculation methods designed to mimic natural field infection as closely as possible, enabling interactions to occur between the crop canopy, wheat ears and pathogen inoculum, potentially passively influencing their movement. The experiments used a range of DH lines, derived from a cross between Rialto and a CIMMYT wheat of long ear phenotype, to provide a wide variation in physiological traits, while providing a limited basis for genetic variation. Limited genetic variation was important to ensure that the observed variation in FHB resistance between genotypes was due in part to physiological traits, and not wholly due to the effects of resistance genes.

In this chapter, a range of physiological traits were assessed via growth analysis using a selection of UK elite and doubled-haploid wheat genotypes, selected to provide wide variation within the assessed physiological traits. Several physiological traits in wheat have been previously suggested to correlate with FHB resistance, including plant height (Buerstmayr *et al.* 2012; He *et al.* 2014), ear density (Suzuki *et al.* 2012), heading date (Steiner *et al.* 2004; Suzuki *et al.* 2012), awn length (Ban and Suenaga. 2000; Lui *et al.* 2013) and anther extrusion (Lu *et al.* 2013; He *et al.* 2014). These traits were therefore all selected for inclusion into the experiment, in addition to multiple other physiological traits relevant to the experiment. Assessment of these traits provided a basis against which to compare FHB resistance data to enable the identification of significant relationships. All assessments of physiological traits and visual disease scoring made during the 2010 field

experiment were undertaken by an undergraduate student, prior to the start of this PhD study. This enabled a larger number of field trials to be included within the study than would otherwise have been possible.

FHB resistance between genotypes was assessed using visual diseases assessments, pathogen DNA quantification using real-time PCR and grain mycotoxin content using liquid chromatography mass spectrometry (LC-MS). Visual disease assessments of FHB were made over a 33 day period post-flowering to create an AUDPC curve. Pathogen DNA and mycotoxin quantification were undertaken on harvested grain from the experiment. The AUDPC, pathogen DNA and mycotoxins were used to make comparisons between the FHB resistance levels of the selected genotypes and to identify significant relationships with physiological trait assessments.

Relationships between FHB resistance and assessed physiological traits were identified using principal component analysis and multiple linear regression. This allowed for multiple physiological traits to be analysed simultaneously so that the maximum variation in FHB resistance could be accounted for by a combination of traits.

4.2 OBJECTIVES AND HYPOTHESES

The aims of these field experiments were to assess a range of physiological traits for their ability to confer passive resistance to FHB, so that identified traits could be studied in further detail in later experiments using a wider range of wheat genotypes. The individual objectives of the 2010 and 2011 field experiments were to:

1) Quantify a range of physiological traits within a selection of UK elite and DH wheat genotypes.

2) Quantify the level of FHB resistance in each wheat genotype using a combination of visual disease assessments, pathogen DNA quantification and grain mycotoxin quantification.

3) Identify significant relationships between physiological traits and variation in the AUDPC, DNA and mycotoxin data between genotypes.

The specific experimental hypotheses tested within this chapter are:

i) Multiple canopy architecture traits will be significantly related to FHB resistance.

ii Multiple ear morphology traits will be significantly related to FHB resistance.

iii) The proportion of trapped anthers will be more strongly related to FHB than either the proportion of retained or extruded anthers.

iv) The quantity of *Fusarium* spp. and *Microdochium* spp. spores on the flag and second leaves will be negatively related to plant height.

v) UK elite genotypes will have a lower AUDPC, mycotoxin contamination and pathogen DNA content than the DH lines.

vi) Physiological traits assessed at GS65 will account for more FHB variation than GS39 traits in the 2011 experiment.

4.3 MATERIALS AND METHODS

4.3.1 Experimental design

Field experiments using a randomised block design were undertaken in 2010 and 2011 at experiment sites located at the University of Nottingham, Sutton Bonington Campus, UK (latitude 52.83368, longitude -1.24638). Each experiment examined 5 UK winter wheat varieties and 10 doubled-haploid lines derived from a cross between a spring wheat advanced line of large-ear phenotype from CIMMYT, Mexico and the UK winter wheat, Rialto (Table 4.1), with two examples shown in Figure 4.1. Each plot was 2 x 6m and there were three replicates, with Figure 4.2 giving an overview of the experiment. Field experiment layouts for 2010 and 2011 are shown in Appendix 4A and 4B, respectively. Plants were ground-inoculated with infected oat grains produced using three isolates of each *Fusarium graminearum, F. culmorum, F. avenaceum, F. langsethiae, F. poae*, *Microdochium majus* and two isolates of *M. nivale,* at GS31 (Appendix 2A). Misting to stimulate infection during anthesis was not used within the experiment.

Table 4.1. Wheat genotypes used within the 2010 and 2011 field experiments

Genotype	Origin	Breeding method
Ambrosia	UK Elite Variety	Conventional
Claire	UK Elite Variety	Conventional
Grafton	UK Elite Variety	Conventional
Rialto	UK Elite Variety	Conventional
Solstice	UK Elite Variety	Conventional
Line 14	L8 x Rialto	Doubled-haploid
Line 16	L8 x Rialto	Doubled-haploid
Line 18	L8 x Rialto	Doubled-haploid
Line 21	L8 x Rialto	Doubled-haploid
Line 24	L8 x Rialto	Doubled-haploid
Line 38	L8 x Rialto	Doubled-haploid
Line 39	L8 x Rialto	Doubled-haploid
Line 51	L8 x Rialto	Doubled-haploid
Line 61	L8 x Rialto	Doubled-haploid
Line 77	L8 x Rialto	Doubled-haploid

Figure 4.1. Differences in ear morphology between Rialto (L) and DH line 61 (R).

Figure 4.2. An overview of the 2011 field experiment, displaying differences in genotype morphology.

4.3.2 Experiment agronomy

Drilling of the 2010 and 2011 field experiments was carried out on the 15th October 2010 and 11th October 2011, respectively, using a seed rate of 375 seeds/m^2. Crop protection measures followed standard agronomic practice except for fungicide applications which aimed to give robust crop protection from foliar and stem based disease while minimising the effect on FHB development. The agronomic practices used within the 2010 and 2011 experiments are detailed in Appendix 4C. Plant growth regulator (PGR) use was omitted to allow each wheat genotype to express its full height potential.

4.3.3 Ground inoculum production

Refer to section 2.2 for full method details.

4.3.4 Traits assessed in the 2010 field experiment

A small number of physiological traits were chosen for assessment at GS89 in 2010 (Table 4.2) using pre-harvest grab samples. The assessed traits were chosen as they represented the more prominent physiological differences expressed between wheat genotypes studied within the experiment. Due to the assessments being carried out at GS89, flag leaves had to be unrolled to assess flag leaf width. Trait assessments carried out during the 2010 field experiment were undertaken by an undergraduate student, prior to the start of this PhD.

4.3.5 Traits assessed in the 2011 field experiment

A wide range of crop traits were assessed during the 2011 field experiment, at both GS39 and GS65. These additional physiological traits and growth stages were chosen for analysis in 2011 to give a much expanded range of data than that collected in the 2010 experiment. The full range of crop traits assessed in the 2011 field experiment at both growth stages are displayed in Table 4.2. Additional information detailing how specific traits were assessed can be found in Sections 2.3 and 2.3.1.

Table 4.2. Physiological traits assessed during the 2010 and 2011 field experiments

Trait name	2010 GS89	2011 GS39	2011 GS65
Plant number (m^2)		×	×
Fertile shoots (m^2)		×	×
Potentially infertile shoots (m^2)		×	×
Dead and dying shoots (m^2)		×	×
Fertile shoots (per plant)	×	×	×
Potentially infertile shoots (per plant)		×	×
Dead and dying shoots (per plant)		×	×
Flag leaf length (cm)	×	×	×
Flag leaf width (cm)	×	×	×
Flag leaf height (cm)		×	×
Distance between flag and second leaf (cm)		×	×
Distance between second and third leaf (cm)		×	×
Plant height (cm)	×		×
Peduncle length (cm)			×
Total fresh weight (g/m^2)		×	×
Flag leaf area of fertile shoots (cm^2/m^2)		×	×
Second leaf area of fertile shoots (cm^2/m^2)		×	×
All remaining leaf area of fertile shoots (cm^2/m^2)		×	×

Total leaf area (cm^2/m^2)		×	×
Dry weight of flag leaves on fertile shoots (g/m^2)		×	×
Dry weight of second leaves on fertile shoots (g/m^2)		×	×
Dry weight of all remaining leaf on fertile shoots (g/m^2)		×	×
Total dry weight of all leaf on fertile shoots (g/m^2)		×	×
Dry weight of all stems (g/m^2)		×	×
Dry weight of ears (g/m^2)			×
Dry weight of all dead/yellow leaf (g/m^2)		×	×
Dry weight of fertile shoots (g/m^2)		×	×
Dry weight of potentially infertile tillers (g/m^2)		×	×
Dry weight of dead/dying tillers (g/m^2)		×	×
Total dry weight (g/m^2)		×	×
Flag leaf rolling (rolled/unrolled)	×		×
Flag leaf angle (0-30^o, 31-60^o, 61-90^o)			×
Ear length (cm)	×		×
Awn presence (present/absent)	×		
Awn length (cm)			×
Number of spikelets per ear	×		×
Spikelet density (spikelets/cm)	×		×
Hair on ear surface (yes/no)	×		×
Brown foot rot (1-3 scale)	×		×
Stem diameter (cm)	×		×
Retained anthers (%)			×
Trapped anthers (%)			×
Extruded anthers (%)			×
Harvest index (%)			×
Grain yield (t/ha)	×		×
Specific weight (kg/hl)	×		×
Thousand grain weight (g)	×		
Days from sowing to mid-anthesis (days)			×
Canopy fractional light interception (fraction)			×
Fusarium and *Microdochium* spores on flag and second leaf surface (pg/ng DNA)			×

4.3.6 Canopy light interception assessment

In 2011, a Sunfleck SF-80 ceptometer (Decagon Devices, Pullman, USA) was used to measure canopy light interception at GS65. Two readings were taken to measure total photosynthetically active radiation (400 – 700; PAR) above the canopy, then immediately measuring PAR below the canopy, towards the near, middle and far end of each plot. The ceptometer was inserted into each plot at 90^{o} to the direction of drilling, to ensure an accurate measurement across the width of the plot. The Fractional Interception (FI) of each plot was calculated as per equations 2 and 3.

PAR interception = PAR above canopy – PAR below canopy (Equation 2)

Fractional interception = (1 ÷ PAR above canopy) * PAR interception (Equation 3)

4.3.7 Anther extrusion assessment

Anther extrusion was measured by sampling five ears per plot ten days after GS65, after which three spikelets per ear (the middle spikelet, plus the fifth spikelet above and below) were marked for assessment. The first three florets within each spikelet were assessed for the position of the three anthers that naturally occur within a wheat floret, assessing whether the anthers were present (retained), trapped (between the lemma and palea) or fully extruded/missing from the ear (missing was deemed to have been fully extruded). For example, since a floret always produces three anthers, if only two were present in the floret during the assessment then it was assumed that the other anther was missing as it had been fully extruded. The count data were transformed into the percentage of retained anthers, trapped anthers and extruded anthers in each plot.

4.3.8 Flag leaf angle

Flag leaf angle was assessed using an enlarged protractor to measure the distance from vertical of the flag leaf on the plant. The protractor was split into three sections consisting of 0-30^{o}, 31-60^{o} and 61-90^{o} from vertical. Assessments were made visually and were scored based on the flag leaf angle of the majority of the plants within the plot.

4.3.9 Harvest index

Grab samples of approximately 100 ear-bearing shoots were collected from each plot upon maturity. Ears from each plot were removed and threshed to collect the grain and chaff separately. Roots were removed from the remaining plants and straw placed into a paper bag, as were the grain and chaff, and oven dried for 48 hours. Dry weights (DW) were recorded and the harvest index calculated using the following equation:

$$HI = 100 / (\text{grain DW} + \text{chaff DW} + \text{straw DW}) * \text{grain DW} \qquad \text{(Equation 4)}$$

4.3.10 Quantification of *Fusarium* DNA on flag leaves

Fusarium spores on the surfaces of the flag leaf were quantified using real-time PCR. Leaves were sampled at GS65 and carefully placed into paper bags before being frozen until required for extraction.

For extraction, 0.5g of tissue was sampled from the middle of each leaf and placed into a 50ml tube containing 15ml of CTAB buffer plus two tubes of lysing matrix A (MP Bio, Santa Ana, USA). Samples were lysed using a FastPrep (MP Bio, Santa Ana, USA) set at 4.0m/s for 120 seconds before being placed in a water bath at 65^{o}C for two hours. Samples

were cooled rapidly on ice for 15 minutes before having 5ml of potassium acetate solution added. Samples were frozen until the process was continued as per section 2.6.1 onwards.

4.3.11 Visual disease assessments and AUDPC calculation

Refer to section 2.4 for full method details. Visual disease assessments carried out in 2010 field experiment were undertaken by an undergraduate student, prior to the start of this PhD.

4.3.12 Grain harvest

Grain harvest of the 2010 experiment was performed at GS92 using a Sampo 2010 plot combine and was collected into paper bags for later analysis. For grain harvest of the 2011 experiment, refer to section 2.5 for full method details.

4.3.13 Pathogen DNA quantification

Refer to section 2.6 for full method details. Quantification of pathogen DNA within grain samples collected during the 2010 experiment were not included as part of the prior undergraduate study, but were subsequently analysed as part of this PhD.

4.3.14 Mycotoxin quantification

Refer to section Appendix 3A for full method details. Quantification of mycotoxin content within grain samples collected during the 2010 experiment were not included as part of the prior undergraduate study, but were subsequently analysed as part of this PhD.

4.3.15 Statistical analysis

All data were analysed using Genstat® Version 12.1 for Windows (VSN International Ltd, UK). Data collected in 2010 as part of the prior undergraduate study was assessed for accuracy and fully reanalysed to suit the objectives of this PhD. Where required, DNA and mycotoxin data were Log_{10} transformed to normalise residuals. All DNA data had 0.001 added to enable Log_{10} transformation of zero values, and where mycotoxin data was below the limit of quantification, the corresponding limit of quantification was added to the individual measurement. While the 2010 and 2011 field experiments were similar in their design, the 2011 experiment contained a wider assessment of physiological traits over the growing season, therefore separate statistical analyses was carried for each individual year to allow the full array of traits to be separately assessed in each year's experiment. Analysis of variance was carried out on AUDPC, physiological traits, pathogen DNA and mycotoxin concentrations to determine significant differences between genotypes. The associations between measured variables were explored using Principal Component Analysis (PCA) to create biplots. Stepwise Multiple Linear Regression (MLR) was used to model relationships between multiple physiological traits and variation in the AUDPC and grain mycotoxin concentration. This was achieved by including all traits within the MLR at the start of the analysis and then removing the least significant trait from each step, until only statistically significant traits were remaining. Strong multicolinearity between physiological traits ($r > 0.70$) within the 2011 field experiment was avoided by grouping traits together (Section 4.5.12) to ensure results were not confounded within the PCA and MLR.

4.4 FIELD EXPERIMENT 2010 RESULTS

4.4.1 Visual disease symptoms

Lesion and bleaching symptoms were both present in every plot of the 2010 field trial, from which an AUDPC was used to assess the development of FHB disease over time. Significant differences (P<0.001) in the AUDPC were present between genotypes used within the experiment showing a continuous distribution of values (Fig. 4.3). The largest mean AUDPC was present in Line 16 (106.64) while the lowest was present in Ambrosia (1.76). The UK elite genotypes generally had lower AUDPC than the DH genotypes, with a mean AUDPC of 4.31 and 39.36, respectively, showing significant differences between the two groups of genotypes.

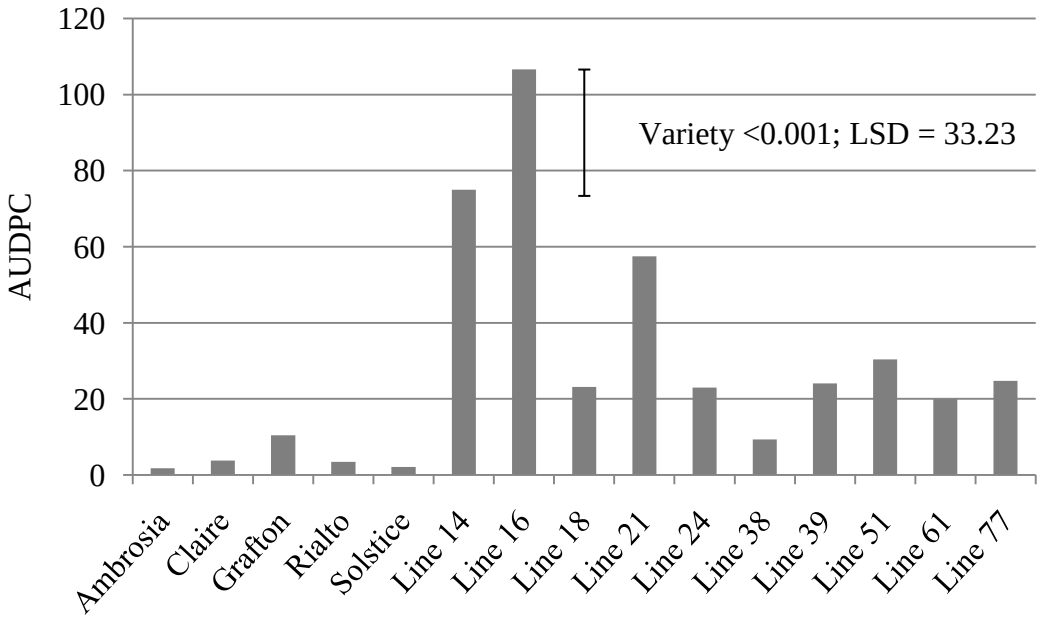

Figure 4.3. Genotypic differences in AUDPC within the 2010 field experiment.

4.4.2 Pathogen DNA

Real-time PCR showed that DNA of each *Fusarium* and *Microdochium* species used for ground inoculation was quantifiable within harvested grain samples, albeit at low concentrations. *F. graminearum* DNA was most commonly found in harvested grain samples, being quantified in 87% of all plots, followed by *F. avanaceum*, *F. poae*, *F. langsethiae* and *F. culmorum* DNA, with *M. majus* and *M. nivale* DNA bring the least frequently quantifiable. *F. culmorum* DNA was found in the greatest concentration of pathogen species DNA in harvested grain samples, followed by *F. graminearum, F. langsethiae, F. poae, F. langsethiae, M. majus* and *M. nivale* (Table 4.3).

Differences between genotypes in pathogen DNA concentrations of harvested grain were found only for *F. culmorum* and *F. poae* ($P<0.001$ and $P=0.003$, respectively) (Table 4.3). The largest quantity of *F. culmorum* DNA was found in Line 14 (0.258 pg/ng), followed by Line 16 (0.133 pg/ng) and Line 39 (0.122 pg/ng) after which concentrations continue to reduce rapidly down to L61 and Grafton in which no *F. culmorum* DNA was detected. Rialto had the highest quantity of *F. poae* DNA (0.045 pg/ng), followed by Ambrosia (0.032 pg/ng) and Claire (0.029 pg/ng) after which concentrations further decrease down to Line 51 in which no *F. poae* DNA was detected. Overall, Line 14 and Ambrosia both contained the highest mean concentration of pathogen DNA with 0.044 pg/ng and 0.042 pg/ng respectively, and Line 77 the lowest concentration of 0.002 pg/ng, showing a wide range of grain pathogen concentrations between genotypes.

Simple linear regression between AUDPC and total mean FHB pathogen DNA content showed that no relationship was present between the two variables ($R^2=0.0008$) (Fig 4.4).

Table 4.3. Differences between genotypes in the amount of *Fusarium spp*. and *Microdochium spp*. DNA present within harvested grain samples collected from the 2010 field experiment, expressed as Log_{10} of total extracted DNA (pg/ng).

	F. graminearum	*F. culmorum*	*F. poae*	*F. avenaceum*	*F. langsethiae*	*M. majus*	*M. nivale*	Mean
Ambrosia	-1.032 (0.093)	-1.175 (0.0668)	-1.493 (0.0321)	-2.034 (0.0092)	-1.119 (0.0760)	-1.817 (0.0152)	-2.520 (0.0030)	-1.599 (0.042)
Claire	-3.150 (0.001)	-3.165 (0.0007)	-1.541 (0.0288)	-2.704 (0.0020)	-2.023 (0.0095)	-3.354 (0.0004)	-2.849 (0.0014)	-2.684 (0.006)
Grafton	-2.354 (0.004)	-4.000 (0.0001)	-1.590 (0.0257)	-1.958 (0.0110)	-1.508 (0.0310)	-3.420 (0.0004)	-1.962 (0.0109)	-2.399 (0.012)
Rialto	-1.365 (0.043)	-2.339 (0.0046)	-1.349 (0.0448)	-2.575 (0.0027)	-1.377 (0.0420)	-3.334 (0.0005)	-2.500 (0.0032)	-2.120 (0.020)
Solstice	-1.592 (0.026)	-3.167 (0.0007)	-2.589 (0.0026)	-4.000 (0.0001)	-1.570 (0.0269)	-2.730 (0.0019)	-4.000 (0.0001)	-2.807 (0.008)
Line 14	-1.461 (0.035)	-0.589 (0.2576)	-2.549 (0.0028)	-2.371 (0.0043)	-2.455 (0.0035)	-3.374 (0.0004)	-2.389 (0.0041)	-2.170 (0.044)
Line 16	-1.489 (0.032)	-0.875 (0.1334)	-1.887 (0.0130)	-2.045 (0.0090)	-2.743 (0.0018)	-3.234 (0.0006)	-3.254 (0.0006)	-2.218 (0.027)
Line 18	-1.800 (0.016)	-1.193 (0.0641)	-2.614 (0.0024)	-1.967 (0.0108)	-2.775 (0.0017)	-3.157 (0.0007)	-4.000 (0.0001)	-2.501 (0.014)
Line 21	-1.779 (0.017)	-2.169 (0.0068)	-1.812 (0.0154)	-3.248 (0.0006)	-3.391 (0.0004)	-2.621 (0.0024)	-3.191 (0.0006)	-2.602 (0.006)
Line 24	-2.137 (0.007)	-1.494 (0.0321)	-2.085 (0.0082)	-2.700 (0.0020)	-2.364 (0.0043)	-3.274 (0.0005)	-4.000 (0.0001)	-2.579 (0.008)
Line 38	-1.898 (0.013)	-3.201 (0.0006)	-2.588 (0.0026)	-2.676 (0.0021)	-1.661 (0.0218)	-4.000 (0.0001)	-3.556 (0.0003)	-2.797 (0.006)
Line 39	-1.435 (0.037)	-0.915 (0.1216)	-1.858 (0.0139)	-3.233 (0.0006)	-2.319 (0.0048)	-2.618 (0.0024)	-2.536 (0.0029)	-2.131 (0.026)
Line 51	-2.667 (0.002)	-1.031 (0.0931)	-4.000 (0.0001)	-1.952 (0.0112)	-2.549 (0.0028)	-2.696 (0.0020)	-3.183 (0.0007)	-2.583 (0.016)
Line 61	-2.555 (0.003)	-4.000 (0.0001)	-1.834 (0.0147)	-3.331 (0.0005)	-2.340 (0.0046)	-4.000 (0.0001)	-3.485 (0.0003)	-3.078 (0.003)
Line 77	-2.694 (0.002)	-2.226 (0.0059)	-3.316 (0.0005)	-2.799 (0.0016)	-2.971 (0.0011)	-4.000 (0.0001)	-3.314 (0.0005)	-3.046 (0.002)
Mean	-1.961 (0.022)	-2.103 (0.0525)	-2.207 (0.0138)	-2.640 (0.0045)	-2.211 (0.0155)	-3.175 (0.0018)	-3.116 (0.0019)	-2.487 (0.016)
P-value	0.111	<0.001	0.003	0.333	0.177	0.441	0.337	-
LSD (5%)	1.344	1.613	1.154	1.628	1.542	1.710	1.7034	-
CV (%)	41.0	45.9	31.3	36.9	41.7	32.2	32.7	-

Back transformed means in parenthesis.

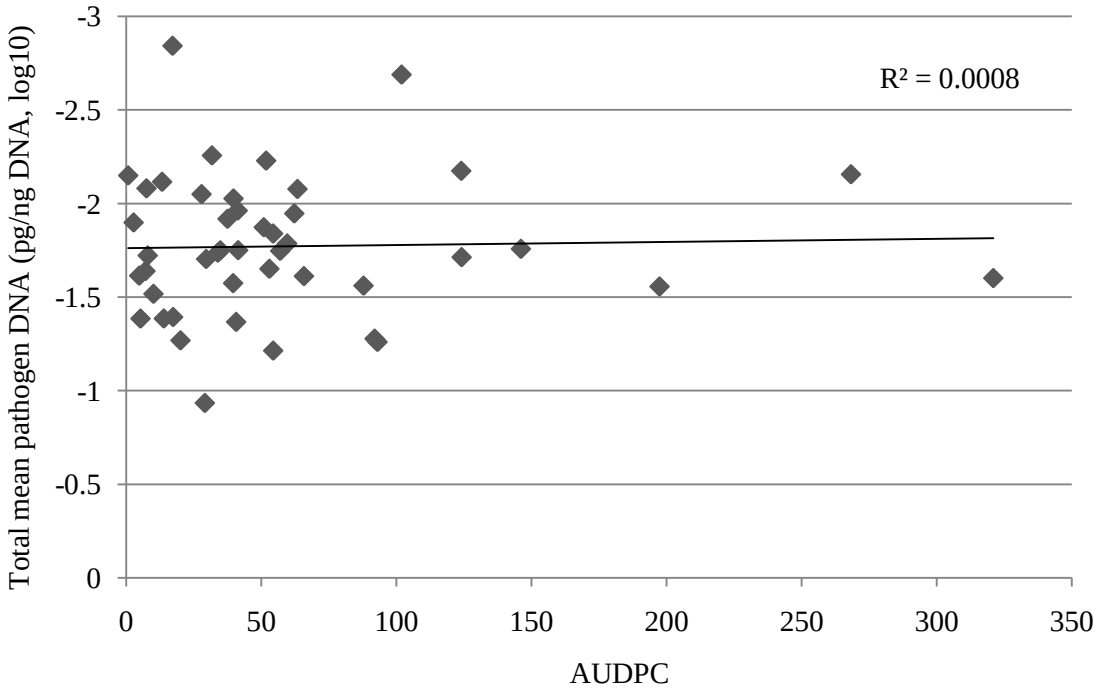

Figure 4.4. Regression between AUDPC vs total mean FHB pathogen DNA content in harvested grain from the 2010 field experiment.

4.4.3 Mycotoxin contamination

Harvested grain mycotoxin contents showed significant differences between genotypes for DON ($P<0.001$) and HT2+T2 ($P=0.002$) (Table 4.4). Ambrosia had the highest DON grain concentration of all genotypes (1128.0 ppb), being significantly higher than Rialto, with the second highest DON content (695.2 ppb). Within the DH population, Line 39, Line 16 and Line 14 had the highest DON grain concentrations, all > 430 ppb, in comparison to the next highest being Line 51 (169.7 ppb). In addition to having the highest grain DON content, Ambrosia also had the highest HT2+T2 concentration (167.5 ppb), being significantly different to all other genotypes excluding Rialto. Despite non-significant differences between genotypes, Ambrosia again had the highest grain mycotoxin concentration for ZEAR (23.0 ppb), followed by Rialto (15.9 ppb) and Line 39 (15.4 ppb). Overall, Ambrosia and Rialto consistently had the highest DON, HT2+T2 and ZEAR contents of the selected genotypes. Strong relationships were present amongst genotypes between the DON and HT2+T2 content of grain and their respective producer species *F. graminearum* and *F. langsethiae* ($R^2=0.50$ and $R^2=0.65$, respectively) (Fig. 4.5 and 4.6). Several outliers were present within each regression, which were due to zero values within DNA quantification. This, however, does not show a complete absence of pathogen DNA within these samples, but that the quantity of DNA was below the limit of detection.

Table 4.4. Differences between genotypes in the amount of DON (ppb), HT2+T2 (ppb) and ZEAR (ppb) present within harvested grain samples collected from the 2010 field experiment, described as log_{10} values.

	DON	HT2+T2	ZEAR
Ambrosia	3.052 (1128.0)	2.224 (167.5)	1.361 (23.0)
Claire	2.420 (263.3)	2.007 (101.6)	1.078 (12.0)
Grafton	2.126 (133.7)	1.804 (63.6)	1.000 (10.0)
Rialto	2.842 (695.2)	1.975 (94.4)	1.201 (15.9)
Solstice	2.635 (431.1)	1.742 (55.2)	1.124 (13.3)
Line 14	2.638 (434.6)	1.704 (50.6)	1.093 (12.4)
Line 16	2.645 (441.4)	1.354 (22.6)	1.066 (11.7)
Line 18	2.021 (105.0)	1.391 (24.6)	1.000 (10.0)
Line 21	2.008 (102.0)	1.771 (59.0)	1.026 (10.6)
Line 24	2.021 (104.9)	1.663 (46.0)	1.000 (10.0)
Line 38	2.045 (110.9)	1.529 (33.8)	1.116 (13.1)
Line 39	2.657 (453.4)	1.909 (81.2)	1.189 (15.4)
Line 51	2.230 (169.7)	1.022 (10.5)	1.000 (10.0)
Line 61	2.228 (169.0)	1.885 (76.7)	1.000 (10.0)
Line 77	2.051 (112.4)	1.340 (21.9)	1.000 (10.0)
Mean	2.375 (323.6)	1.688 (60.6)	1.072 (12.5)
P-value	<.001	0.002	0.384
LSD (5%)	0.476	0.476	0.332
CV (%)	12.0	16.9	18.5

Back transformed means in parenthesis.

Limit of quantification: DON 20ppb; HT2 + T2 10ppb; ZEAR 10ppb.

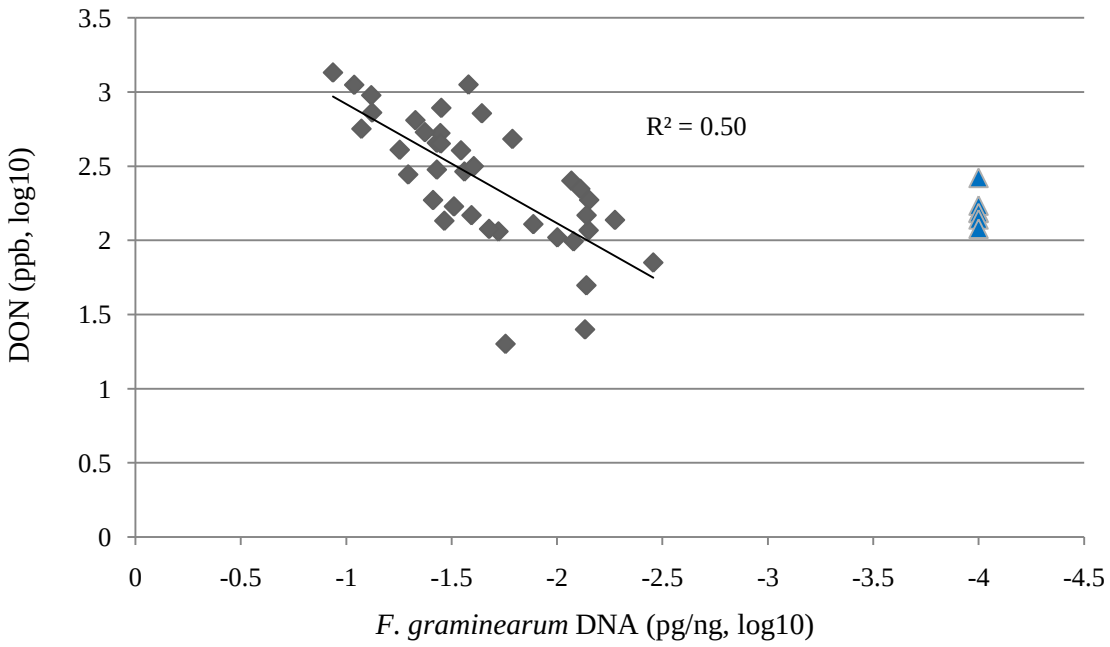

Figure 4.5. Regression between *F.graminearum* DNA vs DON content in harvested grain from the 2010 field experiment. Zero values excluded from the regression are denoted using ▲.

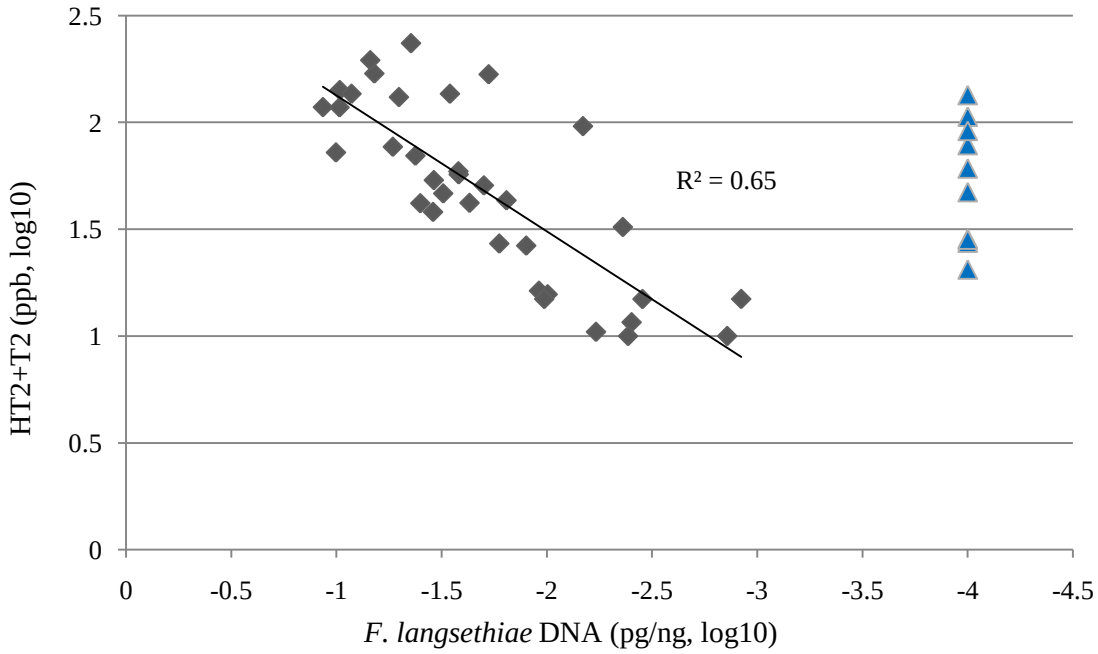

Figure 4.6. Regression between *F.langsethiae* DNA vs HT2+T2 content in harvested grain from the 2010 field experiment. Zero values excluded from the regression are denoted using ▲.

4.4.4 Physiological traits

Significant differences (P<0.001) were present between genotypes for all ear traits (Table 4.5), including ear length, spikelets per ear, spike density, wax presence and awn presence. Line 61 had the longest ears (12.3 cm) and the second highest number of spikelets per ear, and a low spikelet density (1.69 spikelets/cm). Line 21, which had the second longest ears, also had low spikelet density. Grafton had the shortest ears and fewest spikelets per ear (17.2) although this did not result in the highest spikelet density, which was seen in Rialto (2.41 spikelets/cm). Wax on the ears ranged from being present to absent on the genotypes, with some intermediate values resulting in semi waxy ears. Awn presence also ranged from present to absent, with some intermediate values recorded, of which most of the DH population had some form of awn presence, excluding L77. Intermediate values for awn presence indicate an error during assessment, since the genotypes used were either awned or awnless without exception, indicating the presence of contamination with other genotypes. Specific weight ranged from 72.67 - 77.30 kg/hl for Claire and Line 61 respectively (P=0.004), and thousand grain weights ranged from 35.63 - 48.03 g for Line 38 and Line 21 respectively (P<0.001). However, there were no significant differences in grain yield. Flag leaves of Solstice and Line 14 had significantly different levels of rolling compared to the other genotypes (P<0.001), with Solstice showing the highest levels of leaf rolling (0.567), although the majority of genotypes showed either none or very low levels of this trait (Table 4.5). Line 61 had the longest flag leaves (18.37 cm) and Rialto the shortest (12.45) (P<0.001). The UK elite genotypes had shorter flag leaves than the DH lines, except for Solstice, of which several UK genotypes showed significant differences between one another and the DH lines. There were many significant differences between varieties for flag leaf width, ranging between 1.23 - 1.93 cm for Line 38 and Line 51 respectively. Stem diameter ranged from 0.23 - 0.32 cm for Rialto and Line 16 respectively

(P<0.001). A wide variation in plant height was found, ranging from 50.63 - 95.58 cm for Line 18 and Line 61 respectively, showing a continuous variation for these traits (P<0.001). Tiller number per plant ranged from 4.0 - 6.9 for Line 14 and Rialto respectively (P<0.001). There were no significant differences between genotypes in brown foot rot score (Table 4.6).

Table 4.5. Genotypic differences in ear and grain yield traits measured at GS89 in the 2010 field experiment.

	Ear length (cm)	Spikelets per ear (cm)	Spike density (spikelets/cm)	Wax on ear presence	Awn presence	Grain yield (t/ha)	TGW (g)	Specific weight (kg/hl)
Ambrosia	8.63	19.23	2.23	0.00	0.67	8.21	42.61	74.67
Claire	9.73	21.47	2.21	1.00	0.00	8.90	38.93	72.67
Grafton	7.62	17.20	2.26	0.00	1.00	8.61	44.64	76.40
Rialto	8.25	19.87	2.41	0.00	0.67	7.59	43.85	74.93
Solstice	9.85	19.60	1.99	0.00	0.00	8.75	40.92	75.17
Line 14	9.05	19.60	2.17	0.33	0.33	7.45	39.70	75.00
Line 16	8.73	18.33	2.10	1.00	1.00	6.45	44.19	72.90
Line 18	8.68	18.53	2.14	1.00	1.00	7.81	47.59	75.77
Line 21	11.51	19.47	1.69	0.33	0.33	6.63	48.03	75.43
Line 24	8.27	19.07	2.33	0.33	1.00	8.16	40.35	75.80
Line 38	9.55	19.60	2.05	0.00	1.00	7.56	35.63	74.83
Line 39	10.90	20.53	1.89	0.00	1.00	5.99	43.23	76.43
Line 51	9.63	17.80	1.85	1.00	1.00	8.11	45.86	77.30
Line 61	12.25	20.73	1.69	1.00	1.00	6.43	43.30	77.33
Line 77	9.77	20.00	2.05	1.00	0.00	6.66	46.75	75.60
Mean	9.51	19.40	2.07	0.47	0.67	7.55	43.04	75.35
P-value	<0.001	<.001	<.001	<.001	<.001	0.472	<.001	0.004
LSD (5%)	1.04	1.50	0.14	0.43	0.46	2.69	3.71	2.15
CV (%)	6.6	4.6	4.1	55.3	41.4	21.3	5.1	1.7

Table 4.6. Genotypic differences in canopy traits measured at GS89 in the 2010 field experiment.

	Plant height (cm)	Flag leaf length (cm)	Flag leaf width (cm)	Flag leaf rolling	Shoot number per plant	Stem diameter (cm)	Brown foot rot (1-3 scale)
Ambrosia	66.37	14.67	1.62	0.93	6.60	0.25	0.73
Claire	72.55	14.30	1.59	1.00	6.77	0.24	0.83
Grafton	57.90	13.77	1.56	1.00	6.70	0.24	1.00
Rialto	61.84	12.45	1.64	0.97	6.90	0.23	0.97
Solstice	73.82	15.48	1.93	0.57	5.20	0.30	1.00
Line 14	61.33	15.84	1.61	0.87	4.00	0.28	0.90
Line 16	67.79	17.17	1.78	1.00	5.17	0.32	0.97
Line 18	50.63	17.21	1.44	0.90	5.77	0.28	0.83
Line 21	80.40	17.23	1.93	1.00	4.10	0.30	0.97
Line 24	71.61	14.70	1.62	0.97	5.67	0.30	0.93
Line 38	86.48	14.96	1.21	1.00	6.67	0.26	0.93
Line 39	66.32	16.90	1.59	1.00	6.17	0.25	0.77
Line 51	67.74	17.24	1.93	1.00	5.17	0.25	0.83
Line 61	95.58	18.37	1.89	1.00	5.83	0.24	0.87
Line 77	81.38	15.44	1.72	1.00	6.73	0.27	1.00
Mean	70.78	15.72	1.67	0.95	5.83	0.27	0.90
P-value	<.001	<.001	<.001	<.001	<.001	<.001	0.325
LSD (5%)	8.58	1.46	0.13	0.13	1.24	0.03	0.23
CV (%)	7.2	5.6	4.6	8.1	12.7	7.5	15.2

4.4.5 Principal component analysis of the AUDPC, GS89 physiological traits, pathogen DNA and grain mycotoxin content within the 2010 field experiment

Relationships between physiological traits and grain pathogen DNA in the 2010 field trial identified five main groups accounting for 31.4% of the variation (Fig. 4.7). The largest group consisted of tiller number per plant, grain yield, *F. langsethiae* DNA, *F. poae* DNA, *M. nivale* DNA, HT2+T2 and ZEAR. A second group consisted of spike density, *F. graminearum* DNA, *M. majus* DNA and DON. A third group consisted of flag leaf rolling, awn presence, *F. culmorum* DNA and *F. avenaceum* DNA. A fourth group consisted of AUDPC, thousand grain weight, stem diameter, wax presence on ear and brown foot rot score. The fifth group consisted of flag leaf length and flag leaf width, while a sixth group consisted of ear length and plant height. While AUDPC fitted within the fourth group, it was also strongly negatively related to traits found in the first group.

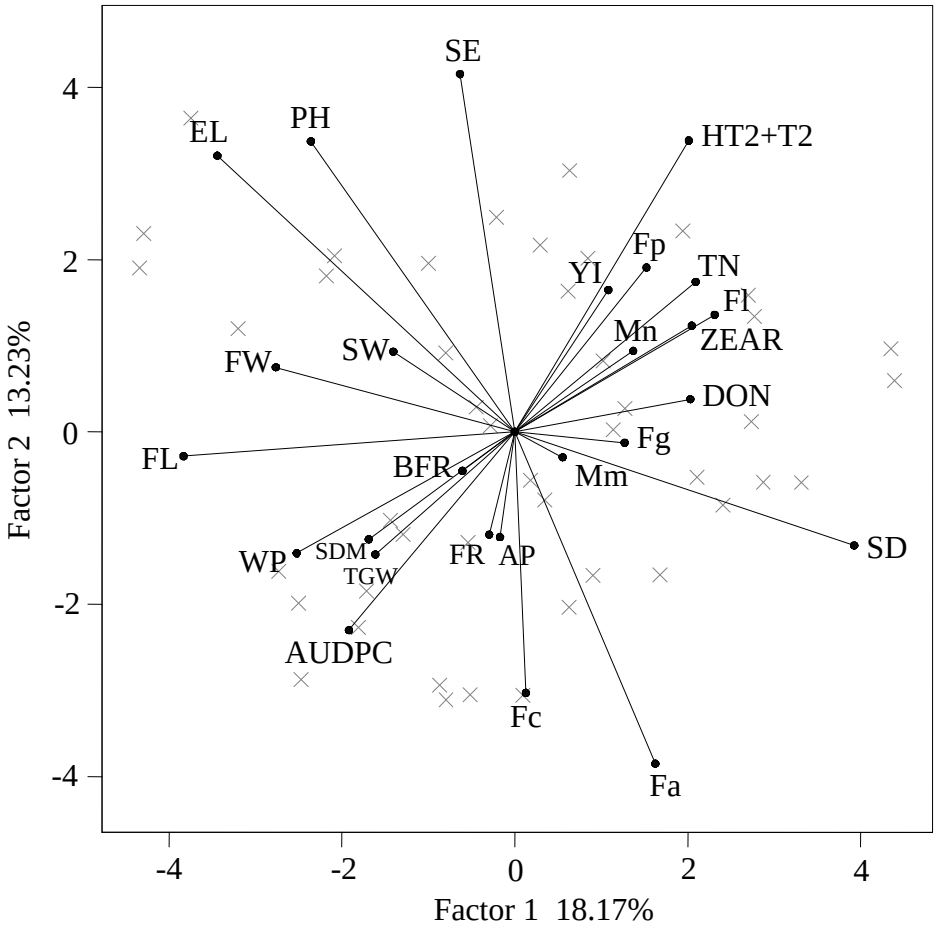

Figure 4.7. Biplot of the principal component analysis for the 2010 field experiment including AUDPC, GS89 physiological traits, pathogen DNA and grain mycotoxin content. Key: AUDPC (Area Under Disease Progress Curve); AP (awn presence, present/absent); BFR (brown foot rot); DON (deoxynivalanol, ppb log10); EL (ear length, cm); FA (flag leaf angle); Fa (*F.avenaceum* DNA, pg/ng log10); Fc (*F.culmorum* DNA, pg/ng log10), Fg (*F.graminearum* DNA, pg/ng log10); Fl (*F.langsethiae* DNA, pg/ng log10) FL (flag leaf length, cm); Fp (*F.poae* DNA, pg/ng log10); FR (rolled flag leaves, rolled/unrolled); FW (flag leaf width, cm); Mm (*M. majus* DNA, pg/ng log10); HT2+T2 (HT2 + T2, pg/ng log10); Mn (*M. nivale* DNA, pg/ng log10); PH (plant height, cm); SE (Spikelets per ear); SD (spikelet density); SDM (stem diameter, cm); SW (specific weight, kg/hl); TGW (thousand grain weight, g); TN (tiller number per plant); WP (wax presence on ear); YI (yield, t/ha); ZEAR (zearalenone, pg/ng log10).

4.4.6 Multiple linear regression of AUDPC and grain mycotoxin content with GS89 physiological traits and grain pathogen DNA

Multiple linear regression (MLR) was used to account for variation in the AUDPC of the 2010 field experiment using physiological traits. MLR showed that 24.8% of the variation could be accounted for ($P<0.001$) using physiological traits with flag leaf length having a positive relationship, and stem diameter having a negative relationship, with the AUDPC (Table 4.5). The addition of grain pathogen DNA into the model did not account for any further variation in the AUDPC.

Multiple linear regression was used to model the variation in mycotoxin content of harvested grain samples using physiological trait and grain pathogen DNA. MLR significantly ($P<0.001$) accounted for 30.7% of the variation of grain DON content, being negatively related to brown foot rot severity, flag leaf rolling and specific weight, and positively related to *F. graminearum* DNA (Table 4.7). No significant relationships between either physiological traits or pathogen DNA were present with ZEAR. Grain concentrations of HT2+T2 were negatively related to flag leaf length and positively related to spikelets per ear accounting for 21.1% of the variation ($P=0.003$).

Table 4.7. Multiple linear regression models of the 2010 field trial accounting for variation in the AUDPC and mycotoxin content between genotypes using physiological traits and pathogen species DNA.

Model	Equation	R^2	P-value
AUDPC	6.80 (FL) - 360 (SDM) - 175	24.8	<0.001
DON	- 0.968 (BFR) - 0.842 (FR) - 0.0737 (SW) + 0.1414 (Fg DNA) + 9.88	30.7	<0.001
ZEAR	-	-	-
HT2+T2	- 0.0735 (FL) + 0.1175 (SE) + 0.563	21.1	0.003

Key: BFR = Brown foot rot; Fg DNA = *F. graminearum* DNA Log_{10}; FL = Flag leaf length (cm); FR = flag leaf rolling; SDM = Stem diameter (cm); SE = Spikelets per ear; SW = specific weight.

4.5 FIELD EXPERIMENT 2011 RESULTS

4.5.1 Visual disease symptoms

Differences (P<0.001) were present between genotypes for AUDPC (Fig. 4.8). Disease pressure and hence visual FHB symptoms were low in 2011. Genotype AUDPCs grouped into two general tiers with Lines 16 and 18 having the highest AUDPCs and were significantly different to all other lines. All remaining lines had reduced AUDPC values and formed the second tier. Lesions were the predominant visual symptom of FHB infection seen throughout the trial, with bleaching symptoms making up a much smaller proportion of the AUDPC, being present in only four out of fifteen wheat genotypes.

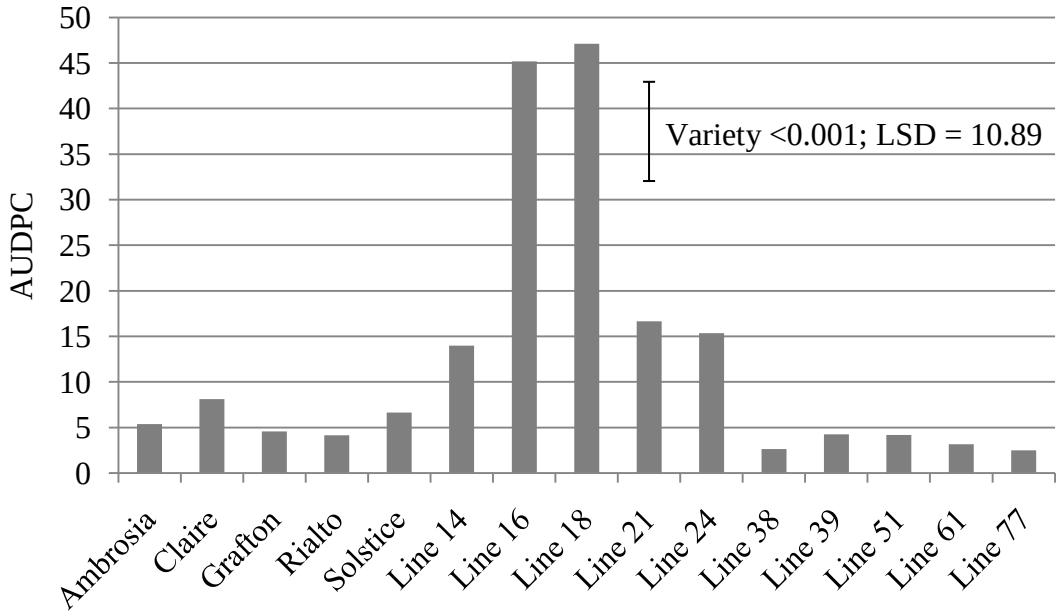

Figure 4.8. Genotypic differences in AUDPC within the 2011 field experiment.

4.5.2 Pathogen DNA

Fusarium DNA was extracted from grain samples and quantified using real-time PCR. *F. avenaceum* had the highest incidence, being detected in 100% of plots, despite its low grain DNA concentration (Table 4.8). *F. poae* had the second highest plot incidence (86.67%), followed by *F. langsethiae* (69.77%) and *F. culmorum* and *F. graminearum* (20%). *Microdochium* species were detected at very low incidences and concentrations.

Significant differences between genotypes for the concentrations of *F. culmorum* DNA ($P<0.001$) and *F. langsethiae* DNA ($P=0.05$) were found in grain samples (Table 4.8). Line 16 had the highest mean *F. culmorum* grain DNA concentration (0.054 pg/ng), exceeding the LSD ($P<0.001$) to all other genotypes except Solstice and Line 14. Ambrosia had the highest *F. langsethiae* grain DNA content (0.0206 pg/ng) compared to Line 51 and Line 61 with the lowest DNA concentration of 0.0001 pg/ng ($P=0.05$). However, due to the inclusion of multiple wheat lines and *Fusarium* species within the study, a high proportion of zero values were present within the analysis, therefore caution should be given to the mean values shown in Table 4.8.

Only a very weak positive relationship was present ($R^2=0.11$) between AUDPC and mean FHB pathogen DNA content in harvested grain samples (Fig. 4.9).

Table 4.8. Differences between genotypes in the amount of *Fusarium spp*. and *Microdochium spp*. DNA present within harvested grain samples collected from the 2011 field experiment, expressed as Log_{10} of total extracted DNA (pg/ng).

	F. graminearum	*F. culmorum*	*F. poae*	*F. avenaceum*	*F. langsethiae*	*M. majus*	*M. nivale*	Mean
Ambrosia	-2.262 (0.0055)	-4.000 (0.0001)	-1.600 (0.0251)	-2.634 (0.0023)	-1.686 (0.0206)	-3.379 (0.0004)	-3.432 (0.0004)	-2.713 (0.0078)
Claire	-3.091 (0.0008)	-4.000 (0.0001)	-1.682 (0.0208)	-2.719 (0.0019)	-3.186 (0.0007)	-4.000 (0.0001)	-4.000 (0.0001)	-3.240 (0.0035)
Grafton	-4.000 (0.0001)	-3.067 (0.0009)	-2.724 (0.0019)	-2.727 (0.0019)	-2.585 (0.0026)	-4.000 (0.0001)	-4.000 (0.0001)	-3.300 (0.0011)
Rialto	-3.235 (0.0006)	-4.000 (0.0001)	-2.569 (0.0027)	-2.547 (0.0028)	-2.995 (0.0010)	-3.426 (0.0004)	-4.000 (0.0001)	-3.253 (0.0011)
Solstice	-3.303 (0.0005)	-2.196 (0.0064)	-1.401 (0.0397)	-2.964 (0.0011)	-2.694 (0.0020)	-4.000 (0.0001)	-3.481 (0.0003)	-2.863 (0.0072)
Line 14	-4.000 (0.0001)	-2.464 (0.0034)	-1.888 (0.0129)	-2.589 (0.0026)	-2.262 (0.0055)	-4.000 (0.0001)	-4.000 (0.0001)	-3.029 (0.0035)
Line 16	-4.000 (0.0001)	-1.268 (0.0540)	-1.411 (0.0388)	-2.508 (0.0031)	-2.849 (0.0014)	-4.000 (0.0001)	-4.000 (0.0001)	-2.862 (0.0139)
Line 18	-4.000 (0.0001)	-4.000 (0.0001)	-1.608 (0.0247)	-2.454 (0.0035)	-1.715 (0.0193)	-4.000 (0.0001)	-4.000 (0.0001)	-3.111 (0.0068)
Line 21	-3.293 (0.0005)	-4.000 (0.0001)	-2.372 (0.0042)	-2.692 (0.0020)	-2.667 (0.0022)	-4.000 (0.0001)	-3.529 (0.0003)	-3.222 (0.0013)
Line 24	-2.517 (0.0030)	-3.299 (0.0005)	-1.123 (0.0753)	-2.894 (0.0013)	-1.891 (0.0129)	-4.000 (0.0001)	-4.000 (0.0001)	-2.818 (0.0133)
Line 38	-4.000 (0.0001)	-4.000 (0.0001)	-3.245 (0.0006)	-2.572 (0.0027)	-3.364 (0.0004)	-4.000 (0.0001)	-4.000 (0.0001)	-3.597 (0.0006)
Line 39	-4.000 (0.0001)	-4.000 (0.0001)	-2.548 (0.0028)	-2.801 (0.0016)	-2.000 (0.0100)	-4.000 (0.0001)	-3.558 (0.0003)	-3.272 (0.0021)
Line 51	-4.000 (0.0001)	-4.000 (0.0001)	-1.928 (0.0118)	-2.143 (0.0072)	-4.000 (0.0001)	-4.000 (0.0001)	-3.692 (0.0002)	-3.398 (0.0028)
Line 61	-3.332 (0.0005)	-4.000 (0.0001)	-1.849 (0.0142)	-2.848 (0.0014)	-4.000 (0.0001)	-4.000 (0.0001)	-4.000 (0.0001)	-3.430 (0.0024)
Line 77	-4.000 (0.0001)	-4.000 (0.0001)	-2.099 (0.0080)	-2.722 (0.0019)	-3.438 (0.0004)	-4.000 (0.0001)	-4.000 (0.0001)	-3.466 (0.0015)
Mean	-3.536 (0.0008)	-3.486 (0.0044)	-2.003 (0.0189)	-2.654 (0.0025)	-2.756 (0.0053)	-3.920 (0.0001)	-3.846 (0.0002)	-3.172 (0.0046)
P-value	0.337	<0.001	0.121	0.220	0.050	0.140	0.767	-
LSD (5%)	1.5610	1.2791	1.3108	0.4966	1.4596	0.4815	0.8080	-
CV (%)	26.4	21.9	39.1	11.2	31.7	7.3	12.6	-

Back transformed means in parenthesis

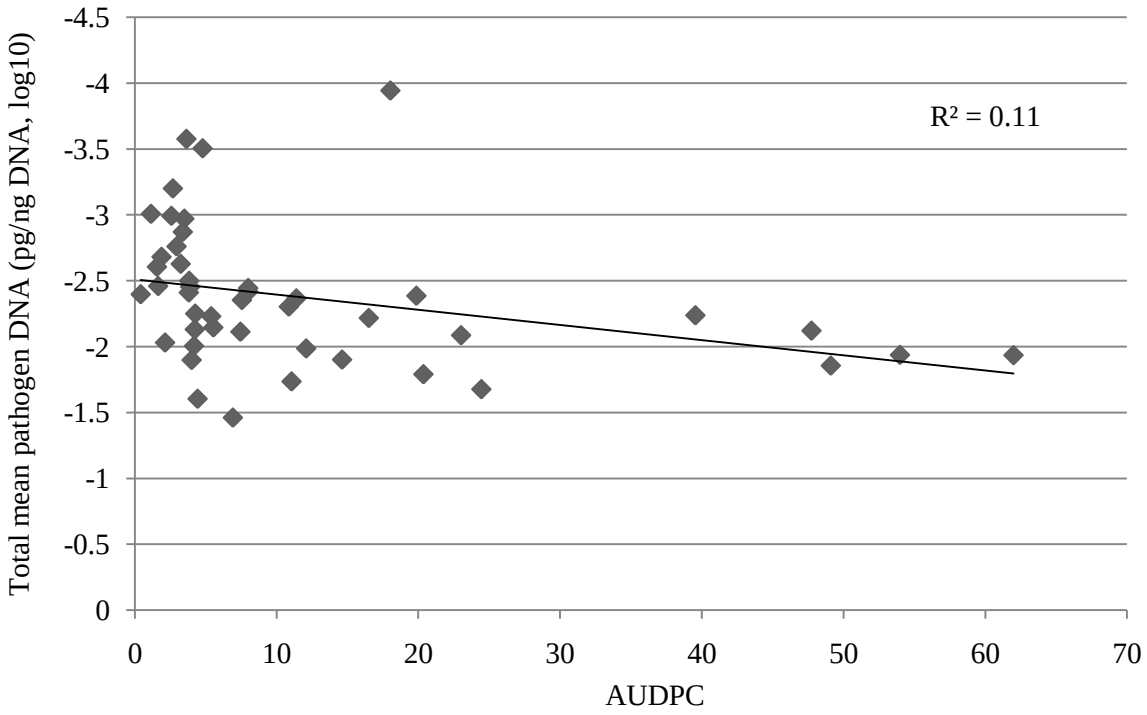

Figure 4.9. Regression between AUDPC vs total mean FHB pathogen DNA content in harvested grain from the 2011 field experiment.

4.5.3 Mycotoxin contamination

Analysis of variance was utilised to identify significant differences between grain mycotoxin content of the wheat genotypes. Log_{10} transformation of DON, HT2+T2 and ZEAR were required to normalise residuals. No significant differences were identified between genotypes for any of the mycotoxins (Table 4.9). However, strong positive relationships between DON and *F. graminearum* DNA (R^2=0.76) as well as DON and *F. culmorum* DNA (R^2=0.71) were present (Fig. 4.10 and Fig. 4.11).

Table 4.9. Differences between genotypes in the amount of DON (ppb), HT2+T2 (ppb) and ZEAR (ppb) present within harvested grain samples collected from the 2011 field experiment, described as log_{10} values.

	DON	HT2+T2	ZEAR
Ambrosia	1.714 (51.76)	1.122 (13.24)	1.000 (10.00)
Claire	1.491 (30.97)	1.105 (12.74)	1.000 (10.00)
Grafton	1.731 (53.83)	1.141 (13.84)	1.000 (10.00)
Rialto	1.388 (24.43)	1.016 (10.38)	1.043 (11.04)
Solstice	1.777 (59.84)	1.028 (10.67)	1.000 (10.00)
Line 14	1.301 (20.00)	1.071 (11.78)	1.000 (10.00)
Line 16	2.142 (138.68)	1.006 (10.14)	1.000 (10.00)
Line 18	1.301 (20.00)	1.101 (12.62)	1.000 (10.00)
Line 21	1.322 (20.99)	1.000 (10.00)	1.000 (10.00)
Line 24	1.509 (32.28)	1.284 (19.23)	1.000 (10.00)
Line 38	1.301 (20.00)	1.000 (10.00)	1.000 (10.00)
Line 39	1.326 (21.18)	1.000 (10.00)	1.000 (10.00)
Line 51	1.301 (20.00)	1.000 (10.00)	1.000 (10.00)
Line 61	1.391 (24.60)	1.000 (10.00)	1.000 (10.00)
Line 77	1.301 (20.00)	1.000 (10.00)	1.000 (10.00)
Mean	1.486 (37.24)	1.058 (11.64)	1.003 (10.07)
P-value	0.163	0.757	0.479
LSD (5%)	0.5829	0.2805	0.0322
CV (%)	23.4	15.8	1.9

Back transformed means in parenthesis

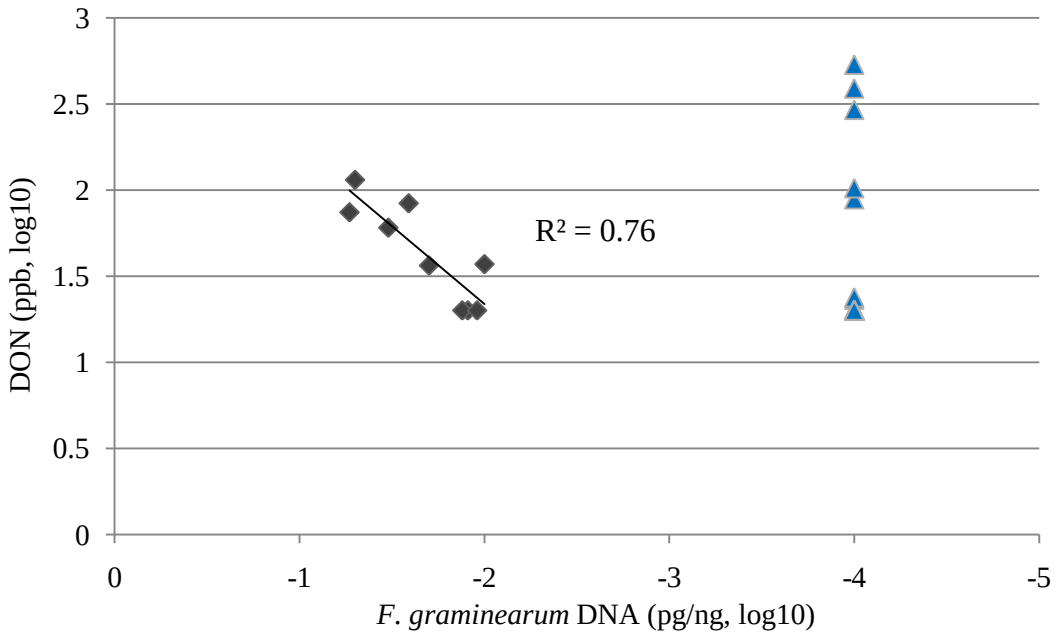

Figure 4.10. Regression between *F. graminearum* DNA vs DON content in harvested grain from the 2011 field experiment. Zero values excluded from the regression are denoted using ▲.

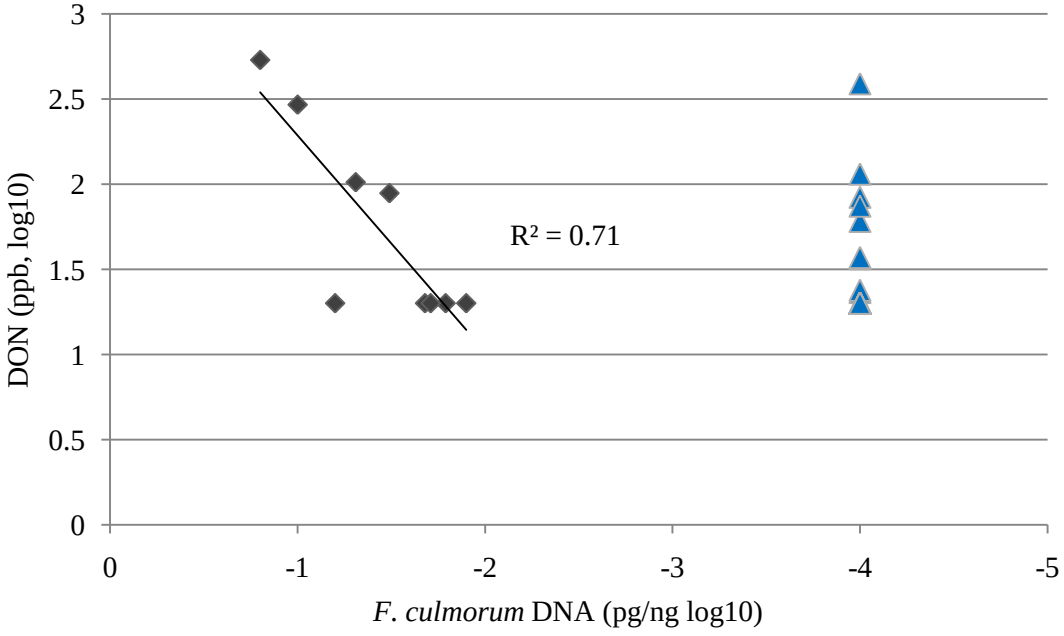

Figure 4.11. Regression between *F. culmorum* DNA vs DON content in harvested grain from the 2011 field experiment. Zero values excluded from the regression are denoted using ▲.

4.5.4 Physiological traits at GS39

Significant differences at GS39 were found between genotypes for several physiological traits (Table 4.10). Key differences identified included the number of plants per unit area (P=0.034) which ranged from 250.7 – 426.7 plants/m^2 and led to significant differences between the number of fertile shoots per m^2 (P=0.043) but not in the number of fertile shoots per plant. There were large differences in the number of dead/dying shoots per genotype, ranging from 128 – 645.3/m^2 (P<0.001) and 0.51 – 1.66 per plant (<0.001). Significant differences were present for all flag leaf traits (P<0.001) including length, width, height, distance from the second leaf and also the distance between second to third leaf. Total fresh weight (g/m^2) had significant differences between genotypes (P=0.006) as did the total leaf fresh weight (g/m^2) (P=0.001), total leaf area (cm^2/m^2) (P<0.001), total dry weight (g/m^2) (P=0.029) and the total leaf dry weight (g/m^2) (P=0.002) of which the measurements of the UK elite genotypes were almost always consistently higher than the DH genotypes.

Table 4.10. Genotypic differences in canopy traits measured at GS39 in the 2011 field experiment *(continued)*

	Plant number (m^2)	Fertile shoots (m^2)	Potentially infertile shoots (m^2)	Dead and dying shoots (m^2)	Number of fertile shoots per plant	Number of potentially infertile shoots per plant
Ambrosia	309.3	501.3	208.0	341.3	1.93	0.70
Claire	325.3	533.3	128.0	304.0	1.93	0.40
Grafton	421.3	576.0	165.3	474.7	1.27	0.43
Rialto	426.7	533.3	112.0	400.0	1.47	0.26
Solstice	410.7	586.7	90.7	213.3	1.60	0.25
Line 14	250.7	368.0	170.7	128.0	1.73	0.69
Line 16	294.1	410.7	69.3	149.3	1.93	0.19
Line 18	394.7	474.7	128.0	138.7	1.58	0.35
Line 21	320.0	437.3	53.3	298.7	1.53	0.17
Line 24	384.0	565.3	48.0	464.0	1.53	0.13
Line 38	394.7	570.7	42.7	645.3	1.53	0.12
Line 39	288.0	405.3	64.0	218.7	1.60	0.22
Line 51	314.7	426.7	42.7	496.0	1.53	0.15
Line 61	314.7	405.3	32.0	154.7	1.40	0.09
Line 77	266.7	400.0	21.3	218.7	1.60	0.09
Mean	341.0	480.0	91.7	309.7	1.61	0.28
P-value	0.031	0.043	0.098	<0.001	0.934	0.060
LSD (5%)	112.2	151.6	125.3	171.9	0.83	0.41
CV (%)	19.6	18.9	81.7	33.2	30.9	86.25

Table 4.10. Genotypic differences in canopy traits measured at GS39 in the 2011 field experiment *(continued)*

	Number of dead and dying shoots per plant	Flag leaf length (cm)	Flag leaf width (cm)	Flag leaf height (cm)	Flag and second leaf distance (cm)	Second and third leaf distance (cm)
Ambrosia	1.10	16.78	1.86	44.13	7.93	12.97
Claire	0.92	16.30	1.70	44.48	6.87	13.25
Grafton	1.16	13.41	1.47	43.97	8.21	11.50
Rialto	0.94	16.89	1.70	54.83	9.86	14.57
Solstice	0.55	16.06	1.83	46.79	7.36	13.34
Line 14	0.51	17.87	1.59	43.48	6.71	12.23
Line 16	0.52	17.97	1.63	45.58	10.41	12.66
Line 18	0.34	21.83	1.53	28.62	5.64	6.93
Line 21	0.94	19.10	1.58	61.67	8.48	16.99
Line 24	1.21	13.35	1.43	43.98	5.77	10.40
Line 38	1.66	13.33	1.23	48.01	6.69	11.09
Line 39	0.77	16.55	1.70	34.74	6.60	8.69
Line 51	1.60	14.57	1.76	45.01	6.53	10.82
Line 61	0.57	16.18	1.45	71.23	11.24	16.97
Line 77	0.83	15.85	1.58	70.32	10.27	17.72
Mean	0.91	16.40	1.60	48.46	7.90	12.67
P-value	< 0.001	<0.001	<0.001	<0.001	<0.001	<0.001
LSD (5%)	0.55	2.85	0.20	7.38	2.51	1.983
CV (%)	35.43	10.4	7.4	9.1	18.0	9.4

Table 4.10. Genotypic differences in canopy traits measured at GS39 in the 2011 field experiment *(continued)*

	Flag leaf area (cm^2/m^2)	Second leaf area (cm^2/m^2)	All remaining leaf area (cm^2/m^2)	Total leaf area (cm^2/m^2)	Flag leaf dry weight (g/m^2)	Second leaf dry weight (g/m^2)
Ambrosia	7839	10530	29079	47448	51.79	62.03
Claire	8789	10793	28649	48231	55.09	63.63
Grafton	6957	9788	27171	43917	45.12	57.97
Rialto	6130	8432	25415	39976	46.61	52.96
Solstice	7448	10377	28899	46724	45.92	55.20
Line 14	3540	6185	11253	20979	24.85	31.95
Line 16	3945	6986	11742	22673	38.40	43.52
Line 18	5305	5492	13666	24464	37.17	37.23
Line 21	5647	6683	15869	28199	41.87	50.51
Line 24	3591	9939	23896	37425	32.11	55.57
Line 38	3696	5927	17075	26698	30.67	40.00
Line 39	3063	5391	9980	18434	34.19	48.00
Line 51	4767	8802	13588	27157	36.64	53.71
Line 61	3446	5742	15546	24734	33.01	45.01
Line 77	3879	6331	11732	21942	32.69	40.27
Mean	5203	7827	18904	31933	39.08	49.17
P-value	<0.001	0.008	<0.001	<0.001	0.040	0.284
LSD (5%)	2430	3479	9501	14570	16.7	24.00
CV (%)	27.9	26.6	30.0	27.3	25.5	29.1

Table 4.10. Genotypic differences in canopy traits measured at GS39 in the 2011 field experiment

	All remaining leaf dry weight (g/m^2)	Total leaf dry weight (g/m^2)	Stem dry weight (g/m^2)	Potentially infertile tiller dry weight (g/m^2)	Dead and dying tiller dry weight (g/m^2)	Total dry weight (g/m^2)
Ambrosia	146.1	259.9	622.1	19.68	31.04	949.4
Claire	135.4	254.1	591.7	18.19	24.05	913.1
Grafton	129.2	232.3	629.1	22.51	34.29	934.0
Rialto	125.2	224.8	625.3	17.92	21.01	909.3
Solstice	128.6	229.7	532.2	19.89	11.25	816.7
Line 14	49.0	105.8	321.4	11.47	8.11	462.2
Line 16	64.1	146.0	409.4	12.85	11.47	602.9
Line 18	32.9	107.3	277.0	15.52	14.99	439.9
Line 21	87.0	179.4	599.4	7.73	22.13	833.6
Line 24	116.7	204.4	593.8	9.33	35.15	879.6
Line 38	91.5	162.1	452.6	8.37	41.55	711.8
Line 39	70.2	152.4	373.3	9.60	19.36	582.5
Line 51	85.1	175.4	472.0	9.55	32.05	732.2
Line 61	89.1	167.1	642.9	14.45	54.03	964.6
Line 77	66.1	139.1	557.0	5.07	26.83	780.6
Mean	94.4	182.6	513.3	13.48	25.82	767.5
P-value	<0.001	0.002	0.031	0.617	<0.001	0.029
LSD (5%)	39.1	76.6	232.6	16.61	12.48	333.1
CV (%)	24.7	25.1	27.1	73.7	28.9	26.0

4.5.5 Principal component analysis of the AUDPC, GS39 physiological traits, pathogen DNA and grain mycotoxin content within the 2011 field experiment

Principal Component Analysis (PCA) showed correlations between several morphological traits, pathogen DNA and AUDPC (Fig. 4.12). Primarily, AUDPC and *F. langsethiae* DNA grouped together. A second group consisted of flag leaf length (cm), infertile shoots/m^2, infertile shoots per plant and *F. culmorum*, *F. poae* and *M. nivale* DNA. A third group consisted of infertile shoot dry weight (g/m^2), infertile shoot fresh weight (g/m^2), flag leaf width (cm) and *M. majus* DNA. A fourth group was made up of flag leaf area (cm^2/m^2), second leaf area (cm^2/m^2), remaining leaf area (cm^2/m^2) and total leaf area (cm^2/m^2), fertile shoots per plant and *F. graminearum* DNA, DON, ZEAR and HT2+T2. A fifth group consisted of fertile shoots/m^2, fertile shoots fresh weight (g/m^2), total fresh weight (g/m^2), fertile shoot dry weight (g/m^2), total dry weight (g/m^2) and plant number/m^2. A sixth group consisted of flag leaf height (cm), distance between flag and second leaf (cm), distance between second and third leaf (cm), dead/dying shoots/m^2, dead/dying shoots per plant, dead/dying shoot fresh weight (g/m^2), dead and dying shoot dry weight (g/m^2), dry weight of dead and yellow leaf (g/m^2). The majority of quantified *Fusarium* DNA was positively associated with the first and morphological traits, except for *F. langsethiae* which was positively associated with AUDPC.

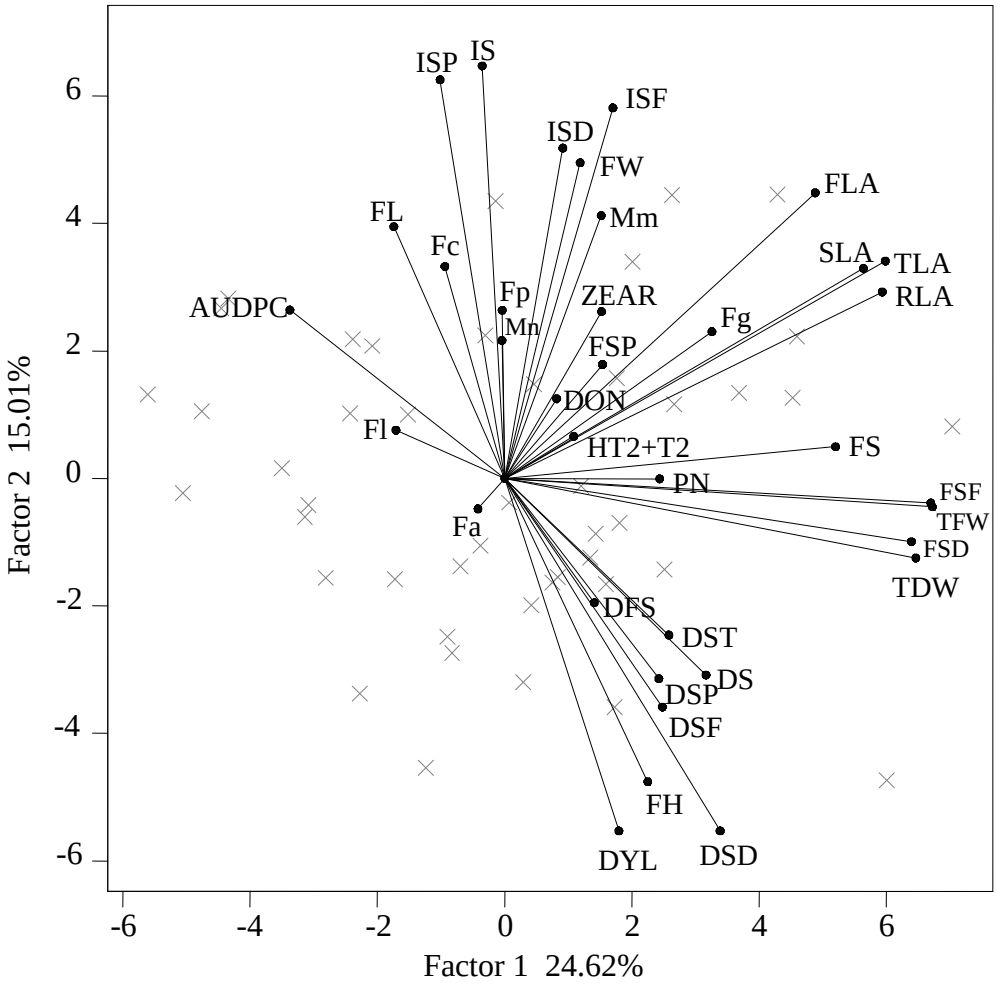

Figure 4.12. Biplot of the principal component analysis of the 2011 field experiment including AUDPC, GS39 physiological traits, pathogen DNA and grain mycotoxin content. Key: AUDPC (Area Under Disease Progress Curve); DFS (distance between flag and second leaf, cm); DON (deoxynivalanol, ppb log10); DS (dead/dying shoots, m^{-2}); DSD (dead and dying shoot dry weight, g/m^{-2}); DSF (dead and dying shoots fresh weight, g/m^{-2}); DSP (dead/dying shoots per plant); DST (distance between second and third leaf, cm); DYL (dry weight of dead/yellow leaf, g/m^{-2}); Fa DNA (*F.avenaceum* DNA); Fc DNA (*F.culmorum* DNA), Fg DNA (*F.graminearum* DNA), Fl DNA (*F.langsethiae* DNA); FL (flag leaf length, cm); FLA (Flag leaf area, cm^{-2}/m^{-2}) FH (flag leaf height, cm); Fp DNA (*F.poae* DNA); FS (fertile shoots, m^{-2}); FSD (fertile shoot dry weight, g/m^{-2}); FSF (fertile shoot fresh weight, g/m^{-2}); FSP (fertile shoots per plant); FW (flag leaf width, cm); HT2+T2 (HT2+T2, ppb log10); IS (potentially infertile shoots, m^{-2}); ISD (infertile shoot dry weight, g/m^{-2}); ISF (infertile shoot fresh weight, g/m^{-2}); ISP (potentially infertile shoots per plant);Mm DNA (*M. majus DNA*); Mn DNA (*M. nivale* DNA); PN (plant number, m^{-2}); RLA (remaining leaf area, cm^{-2}/m^{-2}); SLA (second leaf area, cm^{-2}/m^{-2}); TDW (Total plot dry weight, g/m^{-2}); TFW (total fresh weight, g/m^{-2}); TLA (total leaf area, cm^{-2}/m^{-2}); ZEAR (zearalenone, ppb log10).

4.5.6 Multiple linear regression of AUDPC and mycotoxin concentration with GS39 physiological traits and grain pathogen DNA

Multiple linear regression found significant (P<0.001) relationships between AUDPC, GS39 physiological traits and *Fusarium* DNA, accounting for 51% of the variation in the AUDPC (Table 4.11). AUDPC was positively related to flag leaf length and *F. culmorum* DNA, and negatively related to flag leaf height and flag leaf width. MLR accounted for 19% of the variation in grain DON content (P=0.005), being positively related to plant number per m^2 and *F. culmorum* DNA. Grain HT2+T2 content was not significantly related to any physiological traits in the MLR, however 15% of the variation was accounted for with a positive relationship to *F. langsethiae* DNA (P=0.006). Physiological traits and pathogen DNA were unable to significantly account for variation in grain ZEAR content.

Table 4.11. Multiple linear regression models of the 2011 field experiment accounting for variation in the AUDPC and mycotoxin content (log_{10}) between genotypes using GS39 physiological traits and species DNA.

Model	Equation	R^2	P-value
AUDPC	40.9 + 3.558 (FL) - 34.47 (FW) - 0.397 (FH) + 3.58 (Fc DNA)	0.51	<0.001
DON	1.52 + 0.0013 (PN) + 0.14 (Fc DNA)	0.19	0.005
ZEAR	-	-	-
HT2+T2	1.2393 + 0.0661 (Fl DNA)	0.15	0.006

Key: FL = Flag leaf length (cm); FW = Flag width (cm); FH = Flag leaf height (cm); Fc DNA = *F. culmorum* DNA log_{10}; PN = Plant number (m^2); Fl DNA = *F. langsethiae* DNA log_{10}.

4.5.7 Physiological traits at GS65

Differences between genotypes for plant number per m^2 were close to significance (P=0.055) and ranged from 202.7 - 357.3/m^2. There were significant differences in the number of fertile and dead & dying shoots per m^2 (P=0.037 and P<0.001, respectively), as well as significant differences between the number fertile and dead & dying shoots per plant (P<0.001) (Table 4.12). Significant differences were present for all flag-leaf and plant height traits (P<0.001) including length, width, height, distance from the second leaf, distance between second to third leaf, plant height and peduncle length. Plant height ranged from 40.02 – 118.41 cm for Line 18 and Line 61 respectively, of which the DH genotypes exhibited a much wider variation in plant height than the UK elite genotypes. Total fresh weight (g/m^2) had significant differences between genotypes (P=0.011) as did the total leaf fresh weight (g/m^2) (P=0.033), total leaf area (cm^2/m^2) (P<0.001), total dry weight (g/m^2) (P=0.047) and the total leaf dry weight (g/m^2) (P=0.015) of which the measurements of the UK elite genotypes were usually higher than that of the DH genotypes, however some overlap was present in several situations. No *Fusarium* or *Microdochium* spores could be quantified on the leaves during the canopy spore quantification experiment.

Significant differences (P<0.001) were present between all ear traits (Table 4.13) including ear length, awn length, spikelets per ear, spikelet density and ear dry weight, excluding hair on ear surface of which only Line 61 exhibited this trait. Ear length and the number of spikelets per ear exhibited large variation between genotypes ranging from 9.25 – 13.41 cm and 18.87 – 26.33 spikelets respectively, although differences between UK elite and DH genotypes were not apparent.

Awn length differed greatly between genotypes, with five lines having awn lengths > 5 cm and the remaining ten lines being classed as awnless, having awn lengths of < 0.5 cm. No

lines within the study were truly awnless, as at least a small amount of awn was present on every line.

Significant differences ($P<0.001$) were present between genotypes for the degree of anther extrusion. Ambrosia, Line 18 and Line 21 all had very low levels of anther extrusion, with anthers either being trapped or retained within the floret leading to extrusion levels of 21.6%, 16.7% and 21.6% respectively. L51 however had very high levels of anther extrusion with 92.9% of anthers being fully extruded from the floret.

Significant differences ($P<0.001$) were present between number of days from sowing to mid-anthesis, varying from 219 – 235 days for Line 14 and Claire respectively, with UK elite genotypes taking consistently longer to reach GS65 than DH genotypes.

Harvest traits including harvest index, grain yield and specific weight had significant differences ($P<0.001$) between genotypes (Table 4.14). Harvest index ranged from 40.93 – 57.93% for Line 77 and Grafton respectively, and grain yield ranged from 8.02 – 14.95 t/ha for Line 18 and Grafton, respectively. The UK elite lines had consistently higher harvest indices and grain yields than DH genotypes. However these genotypic differences were not as apparent for specific weight.

Table 4.12. Genotypic differences in canopy traits measured at GS65 in the 2011 field experiment *(continued)*

	Plant number (m^2)	Fertile shoots (m^2)	Potentially infertile shoots (m^2)	Dead and dying shoots (m^2)	Fertile shoots per plant	Potentially infertile shoots per plant
Ambrosia	202.7	405.3	16.00	400.0	2.00	4.00
Claire	288.0	517.3	5.33	400.0	2.00	1.33
Grafton	293.3	608.0	21.33	618.7	2.87	5.33
Rialto	357.3	480.0	21.33	400.0	1.40	5.33
Solstice	282.7	442.7	21.33	352.0	1.80	5.33
Line 14	341.3	485.3	5.33	309.3	1.33	1.33
Line 16	229.3	394.7	0.00	186.7	2.00	0.00
Line 18	357.3	458.7	32.00	256.0	1.67	8.00
Line 21	250.7	378.7	16.00	176.0	1.67	4.00
Line 24	277.3	538.7	5.33	261.3	2.00	1.33
Line 38	304.0	517.3	10.67	533.3	2.07	2.67
Line 39	304.0	480.0	10.67	325.3	1.47	2.67
Line 51	293.3	533.3	21.33	314.7	2.27	5.33
Line 61	202.7	293.3	10.67	261.3	1.67	2.67
Line 77	272.0	442.7	0.00	330.7	1.60	0.00
Mean	283.7	465.1	13.16	341.7	1.85	0.05
P-value	0.055	0.037	0.807	<.001	<.001	0.807
LSD (5%)	98.27	150.66	33.27	144.97	0.55	8.32
CV (%)	20.7	19.4	151.2	25.4	17.7	151.2

Table 4.12. Genotypic differences in canopy traits measured at GS65 in the 2011 field experiment *(continued)*

	Dead and dying shoots per plant	Flag leaf length (cm)	Flag leaf width (cm)	Flag leaf height (cm)	Flag and second leaf distance (cm)	Second and third leaf distance (cm)
Ambrosia	2.03	15.69	1.76	64.41	21.35	15.39
Claire	1.38	16.69	1.72	65.37	23.41	16.22
Grafton	2.07	15.09	1.59	54.31	19.45	12.05
Rialto	1.11	15.96	1.77	68.33	21.64	15.65
Solstice	1.24	15.93	1.75	66.99	24.35	14.62
Line 14	0.92	12.84	1.28	58.78	20.41	12.82
Line 16	0.85	17.39	1.53	61.15	23.65	13.93
Line 18	0.72	20.79	1.58	35.31	12.89	7.63
Line 21	0.69	21.27	1.53	89.6	30.49	21.25
Line 24	0.96	14.09	1.40	65.04	23.67	15.22
Line 38	2.03	14.49	1.26	70.79	24.46	16.54
Line 39	1.09	15.46	1.70	50.1	17.95	9.77
Line 51	1.06	17.81	1.88	61.73	20.65	13.44
Line 61	1.39	19.04	1.54	99.18	31.03	21.57
Line 77	1.24	16.61	1.51	96.54	30.42	22.71
Mean	1.25	16.61	1.59	67.18	23.06	15.25
P-value	<.001	<.001	<.001	<.001	<.001	<.001
LSD (5%)	0.67	2.97	0.16	4.97	1.44	1.36
CV (%)	31.9	10.7	6	4.4	3.7	5.3

Table 4.12. Genotypic differences in canopy traits measured at GS65 in the 2011 field experiment *(continued)*

	Plant height (cm)	Peduncle length (cm)	Flag leaf area (cm^2/m^2)	Second leaf area (cm^2/m^2)	All remaining leaf area (cm^2/m^2)	Total leaf area (cm^2/m^2)
Ambrosia	70.39	7.57	8650	9955	18816	37421
Claire	74.13	8.72	9603	11591	21246	42440
Grafton	61.06	6.71	9243	11837	27929	49009
Rialto	77.73	9.31	6824	7291	16210	30324
Solstice	77.83	10.74	7718	8458	17481	33657
Line 14	66.80	9.01	3817	5543	10304	19664
Line 16	72.87	12.27	5795	7500	10590	23885
Line 18	40.02	5.25	6887	6936	6167	19990
Line 21	106.86	17.30	6444	8372	11967	26782
Line 24	79.16	14.05	6946	11537	20854	39338
Line 38	90.44	19.65	5124	6761	12103	23988
Line 39	58.14	8.68	7809	9869	18347	36025
Line 51	76.97	15.13	9853	13630	17967	41450
Line 61	118.41	19.47	4465	5684	12959	23108
Line 77	116.00	19.31	6137	8396	16983	31516
Mean	79.12	12.21	7021	8891	15995	31907
P-value	<.001	<.001	0.008	0.001	<.001	<.001
LSD (5%)	4.93	2.13	3095	3631	6438	12580
CV (%)	3.7	10.4	26.4	24.5	24.1	23.6

Table 4.12. Genotypic differences in canopy traits measured at GS65 in the 2011 field experiment *(continued)*

	Flag leaf dry weight (g/m^2)	Second leaf dry weight (g/m^2)	All remaining leaf dry weight (g/m^2)	Total leaf dry weight (g/m^2)	Stem dry weight (g/m^2)	Dead and yellow leaf dry weight (g/m^2)
Ambrosia	58.56	58.13	105.97	222.7	1009	19.09
Claire	61.07	64.85	112.00	237.9	999	36.80
Grafton	56.69	63.31	126.40	246.4	604	31.15
Rialto	48.59	49.23	99.47	197.3	1004	27.63
Solstice	47.52	48.64	92.11	188.3	837	28.37
Line 14	29.60	35.89	60.00	125.5	627	21.60
Line 16	42.99	41.49	56.16	140.6	732	22.72
Line 18	54.61	39.73	32.43	126.8	450	20.64
Line 21	46.77	49.07	67.95	163.8	860	23.47
Line 24	41.07	56.00	91.79	188.9	887	34.19
Line 38	40.48	42.77	68.16	151.4	908	44.32
Line 39	52.43	58.24	92.80	203.5	646	25.28
Line 51	62.93	68.96	77.97	209.9	933	39.20
Line 61	35.20	38.35	77.33	150.9	934	26.29
Line 77	39.52	47.31	89.07	175.9	986	30.13
Mean	47.87	50.80	83.31	182.0	828	28.73
P-value	0.073	0.037	<.001	0.015	0.019	0.089
LSD (5%)	20.67	20.18	30.73	68.89	321.1	15.74
CV (%)	25.8	23.8	22.1	22.6	23.2	32.8

Table 4.12. Genotypic differences in canopy traits measured at GS65 in the 2011 field experiment *(continued)*

	Fertile shoot dry weight (g/m^2)	Potentially infertile shoots dry weight (g/m^2)	Dead and dying shoot dry weight (g/m^2)	Total dry weight (g/m^2)	Flag leaf rolling	Flag leaf angle
Ambrosia	1574	6.03	36.16	1616	0.00	2.00
Claire	1590	0.64	36.00	1626	0.00	2.67
Grafton	1119	9.23	52.48	1180	0.00	3.00
Rialto	1525	9.55	27.09	1562	0.00	2.67
Solstice	1282	6.08	30.77	1319	0.00	2.00
Line 14	952	0.80	15.36	968	0.00	2.67
Line 16	1085	0.00	18.61	1104	0.00	2.33
Line 18	809	11.41	15.52	883	0.00	3.00
Line 21	1227	5.01	16.00	1248	1.00	1.17
Line 24	1328	3.57	25.12	1357	0.00	2.00
Line 38	1322	5.01	34.29	1362	1.00	2.00
Line 39	1145	2.56	32.32	1180	0.00	1.67
Line 51	1461	9.81	34.77	1506	0.00	2.83
Line 61	1297	5.87	24.16	1327	0.00	1.00
Line 77	1353	0.00	24.91	1378	0.33	1.83
Mean	1271	5.04	28.24	1308	0.16	2.19
P-value	0.035	0.840	0.002	0.047	<.001	<.001
LSD (5%)	434.1	14.12	15.27	441.8	0.25	0.5247
CV (%)	20.4	167.6	32.3	20.2	95.8	14.3

Table 4.12. Genotypic differences in canopy traits measured at GS65 in the 2011 field experiment

	Brown foot rot score (0-3)	Stem diameter (cm)	Canopy fractional interception	*Fusarium* and *Microdochium* spores on leaf surface (pg/ng DNA)
Ambrosia	0.27	0.51	0.93	0
Claire	0.47	0.53	0.91	0
Grafton	0.47	0.49	0.95	0
Rialto	0.07	0.47	0.91	0
Solstice	0.47	0.48	0.91	0
Line 14	0.00	0.40	0.84	0
Line 16	0.00	0.45	0.89	0
Line 18	0.20	0.47	0.81	0
Line 21	0.00	0.47	0.95	0
Line 24	0.27	0.47	0.94	0
Line 38	0.33	0.44	0.93	0
Line 39	0.27	0.53	0.92	0
Line 51	0.20	0.44	0.95	0
Line 61	0.07	0.44	0.94	0
Line 77	0.07	0.46	0.95	0
Mean	0.21	0.4695	0.92	0
P-value	0.504	<.001	<.001	-
LSD (5%)	0.5071	0.03484	0.043	-
CV (%)	145.2	4.4	2.8	-

Table 4.13. Genotypic differences in ear traits measured at GS65 in the 2011 field experiment *(continued)*

	Ear length (cm)	Awn length (cm)	Spikelets per ear	Spikelet density (spikelets/cm)	Hair on ear surface	Ear dry weight (g/m^2)
Ambrosia	9.97	0.39	21.27	2.13	0	322.6
Claire	11.26	0.31	26.33	2.34	0	315.4
Grafton	9.34	0.35	19.60	2.10	0	237.3
Rialto	10.90	0.32	25.27	2.32	0	296.7
Solstice	10.19	0.37	21.07	2.07	0	228.7
Line 14	9.39	0.27	21.07	2.25	0	178.1
Line 16	9.82	0.51	21.13	2.15	0	189.7
Line 18	9.25	5.19	18.87	2.04	0	212.1
Line 21	13.41	0.54	24.07	1.80	0	180.4
Line 24	9.65	0.38	22.87	2.37	0	217.8
Line 38	10.90	5.87	24.20	2.22	0	218.8
Line 39	12.98	7.51	24.40	1.88	0	270.6
Line 51	11.65	6.27	21.27	1.82	0	279.0
Line 61	12.72	6.17	21.20	1.67	1	185.7
Line 77	11.20	0.31	23.67	2.11	0	160.9
Mean	10.84	2.32	22.42	2.09	0.07	232.9
P-value	<.001	<.001	<.001	<.001	-	<.001
LSD (5%)	0.7976	0.336	1.227	0.109	-	75.53
CV (%)	4.4	8.7	3.3	3.1	-	19.4

Table 4.13. Genotypic differences in ear traits measured at GS65 in the 2011 field experiment

	Retained anthers (%)	Trapped anthers (%)	Extruded anthers (%)	Days from sowing to mid-anthesis
Ambrosia	53.09	25.31	21.60	233.0
Claire	29.63	18.52	51.85	235.0
Grafton	43.83	8.02	48.15	231.0
Rialto	62.96	11.42	25.62	233.7
Solstice	43.52	22.22	34.26	234.0
Line 14	58.64	12.96	28.40	219.0
Line 16	29.94	19.14	50.93	224.0
Line 18	58.64	24.69	16.67	210.0
Line 21	66.05	12.35	21.60	224.0
Line 24	34.88	13.89	51.23	224.0
Line 38	33.64	8.02	58.33	231.0
Line 39	24.69	15.12	60.19	225.0
Line 51	2.16	4.94	92.90	227.7
Line 61	11.73	6.79	81.48	227.3
Line 77	24.07	15.43	60.49	225.7
Mean	38.50	14.59	46.91	227.0
P-value	<.001	<.001	<.001	<.001
LSD (5%)	10.42	7.88	13.61	0.884
CV (%)	16.2	32.3	17.3	0.2

Table 4.14. Genotypic differences in harvest traits measured at GS89 in the 2011 field experiment

	Harvest index (%)	Grain yield (t/ha @ 15% MC)	Specific weight (kg/hl)
Ambrosia	55.10	14.91	85.47
Claire	56.02	14.77	84.70
Grafton	57.92	14.95	85.03
Rialto	51.44	14.17	80.70
Solstice	52.58	13.88	82.37
Line 14	50.36	9.72	82.43
Line 16	48.46	8.77	86.73
Line 18	51.21	8.02	83.13
Line 21	47.09	12.09	84.90
Line 24	49.02	12.11	82.97
Line 38	45.35	10.85	79.10
Line 39	53.60	11.65	86.53
Line 51	46.99	11.37	83.87
Line 61	42.77	8.10	87.17
Line 77	40.93	9.31	86.40
Mean	49.92	11.64	84.10
P-value	<.001	<.001	0.003
LSD (5%)	2.098	1.404	3.651
CV (%)	2.5	7.2	2.6

4.5.8 Correlation between GS65 physiological traits

Plant height at GS65 was strongly correlated with similar traits including peduncle length, flag leaf height, distance between flag and second leaves and distance between second and third leaves (Table 4.15). Plant height was therefore chosen to represent these traits in all further analysis of GS65 traits to prevent multi co-linearity. Shoot dry weights at GS65 were highly correlated with shoot number per m^2 (Table 4.16) and therefore shoot number per m^2 was the selected trait for use in all further analysis. Although the fertile shoot dry weight (g/m^2) was poorly correlated with the number of fertile shoots (m^2), it was highly correlated with total plot dry weight (r=0.99), and was therefore omitted from further analysis to avoid multi co-linearity

Table 4.15. Correlation (r) in 2011 between plant height (cm) at GS65 with peduncle length (cm), flag leaf height (cm), distance between flag and second leaf (cm) and distance between second and third leaf (cm)

	Plant height
Peduncle length	0.88***
Flag leaf height	0.99***
Distance between flag and second leaf	0.96***
Distance between second and third leaf	0.96***

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 4.16. Correlation (r) in 2011 between shoot number (m^2), shoot fresh weight (g/m^2) and shoot dry weight (g/m^2) at GS65.

	Number of fertile shoots	Number of potentially infertile shoots	Number of dead/dying shoots
Fertile shoot dry weight	0.33*	-	-
Potentially infertile shoot dry weight	-	0.93***	-
Dead and dying shoot dry weight	-	-	0.69***

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

4.5.9 Principal component analysis of the AUDPC, GS65 physiological traits, pathogen DNA and grain mycotoxin content within the 2011 field experiment

PCA of GS65 morphological traits, pathogen DNA and mycotoxins showed grouping between traits, although these groups were not well defined (Figure 4.13). AUDPC was grouped together with stem diameter (cm), infertile shoots (m^2), infertile shoots per plant (m^2), trapped anthers (%), retained anthers (%) and as well as *F. culmorum* DNA, *F. poae* DNA and *F. langsethiae* DNA. A second group contained plant number (m^2), flag leaf angle, spikelet density (spikelets/cm), harvest index (%) and as well as and *M. majus* DNA, DON, HT2+T2 and ZEAR. A third group consisted of brown foot rot, grain yield (t/ha), dead/dying shoots per m^2, fertile shoots per plant, fresh weight of ears (g/m^2), flag leaf area (cm^2/m^2), second leaf area (cm^2/m^2), remaining leaf area (cm^2/m^2), total leaf area (cm^2/m^2), flag leaf width (cm), total fresh weight (g/m^2), dead and dying shoots per plant fertile shoots (m^2) as well as *F. graminearum* and *M. nivale* DNA. A fourth group contained flag leaf length (cm), rolled flag leaves, awn length (cm) and hair on ears. AUDPC was additionally strongly negatively associated with spikelets per ear, specific weight and canopy fractional interception.

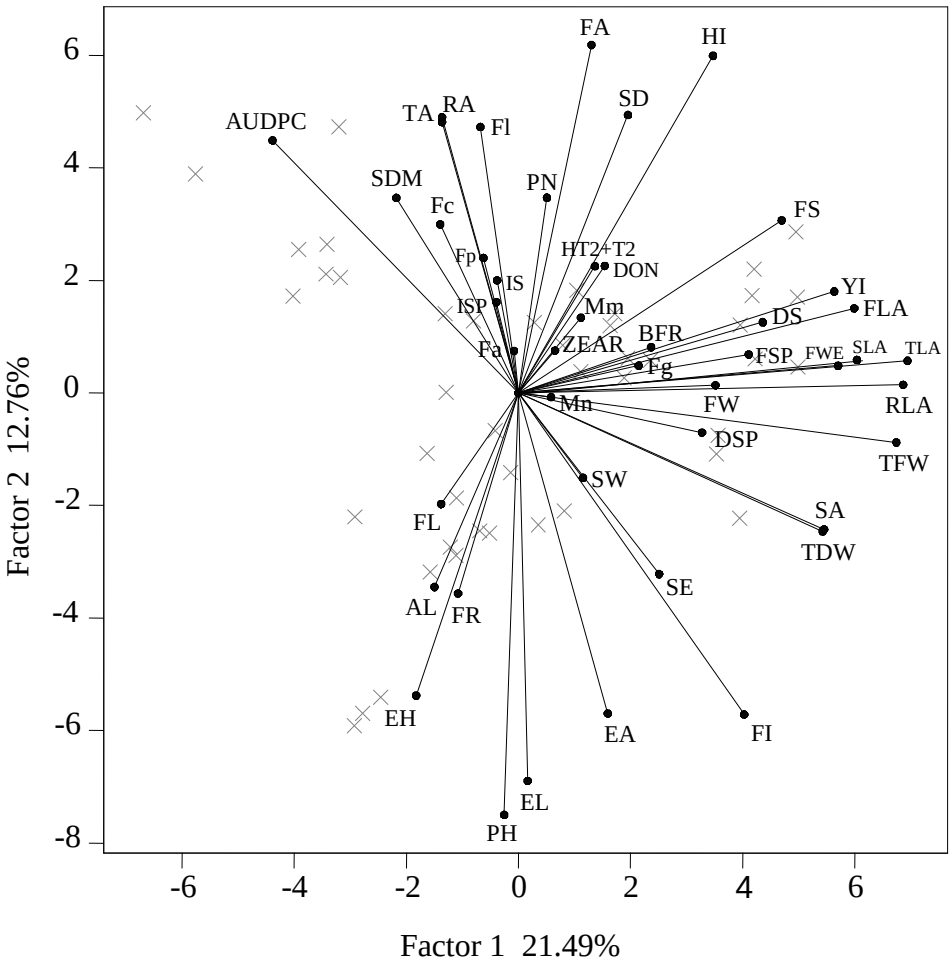

Figure 4.13. Biplot of the principal component analysis of the 2011 field experiment including AUDPC, GS65 physiological traits, pathogen DNA and grain mycotoxin content. Key: AL (awn length, cm); AUDPC (Area Under Disease Progress Curve); BFR (brown foot rot); DON (deoxynivalanol, ppb log10); DS (dead/dying shoots, m^{-2}); DSP (dead/dying shoots per plant); EH (hair on ear); EL (ear length, cm); EA (extruded anthers, %); FA (flag leaf angle); Fa (*F.avenaceum* DNA); Fc (*F.culmorum* DNA), Fg (*F.graminearum* DNA), FI (canopy fractional light interception); Fl (*F.langsethiae* DNA) FL (flag leaf length, cm); FLA (flag leaf area, cm^{-2}/m^{-2}); Fp (*F.poae* DNA); FR (rolled flag leaves), FS (fertile shoots, m^{-2}); FS (fertile shoots per plot); FSP (fertile shoots per plant); FW (flag leaf width, cm); FWE (fresh weight of ears, g/m^{-2}); HI (harvest index); HT2+T2 (HT2+T2 ppb log10); IS (potentially infertile shoots, m^{-2}); ISP (potentially infertile shoots per plant); Mm (*M. majus DNA*); Mn (M. nivale DNA); PH (plant height, cm); PN (plant number, m^{-2}); RA (retained anthers, %); RLA (remaining leaf area); SA (days between sowing and anthesis); SE (Spikelets per ear); SD (spikelet density); SDM (stem diameter, cm); SLA (second leaf area, cm^{-2}/m^{-2}); SW (specific weight, kg/hl); TA (trapped anthers, %); TDW (total plot dry weight, g/m^{-2}); TFW (total fresh weight, g/m^{-2}); TLA (total leaf area, cm^{-2}/m^{-2}); YI (grain yield, t/ha); ZEAR (zearalenone, ppb log10).

4.5.10 Multiple linear regression of AUDPC and mycotoxin concentration with GS65 physiological traits and grain pathogen DNA

Multiple linear regression found significant (P<0.001) relationships between morphological traits and AUDPC, accounting for 49% of the variation in the AUDPC (Table 4.17). AUDPC was positively related to flag leaf length and negatively related to plant height, remaining leaf area and awn length; however no significant relationship with any pathogen DNA was identified.

Specific grain mycotoxin concentrations were significantly related to GS65 physiological traits and grain pathogen DNA content (Table 4.17). DON content was significantly (P<0.001) positively related to the percentage of trapped anthers, remaining leaf area and *F. culmorum* DNA, accounting for 28% of the variation. ZEAR content was not related to any GS65 physiological traits or pathogen DNA. HT2+T2 content was significantly (P=0.006) positively related to *F. langsethiae* DNA.

Table 4.17. Multiple linear regression models of the 2011 field experiment accounting for variation in the AUDPC and mycotoxin content between genotypes using GS65 physiological traits, harvest traits and species DNA

Model	Equation	R^2	P-value
AUDPC	35.2 + 1.652 (FL) - 0.3499 (PH) - 0.0012 (RLA) - 1.445 (AL)	0.49	<0.001
DON	1.445 + 0.0129 (TA) + 0.00002 (RLA) + 0.1374 (Fc DNA)	0.28	0.001
ZEAR	-	-	-
HT2+T2	1.2393 + 0.0661 (Fl DNA)	0.15	0.006

Key: AL = Awn length (cm); FL = Flag leaf length (cm); PH = Plant height (cm); TA = Trapped anthers (%); RLA = Remaining leaf area (cm^2/m^2)

4.5.11 Correlation between GS39 & GS65 physiological traits

Multiple physiological traits were measured at both GS39 and at GS65 to allow for comparisons to be made between the effects of both growth stages on FHB resistance. Several traits measured at both growth stages were highly correlated with one another, however the majority of traits were poorly correlated between growth stages ($r < 0.70$) although they were highly significant (Table 4.18). The poor correlation in plant number per m^2 between growth stages was potentially due to variation in establishment within the plot, or due to difficulties in separating individual plants during analysis.

Table 4.18. Correlation between a selection of morphological traits measured at GS39 and at GS65in the 2011 field experiment

Plant number (m^2)	0.19
Fertile shoots per plant	0.01
Flag leaf height (cm)	0.91^{***}
Flag leaf length (cm)	0.54^{***}
Flag leaf width (cm)	0.68^{***}
Distance between flag leaf and 2nd leaf (cm)	0.54^{***}
Distance between 2nd leaf and 3rd leaf (cm)	0.85^{***}
Fertile shoots (m^2)	0.31^{*}
Potentially infertile shoots (m^2)	0.08
Dead/dying shoots (m^2)	0.46^{**}
Flag leaf area (cm^2/m^2)	0.54^{***}
Second leaf area (cm^2/m^2)	0.49^{***}
Remaining laminar area (g)	0.50^{***}
Total laminar area (cm^2/m^2)	0.50^{***}
Flag leaf dry weight (g/m^2)	0.43^{**}
Second leaf dry weight (g/m^2)	0.47^{***}
Remaining leaf dry weight (g/m^2)	0.64^{***}
Total leaf dry weight (g/m^2)	0.55^{***}

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

4.5.12 Interaction between physiological traits common to both 2010 and 2011 experiments

Significant year*genotype interactions were present between physiological traits common to both the 2010 and 2011 field experiments (Table 4.19). Plant height ranged between 50.6 - 95.6 cm in 2010 and between 40.0 - 118.4 cm in 2011, with Line 18 and Line 61 being the shortest and tallest genotypes in both years, respectively. Mean plant height was around 8 cm greater in 2011 than in 2010 and a year*genotype interaction ($P<0.001$) was present. A wide range of flag leaf lengths were present in both year, varying from 12.45 - 18.37 cm in 2010 and 14.09 - 21.27 cm in 2011, with a year*genotype interaction present ($P=0.002$). UK elite genotypes had very similar flag leaf lengths to each other in 2011 and generally producing intermediate valued compared to DH lines. Mean flag leaf width was similar between years although a year*genotype interaction was present ($P<0.001$). Line 38 had the narrowest flag width in 2010 and 2011 (1.21 and 1.23 cm, respectively) and Line 51 the widest (1.93 and 1.88 cm, respectively) however the widest width in 2010 was also shared by Line 21 and Solstice. UK elite genotypes had intermediate widths which were stable within each year, with the exception of Solstice in 2010. Shoot number per plant varied widely between years with mean values of 5.83 and 1.85 in 2010 and 2011, respectively, with a year*variety interaction ($P<0.001$). Line 14 had the lowest shoot number per plant in both years, while the greatest number of shoots was seen in UK elite genotypes. Mean ear length was 1.35 cm greater in 2011 than in 2010, with a year*genotype interaction present ($P=0.015$). The shortest and longest ears were attributed to different genotypes in each year, ranging from 8.25 - 12.25 cm in 2010 and 9.25 - 13.41 cm in 2011. Mean number of spikelets per ear showed an increase of three spikelets per ear between 2010 and 2011, with a year*genotype interaction ($P<0.001$). Graphton and Line 18 each had the lowest number of spikelets per ear in 2010 and 2011 respectively, while

Claire had the highest spikelet number in both years. UK elite and DH genotypes generally had similar numbers of spikelets per ear. Mean spikelet density was very similar between 2010 and 2011 (2.07 and 2.08, respectively), although a year*genotype interaction was present (P=0.008). Spikelet density ranged from 1.69 - 2.41 in 2010 and 1.67 - 2.37 in 2011. The lowest spikelet densities were attributed to Line 61 in both experimental years, while Rialto and Line 24 had the greatest spikelet density in 2010 and 2011, respectively. UK elite genotypes generally had a greater spikelet density than DH genotypes.

Table 4.19. Interactions between physiological traits common between the 2010 and 2011 field experiments

	Plant height (cm)		Flag leaf length (cm)		Flag leaf width (cm)		Shoot number per plant		Ear length (cm)		Spikelets per ear		Spikelet density (spikelets/cm ear)	
	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011
Ambrosia	66.4	70.4	14.67	15.69	1.62	1.76	6.60	2.00	8.63	9.97	19.23	21.27	2.23	2.13
Claire	72.6	74.1	14.30	16.69	1.59	1.72	6.77	2.00	9.73	11.26	21.47	26.33	2.21	2.34
Grafton	57.9	61.1	13.77	15.09	1.56	1.59	6.70	2.87	7.62	9.34	17.20	19.60	2.26	2.10
Rialto	61.8	77.7	12.45	15.96	1.64	1.77	6.90	1.40	8.25	10.90	19.87	25.27	2.41	2.32
Solstice	73.8	77.8	15.48	15.93	1.93	1.75	5.20	1.80	9.85	10.19	19.60	21.07	1.99	2.07
Line 14	61.3	66.8	15.84	12.84	1.61	1.28	4.00	1.33	9.05	9.39	19.60	21.07	2.17	2.25
Line 16	67.8	72.9	17.17	17.39	1.78	1.53	5.17	2.00	8.73	9.82	18.33	21.13	2.10	2.15
Line 18	50.6	40.0	17.21	20.79	1.44	1.58	5.77	1.67	8.68	9.25	18.53	18.87	2.14	2.04
Line 21	80.4	106.9	17.23	21.27	1.93	1.53	4.10	1.67	11.51	13.41	19.47	24.07	1.69	1.80
Line 24	71.6	79.2	14.70	14.09	1.62	1.40	5.67	2.00	8.27	9.65	19.07	22.87	2.33	2.37
Line 38	86.5	90.4	14.96	14.49	1.21	1.26	6.67	2.07	9.55	10.90	19.60	24.20	2.05	2.22
Line 39	66.3	58.1	16.90	15.46	1.59	1.70	6.17	1.47	10.90	12.98	20.53	24.40	1.89	1.88
Line 51	67.7	77.0	17.24	17.81	1.93	1.88	5.17	2.27	9.63	11.65	17.80	21.27	1.85	1.82
Line 61	95.6	118.4	18.37	19.04	1.89	1.54	5.83	1.67	12.25	12.72	20.73	21.20	1.69	1.67
Line 77	81.4	116.0	15.44	16.61	1.72	1.51	6.73	1.60	9.77	11.20	20.00	23.67	2.05	2.11
Mean	70.8	79.1	15.72	16.61	1.67	1.59	5.83	1.85	9.49	10.84	19.40	22.42	2.07	2.08
P value (Year*Genotype)	<0.001		0.002		<0.001		<0.001		0.015		<.001		0.008	
LSD	6.7		2.26		0.14		0.92		0.91		1.39		0.12	
CV (%)	5.5		8.6		5.2		14.7		5.5		4.1		3.6	

4.6 FIELD EXPERIMENT 2010 AND 2011 WEATHER DATA

Weather data from the University of Nottingham meteorological station, located adjacent to the trial site, was used to compare climatic conditions between the 2010 and 2011 field experiments. Due to the wide range of anthesis and maturity dates between the genotypes, weather data was taken from the date in which the first genotype reached anthesis (GS61), to the date which the last genotype reached maturity (GS91). The start of anthesis occurred on the 30 May and 7 May in 2010 and 2011 respectively, and the final maturity dates were 11 August and 3 of August in 2010 and 2011 respectively.

In 2010, there was a period of wet weather at the very start of anthesis, followed by an extended dry period, before turning wet again during harvest, with a total rainfall of 126.4 mm (Figure 4.14). In contrast, 2011 followed a linear pattern between the start of flowering and GS77, providing rain evenly throughout this period, with a total rainfall of 92.6 mm. The main differences between rainfall patterns in 2010 and 2011 were that 2010 saw two major periods of very wet weather from GS61 onwards, whereas 2011 saw much lighter showers spread evenly over the flowering and harvest period which would have perhaps dried off quickly. Overall, between the start of flowering and the end of harvest, the 2010 experiment received 34.2mm more rain than the 2011 experiment. Humidity patterns in 2010 and 2011 both showed highly linear patterns (Figure 4.15), however lines began to gradually converge, showing differences between years, with an mean relative humidity of 80.48% and 75.57% in 2010 and 2011 respectively, a percentage difference of 6.29%. Temperatures in 2010 and 2011 showed a linear pattern, indicating that periods of temperature extremes did not occur in either year (Figure 4.16). Gradual convergence of the lines showed that between years, 2010 had the warmest period between the start of anthesis and the end of harvest, averaging 16.3^{o}C and 14.56^{o}C in 2010 and 2011, respectively, with a temperature percentage difference between years of 11.07%.

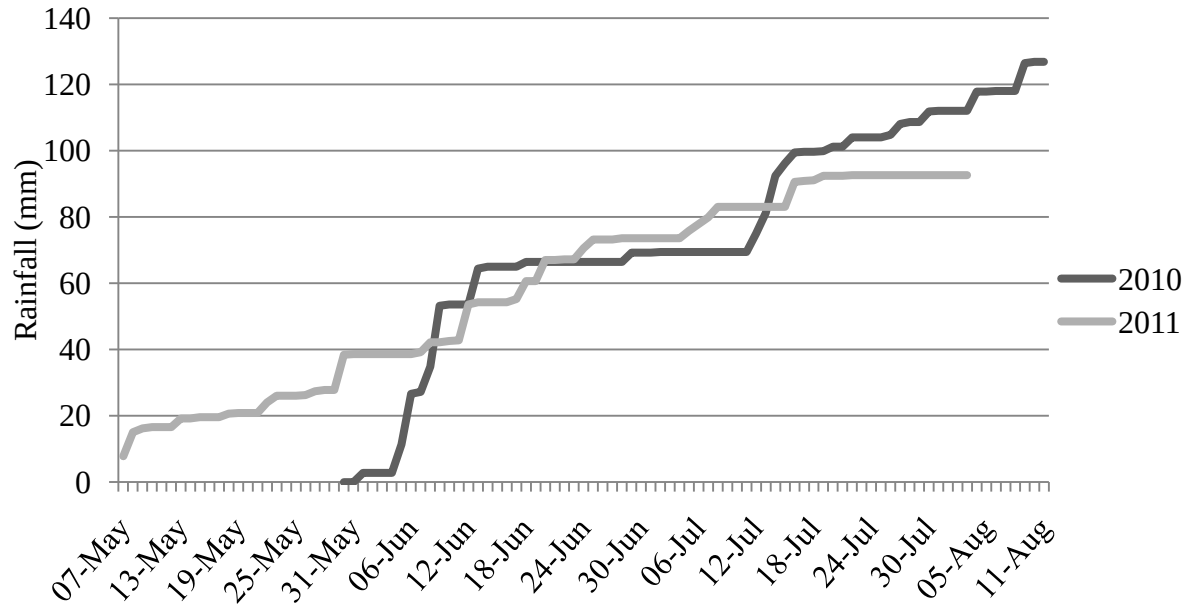

Figure 4.14. Cumulative rainfall between the start of anthesis and the end of harvest in the 2010 and 2011 field experiments.

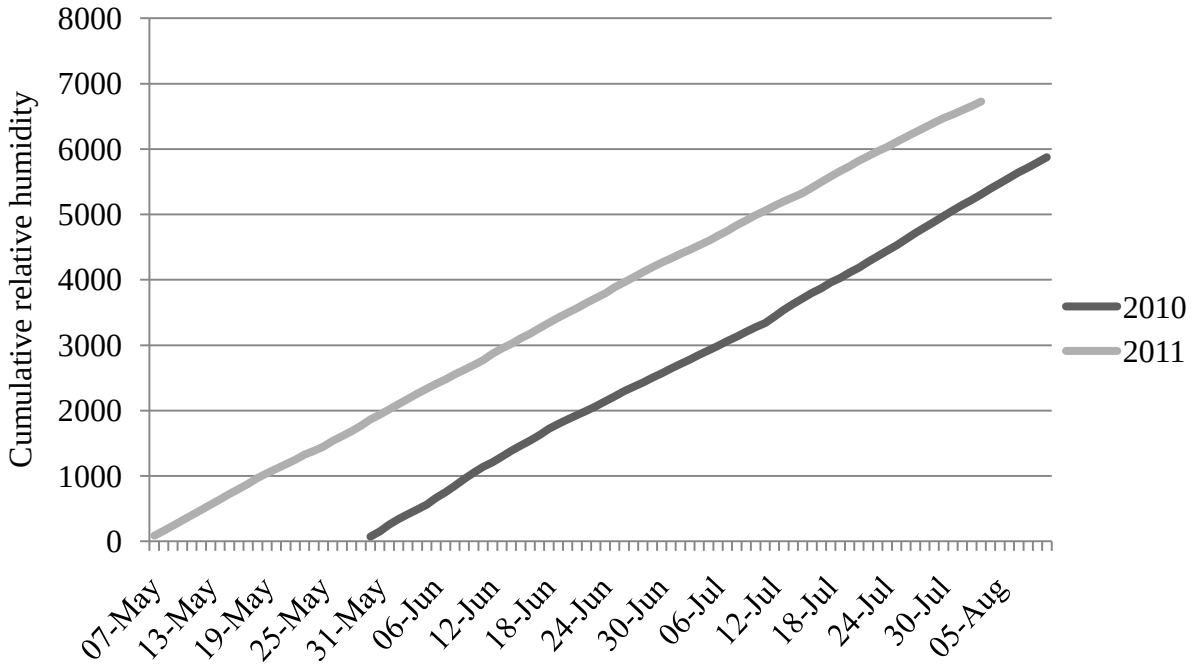

Figure 4.15. Cumulative relative humidity between the start of anthesis and the end of harvest in the 2010 and 2011 field experiments.

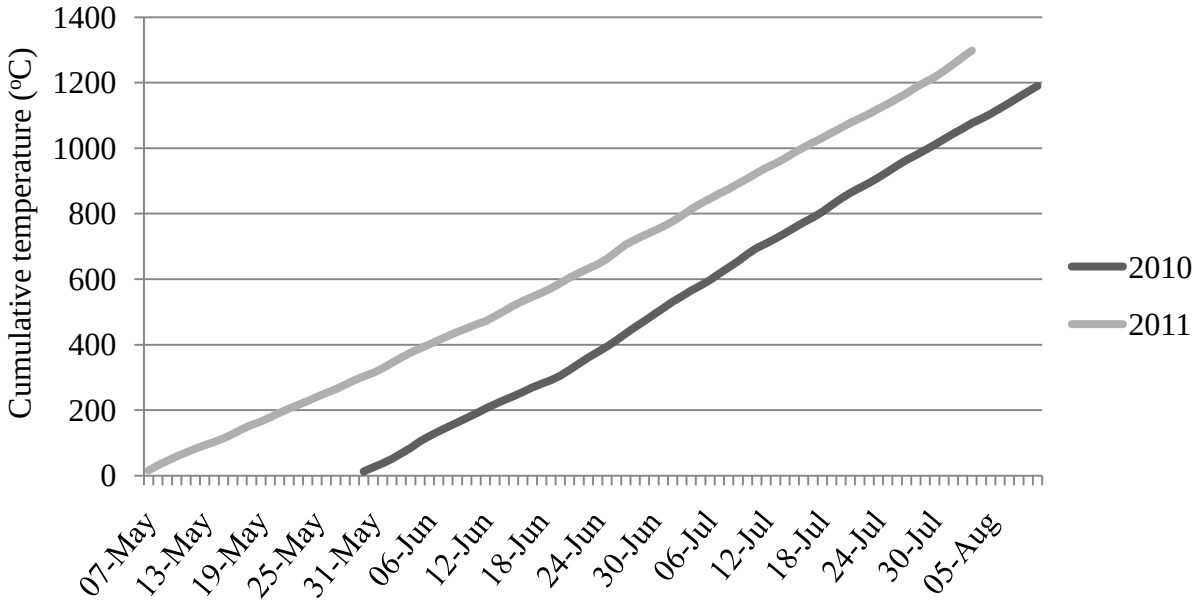

Figure 4.16. Cumulative temperature between the start of anthesis and the end of harvest in the 2010 and 2011 field experiments.

4.7 DISCUSSION

Significant differences (P<0.001) in AUDPC between genotypes were present in both the 2010 and 2011 field experiments, with a significant year*genotype interaction also present (P<0.001, data not shown). Mean AUDPC values were larger in 2010 than in 2011 (27.68 and 12.26 respectively) which could have been due to differences in climatic conditions between years. Weather conditions in 2010 provided a more conducive environment for FHB development than in 2011, with increased rainfall between flowering and harvest likely leading to the differences in AUDPC (Edwards, 2007). Differences in AUDPC values between years was further validated through grain DNA concentrations which were larger in 2010 than in 2011 with grand mean values of 0.016 and 0.005 pg/ng DNA respectively.

F. culmorum was the predominant pathogen species DNA in harvested grain in 2010, while *F. poae* was predominant in 2011. Xu *et al.* (2008) reported that *F. culmorum* prefers cooler, wetter and more humid conditions, while *F. poae* prefers drier and warmer condition. This fits with the differences seen between years as 2010 was wetter and more humid (albeit warmer) than 2011.

A single mycotoxin producer species significantly accounted for additional variation in DON content in both year's MLR analysis, being positively related to *F. graminearum* and *F. culmorum* in 2010 and 2011 respectively, confirming these pathogens as the major DON producing species in each year. Strong relationships between trichothecene producing *Fusarium* species and DON concentrations have also previously been reported (Schnerr *et al.* 2002). Insufficient ZEAR was present to enable MLR to be carried out on the data set. Principal component analysis positively grouped all three mycotoxins together in both experiments. This is as expected for DON and ZEAR as *F. graminearum* and *F. culmorum* are co-producers of these toxins, whereas the relationship between DON and HT2+T2 is

surprising as both DON and HT2+T2 have shown signs of mutual exclusion (Edwards, 2007).

More AUDPC variation was accounted for in the 2011 field experiment using traits assessed at both GS39 and GS65, than for the 2010 experiment using traits assessed at GS89, accounting for 51%, 49% and 25% of the variation, respectively. This may be partially due to the increased number of physiological traits assessed during the 2011 field experiment than the 2010 field experiment; therefore additional traits were available to account for a higher proportion of the AUDPC variation. This also suggests that assessments of physiological traits undertaken at GS39 and GS65 are more useful than assessments carried out later on in the crops development.

Flag leaf length was the only physiological trait to be consistently related to the AUDPC, having a positive relationship. In contrast however, flag leaf length had a significant negative relationship with HT2+T2 in the 2010 field experiment. This is the first report of a significant relationship between flag leaf length and FHB in wheat. It is hypothesized for the AUDPC that, since *F. culmorum* and *F. poae* spores can be continually splashed from the upper leaf surfaces towards wheat ears (Horberg *et al*. 2002) longer flag leaves may provide more overlap of canopy layers for upward spore transmission. On a population level, longer flag leaves may additionally increase spore transmission through increased contact between the leaf surface and the ears of neighbouring plants.

Awn length had a significant negative relationship with AUDPC in the 2011 experiment at GS65. Awn length has been shown to have mixed relationships with FHB, previously being positively (Mesterhazy, 1995; Liu *et al*, 2013), negatively (Ban and Suenaga, 2000; Buerstmayr *et al.* 2000) and unrelated (Buerstmayr *et al.* 2000; Somers *et al.* 2003; Liu *et al.* 2007) to FHB. Therefore, further examination of its relationship with FHB is required.

Flag leaf height and plant height were negatively related to AUDPC at both GS39 and GS65, respectively, in the 2011 field experiment. Flag leaf height at GS39 was very highly correlated with plant height at GS65 (r = 0.99). The negative relationship between plant height and FHB is well documented (Hilton *et al.* 1999; Klahr *et al.* 2007; He *et al.* 2014) and supports the findings of this experiment.

Flag leaf length generally had a stronger influence on AUDPC in terms of unit change (cm) than plant height. However, these differences broadly disappear when considering the greater observed variation in plant height than flag leaf length.

The proportion of trapped anthers at GS65 had a significant positive relationship with grain DON content in 2011. The proportion of trapped anthers was strongly positively related to retained anthers, and strongly negatively related to extruded anthers in the PCA (Fig. 4.13). The strong relationships present between fully extruded, retained and trapped anthers, did not support the hypothesis that anther extrusion was most important of the three scenarios for FHB development, despite the significant relationship between trapped anthers and DON in 2011. Multiple studies have shown similar negative relationships between anther extrusion and FHB infection (Skinnes *et al.* 2008; Graham and Brown, 2009; Skinnes *et al.* 2010; Buerstmayr *et al.* 2012; Lu *et al.* 2013 and He *et al.* 2014). Significant negative relationships between DON content and anther extrusion have also been reported (He *et al.* 2014; Skinnes *et al.* 2008) therefore supporting the findings of this study.

Physiological traits accounting for variation in grain DON content were inconsistent, in agreement with previous studies which have shown inconsistent relationships between plant height and DON contamination (Chu *et al.* 2011). In 2011 plant number and remaining leaf area both had significant positive relationships with DON content at GS39 and GS65, respectively. While previous studies have not identified relationships between

these traits and DON content, relationships have been identified between tiller number and DON content (Gautam *et al.* 2012). This is important as plant number and remaining leaf area in 2011 were both strongly correlated to shoot traits at GS39 and GS65, respectively, indirectly suggesting that the relationship shown by Gautam *et al.* (2012) may be plausible. To further support this statement, grain mycotoxin content was positively grouped with fertile shoots/plant and fertile shoots/m^{-2} at GS39 and GS65, respectively. Furthermore, despite no relationship between tiller number and DON in the 2010 field experiment MLR, tiller number still positively grouped together with mycotoxins in the PCA.

In general, ear morphology traits were not significantly related to FHB. Large differences ($P < 0.001$) were present between genotypes for ear morphology, therefore lack of variation is unlikely to be the causal factor. Ear traits may genuinely have no influence over the development of FHB, although several potential mechanisms can be theorised in which a relationship between ear morphology and FHB would have been expected. These include, a less conducive microclimate for FHB development within lower density ears, or longer ears providing improved Type II resistance by slowing fungal spread within the rachis. An explanation for this lack of relationship may however be that, since *Fusarium* and *Microdochium* inoculum are predominantly splash dispersed through the canopy (Jenkinson and Parry, 1994), canopy traits could influence the pathogen spore load reaching the ear, creating an unfair test on the influence of ear morphology on FHB development.

All assessments during the 2010 field experiment were undertaken by an undergraduate student, for which the assessment of physiological traits was undertaken at GS89. At this growth stage (late dough) it would have been difficult to accurately assess several of the physiological traits, due to the advanced level of senescence within the crop. Additionally, these assessments were carried out after anthesis had taken place, which is the critical

phase for FHB infection. This was perhaps not chronologically correct, since the assessment of physiological traits after infection has taken place would not accurately reflect the state of each traits at the critical period of infection, therefore making it difficult to specifically infer back a disease escape mechanism. This was resolved in further field experiments, by assessing physiological traits at two periods prior to anthesis (GS39 and GS65), as these traits could potentially have an influence on the dispersal of FHB inoculum to the ear, since rain splash is the predominant dispersal mechanism (Jennings and Parry, 1995).

4.8. CONCLUSIONS

The results within this chapter enable the following conclusions to be made, based on the hypotheses in Section 4.2:

i) Flag leaf length was the only canopy trait to be consistently related to AUDPC, of which a positive relationship was present.

ii) Awn length, measured only in the 2012 experiment, was negatively related to AUDPC, while the proportion of trapped anthers was positively related to DON.

iii) Trapped anthers had a stronger relationship with DON than either fully retained or extruded anthers.

iv) Relationships between plant height and the quantity of *Fusarium* and *Microdochium* inoculum on the flag and second leaves could not be determined, due to the lack of sensitivity of real-time PCR to quantify the pathogen inoculum.

v) In general, UK elite genotypes did not have consistently lower AUDPC, mycotoxin or pathogen DNA values than the DH lines. Ambrosia was the exception, producing a low AUDPC, but having pathogen DNA and mycotoxin content that was consistently at the higher end of the spectrum compared to other UK elite genotypes and DH lines.

vi) Similar proportions of variation in the AUDPC were accounted for using physiological traits assessed at both GS39 and GS65, although the traits accounting for this variation differed between growth stage.

Chapter 5.

Field experiment 2012 & 2013

5.1 INTRODUCTION

Flag leaf length, flag leaf height and plant height were the canopy traits most consistently related to FHB in 2010 and 2011 field experiments. These were the first experiments to report relationships between flag leaf length and FHB. It was hypothesised that this relationship may have reflected a passive resistance mechanism, as rainfall dispersed conidia is likely to be transferred via the flag leaf before reaching the ear. Negative relationships were identified between AUDPC and flag leaf height and plant height at GS39 and GS65, respectively. These results concur with the findings of previous studies in which a negative relationship between FHB and height was also found (Hilton *et al*. 1999; Gervais *et al.* 2003; He *et al.* 2014). It is possible that the effect of plant height on FHB resistance may have been due to previously reported genetic linkages between QTL for FHB susceptibility and rht (Srinivasachary *et al.* 2009).

Awn length and the proportion of trapped anthers were the ear traits most consistently related to FHB in 2010 and 2011 field experiments. Awn length was negatively related to AUDPC in 2011, concurring with the findings of other studies (Buerstmayr *et al.* 2000; Ban and Suenaga, 2000). This suggested a potential passive resistance mechanism through longer awns providing a physical barrier to spore deposition on the ear, although QTL on chromosomes 4B and 5A, controlling both awn length and FHB resistance, have previously been reported (Snijders, 1990; Gervais *et al.* 2003; Lui *et al.* 2013). Genotypes with a greater number of trapped anthers and a lower number of extruded anthers accumulated more DON in 2011, agreeing with the findings of previous studies (Skinnes *et al*. 2005; Skinnes *et al.* 2008; Graham and Brown, 2009; Skinnes *et al.* 2010; Buerstmayr *et al.* 2012; Lu *et al.* 2013; He *et al.* 2014).

Two additional field experiments were conducted in 2012 and 2013, as described in this chapter. The inclusion of these experiments would result in field experiments having been carried out across a four year period, with the 2010 experiment having been undertaken by an undergraduate student prior to the start of this PhD study. The 2012 and 2013 experiments utilised a larger selection of genotypes from the Line 8 x Rialto DH population, as well as a reduced number of UK elite genotypes, to increase the amount of genotypic variation within the study. Additional DH genotypes were selected for inclusion based on data provided by Gaju (2007), in which flowering date and plant height were standardised to limit the deleterious effects of these traits on the identification of new potential passive resistance traits. Two highly FHB resistant control genotypes, Frontana and Sumai-3, were also included to act as a reference for genetic resistance. Frontana carries a high level of Type I resistance to FHB (resistance to initial infection), conferred predominantly through a major resistance QTL on chromosome 3A (Steiner *et al.* 2004), while Sumai-3 is a source of Type II resistance (resistance to spread within the ear), conferred predominantly through a major resistance QTL on chromosomes 3BS (Xie *et al.* 2007) and 6BS (Cuthbert *et al.* 2007). Physiological traits were assessed at GS39 and GS65. Misting of the plots was included as a factor at GS65 to stimulate FHB infection and allow for trait comparisons at a range of FHB disease pressures. Misting was envisaged to additionally favour pathogen species that are splash-dispersed and require humid conditions for disease development. Due to the increase in the number of genotypes examined compared to experiments in previous years, the 2012 and 2013 experiments were drilled as 1 x 1m plots, to facilitate management and misting of the experiments.

Key physiological traits related to AUDPC or grain mycotoxin content in the 2010 and 2011 field experiments were assessed within the 2012 and 2013 field experiments. A modified version of the 2011 fractional interception method was used in the 2012 field

experiment, and was undertaken to provide further information on canopy development rate and light interception, since the canopy is likely to influence the splash dispersal of FHB inoculum (Jenkinson and Parry, 1994). The focus of these experiments was to identify novel traits that have not previously been studied, thus several previous traits that were not related to AUDPC or mycotoxin content, or had been previously confirmed by other published literature, were excluded.

5.2 OBJECTIVES AND HYPOTHESES

The objectives of these experiments was to test the previously identified novel traits under low and high disease pressures provided by misting, and to elucidate their role in FHB disease escape caused by diverse range of *Fusarium/Microdochium* species. The objectives of these field experiments were to:

1) Further quantify key physiological traits that were related to FHB during the 2010 and 2011 field experiments, within an expanded range of wheat genotypes.

2) Quantify the level of FHB resistance in an expanded range of wheat genotypes, using a combination of visual disease assessment and pathogen DNA quantification.

3) Identify if the physiological traits that significantly account for AUDPC variation were consistent with the 2010 and 2011 field experiments.

4) Identify if the same physiological traits account for variation in AUDPC are the same under misted or natural rainfall conditions.

The specific experimental hypotheses tested within this chapter are:

i) The inclusion of additional DH lines will create increased variation within the assessed physiological traits, compared to the 2011 field experiment.

ii) The highly resistant genotypes Sumai-3 and Frontana will have lower AUDPC and pathogen DNA content than either UK elite or DH lines.

iii) Canopy traits including flag leaf length will be significantly related to the AUDPC, as in Chapter 4.

iv) Ear traits including awn length will be significantly related to AUDPC, as in Chapter 4.

v) Physiological traits related to AUDPC will be different under misted and natural rainfall conditions.

vi) Physiological traits assessed at GS65 will account for more variation in the AUDPC than traits assessed at GS39.

5.3 MATERIALS AND METHODS

5.3.1 Experimental design

Field experiments were undertaken in 2012 and 2013 at trial sites located at the University of Nottingham, Sutton Bonington Campus, UK (latitude 52.83368, longitude -1.24638). Each experiment consisted of 3 UK winter wheat genotypes, 16 doubled-haploid lines derived from an L8 x Rialto cross, and two highly FHB resistant genotypes Sumai-3 and Frontana (Table 5.1). Genotypes were assigned to an early, medium or late flowering group to assist with misting requirements. Additionally, specific genotypes were selected to standardise on crop height, avoiding either very tall or very short genotypes, to minimise the effects of this trait on passive and active resistance. Each plot was $1m^2$ (1m x 1m) in dimension with six blocked replicates, of which three were misted and three were non-misted to create a misting treatment. Field experiment layouts for 2012 and 2013 are shown in Appendix 5A and 5B, respectively. Experiments were ground-inoculated using oat grains infected with *Fusarium graminearum, F. culmorum, F. avenaceum, F. langsethiae, F. poae, Microdochium majus* and *M. nivale* (Appendix 2A) at GS31.

5.3.2 Experiment agronomy

Drilling of the 2012 and 2013 field experiments was carried out on the 26th October 2011 and 15th October 2012, respectively, using a seed rate of 375 seeds/m^2. Refer to Appendix 5C for full crop protection details. In brief, crop protection measures followed standard agronomic practice, except for fungicide applications which aimed to give robust crop protection from foliar and stem-base diseases while minimising the effect on FHB development. Plant growth regulator use was omitted to allow each wheat genotype to express its full height potential.

5.3.3 Ground inoculum production

Refer to section 2.2 for full method details.

Table 5.1. Wheat lines used in the 2012 and 2013 field experiment

Genotype	Origin	Breeding method
Ambrosia	UK Elite Variety	Conventional
Claire	UK Elite Variety	Conventional
Rialto	UK Elite Variety	Conventional
Frontana	Brazilian Spring wheat	Conventional
Sumai-3	Chinese spring wheat	Conventional
Line 8	L8 x Rialto	Doubled-haploid
Line 14	L8 x Rialto	Doubled-haploid
Line 15	L8 x Rialto	Doubled-haploid
Line 16	L8 x Rialto	Doubled-haploid
Line 18	L8 x Rialto	Doubled-haploid
Line 21	L8 x Rialto	Doubled-haploid
Line 24	L8 x Rialto	Doubled-haploid
Line 28	L8 x Rialto	Doubled-haploid
Line 38	L8 x Rialto	Doubled-haploid
Line 39	L8 x Rialto	Doubled-haploid
Line 43	L8 x Rialto	Doubled-haploid
Line 48	L8 x Rialto	Doubled-haploid
Line 51	L8 x Rialto	Doubled-haploid
Line 61	L8 x Rialto	Doubled-haploid
Line 69	L8 x Rialto	Doubled-haploid
Line 77	L8 x Rialto	Doubled-haploid

5.3.4. Trait assessments

The methods used for trait assessments were the same as described in Section 2.3. Traits assessed in the 2012 and 2013 field experiments are shown in Table 5.2. These traits were chosen based on the findings of previous experiments and aimed to examine key physiological traits only, excluding several non-significant traits from the previous

experiments (Sections 4.4.6, 4.5.7 and 4.5.11). Due to relationships between anther extrusion and mycotoxins in the 2011 field experiment confirming the findings of previous studies, it was not felt necessary to include this trait assessment within further studies.

Table 5.2. Physiological traits assessed at GS39 and GS65 during the 2012 and 2013 field experiments

Trait name	2012 GS39	2012 GS65	2013 GS39	2013 GS65
Plant number (m^2)	×	×	×	×
Fertile shoots (m^2)	×	×	×	×
Fertile shoots (per plant)	×	×	×	×
Flag leaf length (cm)	×	×	×	×
Flag leaf width (cm)	×	×	×	×
Flag leaf height (cm)	×	×	×	×
Plant height (cm)		×		×
Total fresh weight (g/m^2)	×	×	×	×
Fresh weight of flag leaves on fertile shoots (g/m^2)	×	×	×	×
Fresh weight of second leaves on fertile shoots (g/m^2)	×	×	×	×
Fresh weight of all remaining leaf on fertile shoots (g/m^2)	×	×	×	×
Total fresh weight of all leaf on fertile shoots (g/m^2)	×	×	×	×
Flag leaf area of fertile shoots (cm^2/m^2)	×	×	×	×
Second leaf area of fertile shoots (cm^2/m^2)	×	×	×	×
All remaining leaf area of fertile shoots (cm^2/m^2)	×	×	×	×
Total leaf area (cm^2/m^2)	×	×	×	×
Fresh weight of all stems (g/m^2)	×	×	×	×
Ear length (cm)		×		×
Number of spikelets per ear		×		×
Spikelet density (spikelets/cm)		×		×
Awn length (cm)		×		×
Fresh weight of ears (g/m^2)		×		×
Fractional interception	×			

5.3.5 Canopy light interception assessments

In 2012, canopy light interception was measured using a SS1 SunScan Canopy Analysis System (Delta-T, Cambridge, UK). Two readings were taken per plot, with the probe inserted from diagonally across the plot, passing through the centre. Readings were taken from each plot at 7 day intervals from GS31 until GS65. Each individual reading was used to plot a fractional interception progress curve using the same formula as used to calculate the AUDPC in section 2.4.1. This curve was used to compare the integrated fractional interception over time between genotypes over time. The fractional interception of each plot at each individual reading from GS31 to GS65 was calculated using the same formula as in Section 4.3.6.

5.3.6 Misting treatment

Misting was carried out on the appropriate plots from GS65 onwards for 10 days to simulate high disease pressure. To achieve this, mains irrigation pipes were laid to the trial site in early spring and then connected through smaller pipes the length of the trial to misting risers so that water could be supplied to each plot. Within the centre of each misted plot, risers were used to hold spray nozzles which delivered a fine mist of water in a 360^{o} angle around the nozzle. Each nozzle was maintained at 20cm above ear height as this was found to produce an even misting throughout the plot. Flexible plastic tubing was used to connect the misting nozzles to the water pipes which ran between the plots.

Misting was started manually each day at 1pm and was maintained until the wheat heads in each misted plot were thoroughly wetted. It was important to survey each nozzle during misting to ensure that any blocked or damaged nozzles were replaced (Fig. 5.1).

Figure 5.1. Assistance received from an MSc student to help clear blocked nozzles during misting.

5.3.7 Visual disease assessment and AUDPC calculation

Refer to section 2.4 for full method details.

5.3.8 Grain harvest

Due to the wide range of maturity dates between genotypes, each genotype was harvested individually at the date its grain had ripened (GS93) so that mycotoxin and *Fusarium* infection did not continue to increase in the field once physiological maturity had been

reached. As each line reached GS93, whole $1m^2$ plots were harvested using grab samples, avoiding the edge of the plot, and placed into paper bags. Ears were threshed using a stationary thresher and the grain was milled to a fine flour using a Krups F203 grinder (Krups, Windsor). Grain yield was unable to be measured due to variation in sampling between plots.

5.3.9 Pathogen DNA quantification

Refer to section 2.6 for full method details.

5.3.10 Statistical analysis

All data was analysed using Genstat® Version 12.1 for Windows (VSN International Ltd, UK). Where required, DNA and mycotoxin data was Log_{10} transformed to normalise residuals. Several plots needed to be discarded due to drilling errors in which genotypes became mixed within the plot. The non-misted plots of Ambrosia and L61 in the 2013 experiment were omitted from ANOVA due to insufficient replicates being present, while genotypes with a single missing replicate were assigned a missing value, in which groupings were used to detect significant year*genotype*misting interactions. The visualisation of groupings between measured variables was explored using Principal Component Analysis (PCA) to create biplots. Stepwise Multiple Linear Regression (MLR), used as per Section 4.3.15, was used to account for variation in the AUDPC, using assessed physiological traits and quantified pathogen DNA. Due to large differences in AUDPC and pathogen DNA between each experimental year, MLR and PCA analysis was performed separately for each years data to avoid confounding results.

5.4 FIELD EXPERIMENT 2012 & 2013 RESULTS

5.4.1 Visual disease symptoms

A wide range of AUDPCs were found in the wheat genotypes studied in the 2012 and 2013 field experiments (Table 5.3). The mean AUDPC in 2012 was 290.9 with Frontana and Sumai-3 exhibiting the highest levels of FHB resistance (AUDPC 29.9 and 30.9, respectively). UK elite lines showed the lowest levels of FHB resistance of the genotypes, with mean AUDPC values of 726, 672 and 663 for Rialto, Ambrosia and Claire respectively, being higher than all other genotypes ($P=0.005$). In 2013 the disease pressure was much lower than in 2012, with the range of AUDPC greatly reduced. In 2013 Frontana and Sumai-3 had low mean AUDPC scores of 0 and 0.3 respectively, with only L43 (0.20) having a lower AUDPC than Sumai-3. AUDPC values for elite genotypes were not significantly higher than the rest of the genotypes, in contrast to 2012. Overall, there was an interaction ($P<0.001$) between year and genotype for AUDPC, with elite genotypes and DH lines 14 and 38 having the greatest interaction between years. Misted plants in 2012 had higher AUDPC than non-misted plants (273 and 249, respectively), but the difference was not significant at $P<0.05$ and no differences were observed in 2013.

In both 2012 and 2013 symptoms of water soaked lesions made up the majority of the AUDPC (Fig. 5.2 and 5.3). The UK elite varieties Ambrosia, Claire and Rialto, however, showed bleaching symptoms contributing to a greater proportion of their total AUDPC than other genotypes, indicating poor Type II resistance (resistance to disease spread within the ear). In 2012, a mean value of 26% of Ambrosia, Claire and Rialto's AUDPC was made up of bleaching symptoms, compared to 6.6% of the other genotypes included in the study. In 2013 however, these figures increased to 63.9% and 13.78%, respectively. The highly resistant lines Frontana and Sumai-3 both showed high levels of disease

resistance, although Frontana had less lesion symptoms than Sumai-3 in 2013, potentially reflecting Frontana's Type I resistance (resistance to initial infection).

Table 5.3. Genotypic differences in AUDPC between misted and non-misted treatments within the 2012 and 2013 field experiments

	2012			2013		
	Misted	Non-misted	Mean	Misted	Non-misted	Mean
Ambrosia	679.5	664.3	671.9	3.6	-	3.60
Claire	725.0	601.7	663.4	2.0	2.3	2.15
Rialto	680.0	772.4	726.2	0.5	2.0	1.25
Frontana	36.6	23.1	29.9	0.0	0.0	0.00
Sumai-3	27.4	34.3	30.9	0.6	0.0	0.30
L8	259.6	250.8	255.2	1.9	3.5	2.70
L14	133.9	201.1	167.5	4.6	5.7	5.15
L15	584.1	353.0	468.6	1.4	1.5	1.45
L16	276.9	227.5	252.2	3.5	4.4	3.95
L18	157.3	107.9	132.6	3.1	1.2	2.15
L21	303.1	309.4	306.3	1.2	1.4	1.30
L24	262.9	205.8	234.4	3.2	2.8	3.00
L28	84.0	77.4	80.7	0.5	1.0	0.75
L38	401.8	439.1	420.5	0.4	1.3	0.85
L39	159.0	147.6	153.3	0.4	1.5	0.95
L43	223.8	219.9	221.9	0.2	0.2	0.20
L48	92.1	90.7	91.4	1.3	1.0	1.15
L51	226.3	139.9	183.1	0.4	1.2	0.80
L61	194.0	125.6	159.8	0.3	-	0.30
L69	50.7	59.4	55.1	0.6	1.2	0.90
L77	175.7	172.6	174.2	1.7	1.4	1.55
Mean	273.0	248.7	290.9	1.5	1.6	1.60
Year			<.001			
Genotype			<.001			
Misting			0.192			
Year*Genotype			<.001			
Year*Misting			0.118			
Genotype*Misting			0.768			
Year*Genotype*Misting			0.759			
LSD (5%)			110.02			
CV (%)			50.64			

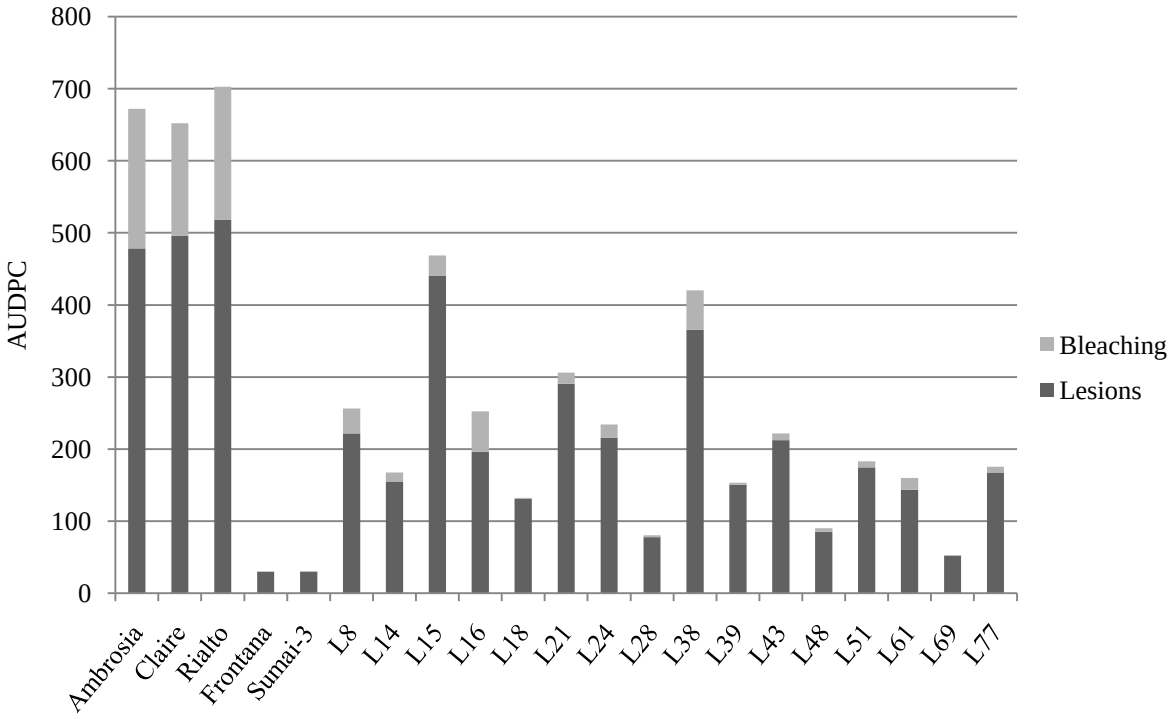

Figure 5.2. Proportion of AUDPC accounted for by lesion and bleaching symptoms in the 2012 field experiment.

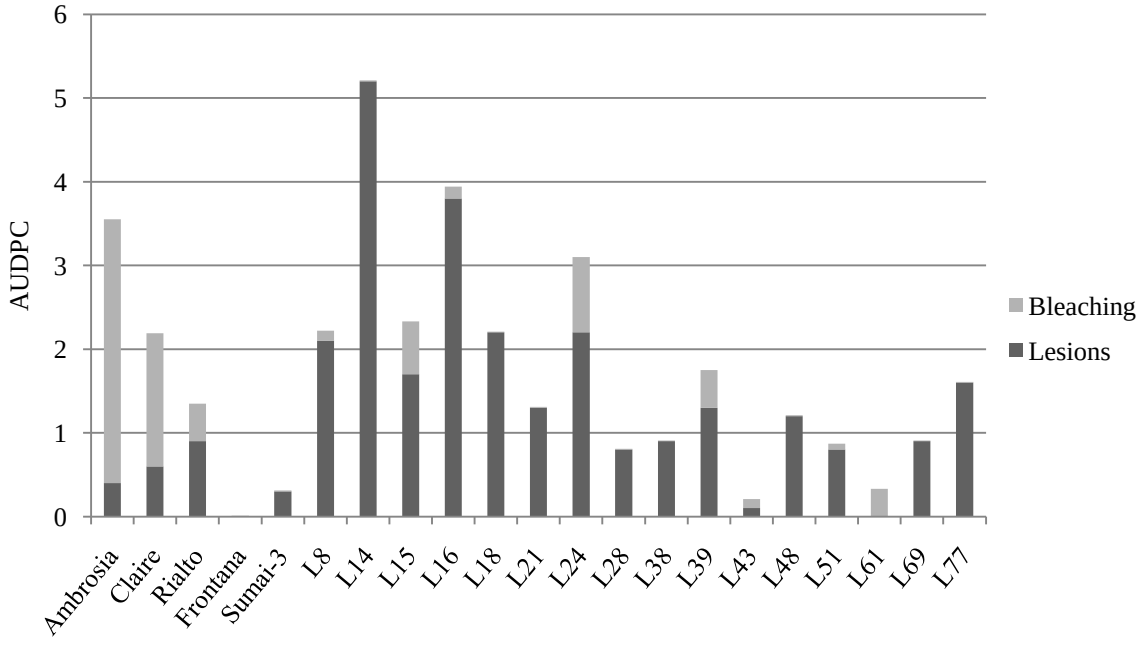

Figure 5.3. Proportion of AUDPC accounted for by lesion and bleaching symptoms in the 2013 field experiment.

5.4.2 Pathogen DNA

Comparisons between experimental years revealed significantly (P<0.001) higher concentrations of *F. graminearum, F. culmorum, F. avenaceum, M. majus* and *M. nivale* DNA in 2012 than in 2013. *F. langsethiae* and *F. poae* DNA were the exception to this, with significantly higher (P<0.001) DNA concentrations in 2013 than in 2012, although both species were present at very low concentrations (Table 5.4 – 5.7).

In 2012 *F. graminearum* and *M. nivale* were the predominant species within the harvested grain samples, detected at DNA concentrations of 9.30 pg/ng and 6.42 pg/ng respectively. Other pathogen species including *F. culmorum, F. avenaceum F. poae, F. langsethiae* and *M. majus* were detected in the 2012 grain samples, however their DNA was detected at lower concentrations.

In 2013, *F. graminearium* predominated with the highest DNA at 0.31 pg/ng of total DNA, accounting for 85% of the combined quantity of *Fusarium* and *Microdochium* spp. DNA in that year (Table 5.4 – 5.7). *F. langsethiae* was the second most predominant pathogen species with a mean DNA content of 0.02 pg/ng. The DNA of the remaining *Fusarium* spp. and *Microdochium* spp. was present in the grain at very low mean concentrations ranging from 0.001 – 0.008 pg/ng DNA with species in a descending order of *M. nivale, F. avenaceum, M. majus, F. culmorum* and *F. poae.*

Misting created a significant difference only for *F.langsethiae,* in which non-misted plots had a significantly higher DNA concentration than misted plots in 2012 (0.012 and 0.009 pg/ng, respectively), with the same situation occurring again in 2013 (0.022 and 0.017 pg/ng, respectively).

Significant differences were present between wheat genotypes for *F. graminearum, F. avenaceum, F. langsethiae, M. majus* and *M. nivale* DNA and significant year*genotype

interactions were present for *F. graminearum*, *F. avenaceum*, *F. langsethiae* and *M. nivale* DNA.

UK elite genotypes showed no consistent significant differences to DH lines for pathogen DNA concentration, with the exception of Ambrosia, Claire and Rialto all having higher *F. langsethiae* DNA concentrations than DH or control genotypes in the 2012 field experiment (Table 5.5). The pathogen DNA concentration of the resistant control genotypes Sumai-3 and Frontana, was not consistently lower than either UK elite or DH lines.

Relationships between AUDPC and total mean grain pathogen DNA were not present in either the 2012 or 2013 field experiment (Fig. 5.4 and 5.5)

Due to the inclusion of multiple wheat lines and *Fusarium* species within the study however, a high proportion of zero values were present within the analysis, therefore caution should be given to the mean values represented in Tables 5.4 - 5.7.

5.4. Differences between genotypes and misting treatment in the amount of *F. graminearum* and *F. culmorum* DNA present within harvested grain samples collected from the 2012 and 2013 field experiment, expressed as Log_{10} of total DNA

	F. graminearum DNA (pg/ng)				*F. culmorum* DNA (pg/ng)			
	2012		2013		2012		2013	
	Misted	Non-misted	Misted	Non-misted	Misted	Non-misted	Misted	Non-misted
Ambrosia	1.16 (14.52)	1.15 (13.96)	-0.14 (0.72)	-	-3.09 (0.0008)	-2.44 (0.004)	-2.65 (0.002)	-
Claire	0.97 (9.23)	0.80 (6.31)	-1.41 (0.04)	-0.73 (0.19)	-2.83 (0.002)	-2.64 (0.002)	-4.00 (0.0001)	-2.03 (0.009)
Rialto	1.16 (14.39)	1.07 (11.78)	-0.52 (0.30)	-0.32 (0.48)	-1.39 (0.041)	-3.89 (0.0001)	-2.96 (0.001)	-3.17 (0.001)
Frontana	0.81 (6.41)	0.28 (1.88)	-0.31 (0.49)	-0.43 (0.37)	-1.11 (0.078)	-1.23 (0.059)	-3.21 (0.001)	-2.78 (0.002)
Sumai-3	0.38 (2.39)	0.61 (4.06)	-1.61 (0.02)	-1.51 (0.03)	-1.35 (0.045)	-2.02 (0.009)	-3.13 (0.001)	-3.01 (0.001)
L8	1.09 (12.30)	1.00 (10.02)	-0.25 (0.56)	-0.23 (0.59)	-2.86 (0.001)	-2.57 (0.003)	-3.21 (0.001)	-1.47 (0.034)
L14	0.87 (7.36)	0.99 (9.75)	-1.81 (0.02)	-1.08 (0.08)	-2.86 (0.001)	-2.01 (0.010)	-3.27 (0.001)	-3.44 (0.0004)
L15	1.24 (17.38)	1.64 (43.75)	-0.12 (0.76)	-0.51 (0.31)	-2.63 (0.002)	-1.70 (0.020)	-1.99 (0.010)	-2.12 (0.008)
L16	1.19 (15.35)	1.19 (15.60)	-0.37 (0.42)	-0.47 (0.34)	-1.73 (0.019)	-3.34 (0.001)	-3.41 (0.0004)	-2.07 (0.009)
L18	1.06 (11.35)	0.91 (8.18)	-0.47 (0.34)	-0.69 (0.20)	-1.12 (0.076)	-2.02 (0.010)	-2.56 (0.003)	-2.80 (0.002)
L21	0.87 (7.33)	1.23 (17.02)	-0.26 (0.55)	-1.15 (0.07)	-1.98 (0.011)	-1.74 (0.018)	-2.59 (0.003)	-4.00 (0.0001)
L24	0.72 (5.19)	0.70 (4.95)	-1.40 (0.04)	-1.47 (0.03)	-1.71 (0.019)	-2.24 (0.006)	-3.01 (0.001)	-3.33 (0.001)
L28	0.99 (9.68)	0.87 (7.36)	-0.65 (0.22)	-0.26 (0.55)	-1.51 (0.031)	-2.96 (0.001)	-2.26 (0.006)	-1.23 (0.059)
L38	0.88 (7.62)	1.02 (10.45)	-0.72 (0.19)	-0.88 (0.13)	-4.00 (0.0001)	-1.18 (0.066)	-2.47 (0.003)	-3.49 (0.0003)
L39	0.89 (7.73)	-0.47 (0.34)	-0.32 (0.48)	-0.30 (0.50)	-2.35 (0.005)	-1.70 (0.020)	-2.70 (0.002)	-1.82 (0.015)
L43	1.08 (11.94)	0.95 (8.99)	-0.21 (0.62)	-0.83 (0.15)	-2.02 (0.009)	-4.00 (0.0001)	-2.69 (0.002)	-1.92 (0.012)
L48	0.97 (9.31)	1.05 (11.27)	-0.10 (0.79)	-0.70 (0.20)	-1.47 (0.034)	-0.93 (0.118)	-3.05 (0.001)	-2.04 (0.009)
L51	0.29 (1.97)	0.41 (2.55)	-0.53 (0.29)	-0.37 (0.43)	-3.33 (0.0005)	-3.05 (0.001)	-2.90 (0.001)	-3.25 (0.001)
L61	0.77 (5.94)	0.60 (3.99)	-3.88 (0.0001)	-	-1.98 (0.011)	-2.19 (0.007)	-3.89 (0.0001)	-
L69	0.92 (8.26)	0.63 (4.24)	-0.50 (0.32)	-1.45 (0.04)	-2.23 (0.006)	-0.45 (0.355)	-4.00 (0.0001)	-3.27 (0.001)
L77	0.46 (2.88)	0.75 (5.62)	-0.60 (0.25)	-1.00 (0.10)	-2.34 (0.005)	-3.08 (0.001)	-2.89 (0.001)	-2.32 (0.005)
Mean	0.89 (8.98)	0.83 (9.62)	-0.77 (0.35)	-0.88 (0.26)	-2.19 (0.019)	-2.26 (0.034)	-2.99 (0.002)	-2.60 (0.009)
Year		<.001				<.001		
Genotype		<.001				0.736		
Misting		0.400				0.355		
Year*Genotype		<.001				0.227		
Year*Misting		0.825				0.182		
Genotype*Misting		0.998				0.750		
Year*Genotype*Misting		0.975				0.271		
LSD (5%)		1.342				2.201		
CV (%)		5004.6				54.4		

Back transformed means in parenthesis

Table 5.5. Differences between genotypes and misting treatment in the amount of *F. poae* and *F. langsethiae* DNA present within harvested grain samples collected from the 2012 and 2013 field experiment, expressed as Log_{10} of total DNA (pg/ng)

	F. poae DNA (pg/ng)				*F. langsethiae* DNA (pg/ng)			
	2012		2013		2012		2013	
	Misted	Non-misted	Misted	Non-misted	Misted	Non-misted	Misted	Non-misted
Ambrosia	-3.88 (0.0001)	-4.00 (0.0001)	-3.33 (0.0005)	-	-1.40 (0.040)	-1.59 (0.026)	-1.27 (0.054)	-
Claire	-3.08 (0.0008)	-2.94 (0.0011)	-3.21 (0.0006)	-3.38 (0.0004)	-1.38 (0.042)	-1.38 (0.042)	-2.47 (0.003)	-2.12 (0.008)
Rialto	-3.35 (0.0004)	-2.92 (0.0012)	-2.73 (0.0019)	-3.33 (0.0005)	-1.41 (0.039)	-1.06 (0.087)	-2.24 (0.006)	-1.90 (0.013)
Frontana	-4.00 (0.0001)	-4.00 (0.0001)	-3.13 (0.0007)	-3.03 (0.0009)	-4.00 (0.0001)	-4.00 (0.0001)	-2.49 (0.003)	-1.90 (0.013)
Sumai-3	-2.60 (0.0025)	-4.00 (0.0001)	-3.08 (0.0008)	-2.82 (0.0015)	-2.89 (0.001)	-3.21 (0.001)	-2.41 (0.004)	-2.28 (0.005)
L8	-4.00 (0.0001)	-3.23 (0.0006)	-3.23 (0.0006)	-2.86 (0.0014)	-2.78 (0.002)	-2.30 (0.005)	-1.52 (0.030)	-1.35 (0.045)
L14	-3.20 (0.0006)	-4.00 (0.0001)	-3.22 (0.0006)	-3.47 (0.0003)	-2.96 (0.001)	-2.50 (0.003)	-3.09 (0.001)	-2.84 (0.001)
L15	-3.19 (0.0006)	-4.00 (0.0001)	-3.24 (0.0006)	-2.88 (0.0013)	-1.83 (0.015)	-1.95 (0.011)	-1.59 (0.026)	-1.16 (0.069)
L16	-3.64 (0.0002)	-3.06 (0.0009)	-2.79 (0.0016)	-2.51 (0.0031)	-2.18 (0.007)	-2.34 (0.005)	-2.17 (0.007)	-1.90 (0.013)
L18	-4.00 (0.0001)	-2.41 (0.0039)	-3.42 (0.0004)	-3.09 (0.0008)	-4.00 (0.0001)	-3.47 (0.0003)	-2.23 (0.006)	-1.38 (0.042)
L21	-4.00 (0.0001)	-3.64 (0.0002)	-2.93 (0.0012)	-2.29 (0.0051)	-2.75 (0.002)	-1.95 (0.011)	-1.35 (0.045)	-3.06 (0.001)
L24	-4.00 (0.0001)	-3.18 (0.0007)	-3.44 (0.0004)	-3.02 (0.0010)	-2.10 (0.008)	-1.92 (0.012)	-2.61 (0.003)	-2.30 (0.005)
L28	-3.64 (0.0002)	-3.64 (0.0002)	-3.23 (0.0006)	-2.31 (0.0049)	-4.00 (0.0001)	-3.44 (0.0004)	-1.57 (0.027)	-1.29 (0.051)
L38	-3.56 (0.0003)	-4.00 (0.0001)	-2.32 (0.0048)	-2.72 (0.0019)	-1.88 (0.013)	-2.18 (0.007)	-2.45 (0.004)	-1.93 (0.012)
L39	-3.07 (0.0009)	-3.90 (0.0001)	-2.27 (0.0054)	-3.05 (0.0009)	-2.14 (0.008)	-1.95 (0.011)	-1.52 (0.030)	-1.11 (0.078)
L43	-4.00 (0.0001)	-3.70 (0.0002)	-3.84 (0.0001)	-3.48 (0.0003)	-3.41 (0.0004)	-2.12 (0.008)	-2.33 (0.005)	-1.56 (0.028)
L48	-3.49 (0.0003)	-4.00 (0.0001)	-2.70 (0.0020)	-3.24 (0.0006)	-2.00 (0.010)	-2.03 (0.009)	-1.15 (0.071)	-1.45 (0.036)
L51	-4.00 (0.0001)	-4.00 (0.0001)	-4.00 (0.0001)	-3.23 (0.0006)	-3.03 (0.001)	-2.87 (0.001)	-1.94 (0.012)	-2.21 (0.006)
L61	-3.70 (0.0002)	-4.00 (0.0001)	-3.95 (0.0001)	-	-3.44 (0.0004)	-2.95 (0.001)	-3.76 (0.0002)	-
L69	-4.00 (0.0001)	-3.66 (0.0002)	-2.50 (0.0032)	-2.94 (0.0011)	-2.68 (0.002)	-2.21 (0.006)	-1.64 (0.023)	-2.23 (0.006)
L77	-4.00 (0.0001)	-3.46 (0.0003)	-3.68 (0.0002)	-2.83 (0.0015)	-2.99 (0.001)	-2.03 (0.009)	-2.54 (0.003)	-1.86 (0.014)
Mean	-3.64 (0.0004)	-3.61 (0.0005)	-3.15 (0.0013)	-3.05 (0.0014)	-2.63 (0.009)	-2.35 (0.012)	-2.11 (0.017)	-1.94 (0.022)
Year	<.001				<.001			
Genotype	0.070				<.001			
Misting	0.431				0.026			
Year*Genotype	0.373				<.001			
Year*Misting	0.673				0.609			
Genotype*Misting	0.141				0.911			
Year*Genotype*Misting	0.714				0.841			
LSD (5%)	1.159				1.283			
CV (%)	21.4				35.2			

Back transformed means in parenthesis

Table 5.6. Differences between genotypes and misting treatment in the amount of *M. nivale* and *M. majus* DNA present within harvested grain samples collected from the 2012 and 2013 field experiment, expressed as Log_{10} of total DNA (pg/ng)

	M. nivale DNA (pg/ng)				*M. majus* DNA (pg/ng)			
	2012		2013		2012		2013	
	Misted	Non-misted	Misted	Non-misted	Misted	Non-misted	Misted	Non-misted
Ambrosia	0.96 (9.18)	0.77 (5.92)	-2.19 (0.006)	-	0.17 (1.47)	0.12 (1.32)	-1.81 (0.016)	-
Claire	0.90 (7.99)	0.80 (6.27)	-2.34 (0.005)	-2.16 (0.007)	0.07 (1.17)	-0.02 (0.95)	-2.62 (0.002)	-2.84 (0.001)
Rialto	1.05 (11.09)	0.85 (7.15)	-2.13 (0.007)	-2.45 (0.004)	0.27 (1.88)	-0.47 (0.34)	-2.51 (0.003)	-3.28 (0.001)
Frontana	0.22 (1.66)	0.27 (1.86)	-2.58 (0.003)	-2.06 (0.009)	-0.49 (0.33)	-0.31 (0.49)	-2.71 (0.002)	-2.42 (0.004)
Sumai-3	0.71 (5.07)	0.74 (5.46)	-2.63 (0.002)	-2.55 (0.003)	-0.22 (0.60)	0.05 (1.11)	-2.51 (0.003)	-2.57 (0.003)
L8	0.83 (6.76)	0.89 (7.73)	-2.22 (0.006)	-1.63 (0.023)	0.01 (1.03)	0.25 (1.79)	-3.19 (0.001)	-1.81 (0.015)
L14	0.73 (5.41)	0.86 (7.23)	-3.32 (0.001)	-3.17 (0.001)	-0.07 (0.86)	-0.02 (0.95)	-3.27 (0.001)	-1.92 (0.012)
L15	1.11 (12.82)	0.96 (9.08)	-1.84 (0.014)	-1.66 (0.022)	0.37 (2.32)	0.23 (1.70)	-2.33 (0.005)	-2.77 (0.002)
L16	0.85 (7.15)	0.74 (5.53)	-1.76 (0.017)	-1.88 (0.013)	0.02 (1.05)	0.03 (1.08)	-2.15 (0.007)	-2.62 (0.002)
L18	1.19 (15.39)	1.17 (14.72)	-2.22 (0.006)	-2.01 (0.010)	0.13 (1.34)	0.20 (1.59)	-2.63 (0.002)	-1.87 (0.014)
L21	0.61 (4.08)	0.86 (7.18)	-1.81 (0.016)	-2.56 (0.003)	-0.08 (0.84)	0.18 (1.52)	-2.73 (0.002)	-2.01 (0.010)
L24	0.82 (6.53)	0.78 (5.98)	-2.56 (0.003)	-2.49 (0.003)	-0.03 (0.93)	-0.11 (0.77)	-2.71 (0.002)	-2.37 (0.004)
L28	0.77 (5.82)	0.87 (7.38)	-2.80 (0.002)	-2.40 (0.0001)	-0.07 (0.85)	0.22 (1.67)	-2.87 (0.001)	-2.13 (0.008)
L38	0.76 (5.75)	0.82 (6.61)	-2.76 (0.002)	-2.72 (0.002)	-0.08 (0.82)	0.08 (1.21)	-2.11 (0.008)	-1.86 (0.014)
L39	0.87 (7.43)	0.99 (9.82)	-1.89 (0.013)	-1.63 (0.024)	-1.24 (0.06)	0.09 (1.24)	-2.34 (0.005)	-1.77 (0.017)
L43	0.37 (2.35)	0.72 (5.24)	-1.77 (0.017)	-1.92 (0.012)	0.19 (1.54)	0.08 (1.21)	-1.77 (0.017)	-1.89 (0.013)
L48	0.94 (8.67)	0.48 (3.02)	-2.07 (0.008)	-1.94 (0.012)	0.08 (1.20)	-0.32 (0.48)	-1.83 (0.015)	-1.93 (0.012)
L51	0.52 (3.33)	0.61 (4.06)	-2.14 (0.007)	-1.62 (0.024)	-0.11 (0.78)	0.02 (1.04)	-2.13 (0.007)	-2.15 (0.007)
L61	0.31 (2.05)	0.43 (2.69)	-3.90 (0.0001)	-	-0.19 (0.64)	-0.15 (0.71)	-4.12 (0.0001)	-
L69	0.66 (4.53)	0.80 (6.24)	-2.09 (0.008)	-2.67 (0.002)	-0.12 (0.76)	-0.03 (0.93)	-2.72 (0.002)	-2.44 (0.004)
L77	0.43 (2.68)	0.69 (4.94)	-2.16 (0.007)	-2.26 (0.006)	-0.21 (0.62)	0.02 (1.04)	-2.72 (0.002)	-3.44 (0.0004)
Mean	0.74 (6.46)	0.77 (6.38)	-2.30 (0.007)	-2.27 (0.009)	-0.08 (1.00)	0.01 (1.10)	-2.56 (0.002)	-2.37 (0.008)
Year			<.001				<.001	
Genotype			<.001				0.046	
Misting			0.544				0.142	
Year*Genotype			0.026				0.164	
Year*Misting			0.760				0.554	
Genotype*Misting			0.999				0.570	
Year*Genotype*Misting			0.992				0.974	
LSD (5%)			0.988				1.203	
CV (%)			78.9				59.7	

Back transformed means in parenthesis

Table 5.7. Differences between genotypes and misting treatment in the amount of *F. avenaceum* DNA present within harvested grain samples collected from the 2012 and 2013 field experiment, expressed as Log_{10} of total DNA (pg/ng)

	F. avenaceum DNA (pg/ng)			
	2012		2013	
	Misted	Non-misted	Misted	Non-misted
Ambrosia	-1.80 (0.02)	-1.56 (0.03)	-1.67 (0.021)	-
Claire	-1.89 (0.01)	-2.86 (0.001)	-2.79 (0.002)	-2.25 (0.01)
Rialto	-1.01 (0.10)	-0.71 (0.20)	-2.47 (0.003)	-2.35 (0.006)
Frontana	-0.57 (0.27)	-0.12 (0.76)	-2.25 (0.006)	-2.42 (0.004)
Sumai-3	-1.06 (0.09)	-1.70 (0.02)	-2.89 (0.001)	-2.48 (0.003)
L8	-1.94 (0.01)	-3.93 (0.0001)	-2.33 (0.005)	-2.62 (0.002)
L14	-0.94 (0.11)	-0.24 (0.57)	-2.82 (0.002)	-2.65 (0.002)
L15	0.07 (1.16)	-0.76 (0.18)	-2.52 (0.003)	-2.08 (0.008)
L16	-1.37 (0.04)	-1.01 (0.09)	-2.09 (0.008)	-1.98 (0.011)
L18	-0.38 (0.42)	-0.80 (0.16)	-2.00 (0.010)	-2.22 (0.006)
L21	-0.61 (0.24)	-1.35 (0.04)	-1.64 (0.023)	-2.08 (0.008)
L24	-0.56 (0.28)	-0.83 (0.15)	-2.69 (0.002)	-2.75 (0.002)
L28	-0.80 (0.16)	-1.90 (0.01)	-2.17 (0.007)	-2.11 (0.008)
L38	-0.54 (0.29)	-0.40 (0.40)	-2.37 (0.004)	-3.09 (0.001)
L39	-0.48 (0.33)	-0.35 (0.45)	-2.05 (0.009)	-2.79 (0.002)
L43	-0.71 (0.19)	-2.10 (0.001)	-2.08 (0.008)	-1.94 (0.012)
L48	-1.54 (0.03)	-0.74 (0.18)	-2.64 (0.002)	-1.79 (0.016)
L51	-1.73 (0.02)	-1.51 (0.03)	-2.81 (0.002)	-3.02 (0.001)
L61	-0.25 (0.56)	-3.01 (0.001)	-4.00 (0.0001)	-
L69	-2.18 (0.01)	-1.39 (0.04)	-2.31 (0.005)	-2.73 (0.002)
L77	-2.02 (0.01)	-0.84 (0.14)	-2.80 (0.002)	-2.60 (0.003)
Mean	-1.06 (0.21)	-1.34 (0.16)	-2.45 (0.006)	-2.56 (0.008)
Year		<.001		
Genotype		<.001		
Misting		0.170		
Year*Genotype		0.004		
Year*Misting		0.543		
Genotype*Misting		0.205		
Year*Genotype*Misting		0.925		
LSD (5%)		1.77		
CV (%)		59.3		

Back transformed means in parenthesis

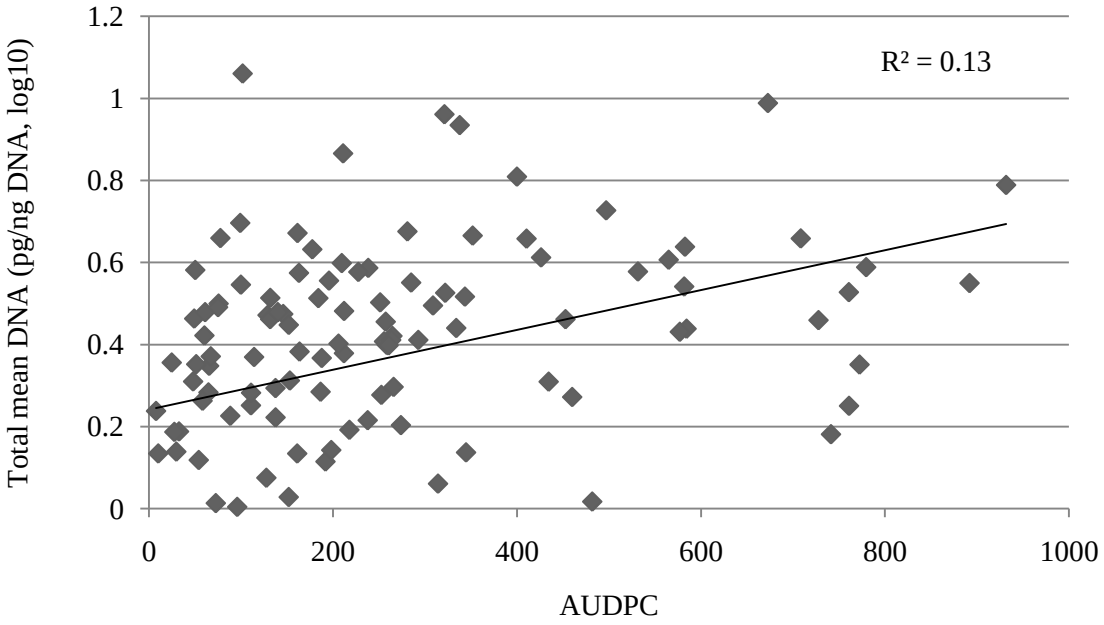

Figure 5.4. Regression between AUDPC vs total mean FHB pathogen DNA content in harvested grain from the 2012 field experiment.

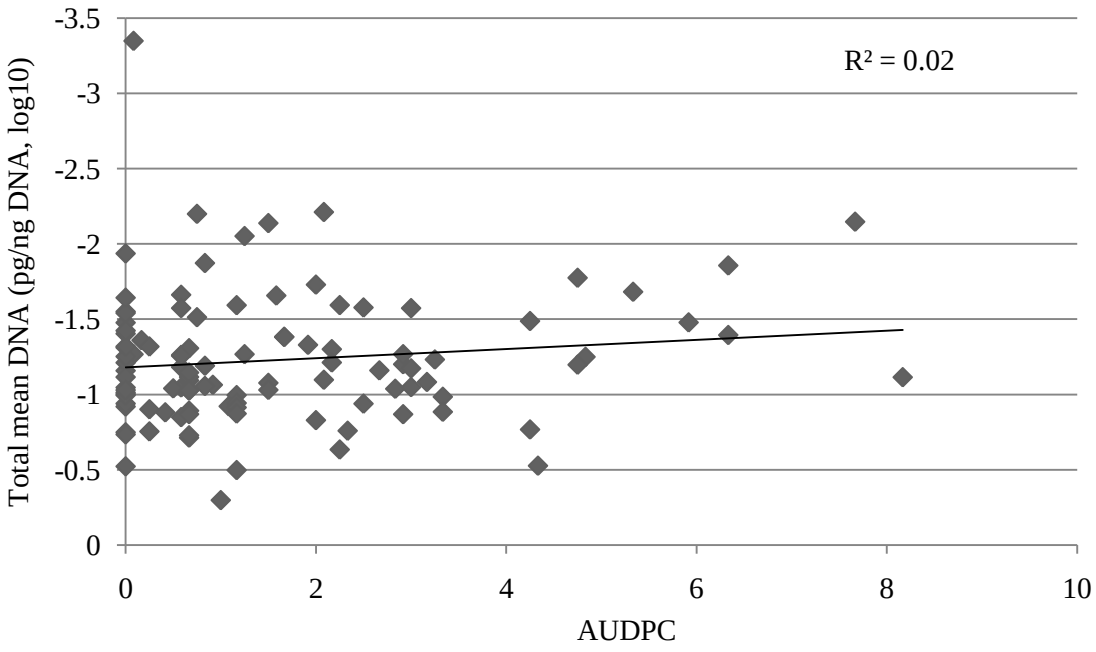

Figure 5.5. Regression between AUDPC vs total mean FHB pathogen DNA content in harvested grain from the 2013 field experiment.

5.4.3 Physiological traits at GS39

In general, the 21 wheat genotypes showed wide variation in physiological traits at GS39 and interactions (P<0.001) were present between year*genotype for most of the traits (Table 5.8).

Mean plant establishment ranged between 207 plants/m^2 and 293 plants/m^2 in 2012, however much more variation in plant establishment was present in the 2013 experiment with means ranging from 136 plants/m^2 to 439 plants/m^2. The much larger variation in plant number in 2013 than in 2012 is the likely cause of the year*genotype interaction (P<0.001).

Further key differences between genotypes included flag leaf height, with the shortest height in both 2012 and 2013 of 48.44 and 47.49 cm, respectively, for L18. L77 and Frontana had the tallest flag leaf heights in 2012 and 2013, respectively. Significant differences (P<0.001) were present between year, with mean flag leaf heights of 75.25cm and 65.76 in 2012 and 2013, respectively. The number of fertile shoots per plant ranged between 2.24 – 4.20 in 2012, and between 2.50 – 7.20 in 2013, for which a year*genotype interaction (<0.001) was present. This interaction was likely caused by the much larger variation in establishment experienced during the 2013 experiment.

Significant differences (P<0.001) were present between years for both flag leaf length and flag leaf width, being larger in 2012 than in 2013, and this was reflected in mean leaf fresh weight and area data between years (Table 5.8). The UK elite genotypes, Ambrosia and Claire had consistently greater leaf fresh weight and areas than the DH genotypes, however Rialto tended to overlap with the DH genotypes. Total plot fresh weight was significantly greater in 2013 than 2012, with UK elite genotypes having consistently greater fresh weights than the DH genotypes.

Table 5.8. Genotypic and year differences in canopy traits measured at GS39 in the 2012 and 2013 field experiments *(continued)*

	Plant number (m^2)		Fertile shoots (m^2)		Fertile shoots per plant		Flag leaf height (cm)		Flag leaf length (cm)		Flag leaf width (cm)	
	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
Ambrosia	259.2	135.8	484.5	422.4	3.80	7.20	67.31	56.98	21.56	19.35	1.92	2.00
Claire	259.2	438.6	461.6	550.2	3.57	2.50	72.15	60.18	22.26	15.02	1.95	1.55
Rialto	235.2	292.0	413.8	484.5	3.47	3.40	80.94	66.38	23.47	14.45	2.01	1.69
Frontana	236.0	327.6	331.4	460.6	2.77	2.85	79.88	83.4	25.86	16.49	1.71	1.22
Sumai-3	293.2	342.0	390.2	521.7	2.67	3.05	71.11	64.94	27.66	20.43	1.70	1.34
L8	214.6	233.2	447.3	404.5	4.20	3.67	72.49	65.76	25.23	17.42	1.99	1.63
L14	220.6	242.6	422.1	403.8	3.86	3.34	75.55	53.75	23.77	17.97	1.84	1.60
L15	234.2	162.2	381.3	315.7	3.26	4.10	87.64	66.21	26.73	20.26	2.02	1.91
L16	206.8	229.2	288.4	389.3	2.81	3.37	67.20	59.75	24.29	18.25	1.92	1.60
L18	291.2	219.8	312.2	376.3	2.24	3.74	48.44	47.49	24.00	17.20	1.75	1.61
L21	229.2	245.2	365.6	466.2	3.24	3.77	93.67	76.16	24.55	18.54	1.89	1.53
L24	247.2	281.2	433.3	444.3	3.54	3.21	71.48	62.02	22.63	14.49	1.76	1.36
L28	228.0	242.6	451.9	436.2	3.94	3.59	72.70	62.91	24.05	16.95	1.86	1.47
L38	253.2	200.0	451.4	498.2	3.57	4.97	74.13	67.60	22.02	15.70	1.54	1.30
L39	249.2	204.4	422.4	446.8	3.44	4.30	60.45	54.62	23.64	18.31	2.00	1.63
L43	217.2	136.0	364.1	348.4	3.34	5.27	84.66	62.26	25.10	20.30	1.96	1.75
L48	255.2	292.0	460.9	474.1	3.61	3.20	54.83	53.21	23.29	16.39	1.98	1.50
L51	271.2	262.6	404.2	438.0	3.00	3.40	71.57	63.65	23.04	16.09	2.13	1.73
L61	214.6	218.8	356.1	311.1	3.34	3.37	103.6	74.25	25.61	19.19	1.75	1.64
L69	266.6	242.8	426.4	433.4	3.20	3.45	69.46	61.13	25.14	17.17	1.77	1.53
L77	218.0	215.0	273.4	388.1	2.52	4.02	101.05	81.29	22.26	18.86	1.89	1.68
Mean	242.8	245.9	397.3	429.2	3.30	3.80	75.25	64	24.10	17.56	1.87	1.58
Year	0.351		0.009		<.001		<.001		<.001		<.001	
Genotype	<.001		<.001		<.001		<.001		<.001		<.001	
Year*Genotype	<.001		0.256		<.001		<.001		0.006		<.001	
LSD (5%)	57.4		112.6		0.91		6.09		2.11		0.11	
CV (%)	19.53		22.84		21.74		7.33		8.48		5.22	

Table 5.8. Genotypic and year differences in canopy traits measured at GS39 in the 2012 and 2013 field experiments *(continued)*

	Total fresh weight (g/m^2)		Flag leaf fresh weight (g/m^2)		Second leaf fresh weight (g/m^2)		Remaining leaf fresh weight (g/m^2)		Total leaf fresh weight (g/m^2)		Flag leaf area (cm^2/m^2)	
	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
Ambrosia	4249	4407	241.2	291.8	316.6	296.8	600.1	552.0	1158.0	1140.4	12524	13368
Claire	4291	4735	268.3	207.2	307.1	221.6	526.7	560.8	1102.0	989.6	13864	8644
Rialto	4004	4570	218.5	179.9	267.6	210.6	488.0	509.6	974.0	900.0	11512	8332
Frontana	2930	3644	194.3	92.9	192.1	128.4	326.2	227.8	712.4	449.2	8976	4856
Sumai-3	3326	4037	233.4	162.2	246.7	168.8	346.2	305.0	826.4	636.0	8824	9040
L8	3341	4111	208.6	181.2	249.3	195.0	420.5	395.9	878.4	772.0	9776	8820
L14	3128	3347	207.2	193.9	201.3	185.6	309.5	323.8	718.0	703.2	10784	8624
L15	3783	3705	202.8	220.5	273.5	225.8	488.3	381.1	964.4	827.2	8900	9704
L16	2641	3634	196.6	168.3	206.0	175.1	292.3	349.0	694.8	692.4	9080	8288
L18	2882	2528	216.1	134.7	224.6	134.7	369.2	211.8	810.0	481.2	9060	6368
L21	3486	3947	185.6	165.4	215.7	171.7	362.6	335.2	764.0	672.4	8956	7836
L24	3621	3566	196.0	107.4	275.6	148.4	470.2	352.0	941.6	608.0	10708	5916
L28	3399	3467	228.8	149.4	272.1	170.1	421.8	313.1	922.8	632.8	11416	7692
L38	3278	3989	190.8	157.6	224.4	170.5	466.2	378.6	881.6	706.8	8464	6964
L39	3421	3534	240.5	215.8	289.5	216.6	462.8	344.9	992.8	777.2	11156	10232
L43	3528	3225	204.8	183.9	257.0	180.2	382.7	281.7	844.4	646.0	10196	8444
L48	3369	3179	276.7	165.0	270.0	160.0	318.6	294.8	865.2	620.0	12664	7844
L51	3444	3879	218.4	163.2	284.2	185.2	436.0	402.7	938.8	751.2	11496	7932
L61	3210	3762	164.0	150.2	192.8	166.8	380.6	359.2	737.2	676.4	7416	7404
L69	2994	3645	196.9	143.5	210.1	167.0	289.5	311.9	696.4	622.4	8180	7028
L77	3216	4118	150.8	155.7	192.4	169.3	368.4	389.4	711.6	714.4	7412	7248
Mean	3407	3763	211.4	170.9	246.1	183.2	406.0	361.0	863.6	715.2	10065	8123
Year	<.001		<.001		<.001		<.001		<.001		<.001	
Genotype	<.001		<.001		<.001		<.001		<.001		<.001	
Year*Genotype	0.037		<.001		0.001		0.004		0.010		<.001	
LSD (5%)	617		35.9		40.2		82.4		146.8		1,804	
CV (%)	14.39		15.69		15.63		17.95		15.53		16.58	

Table 5.8. Genotypic and year differences in canopy traits measured at GS39 in the 2012 and 2013 field experiments

	Second leaf area (cm^2/m^2)		All remaining leaf area (cm^2/m^2)		Total leaf area (cm^2/m^2		Stem fresh weight (g/m^2)	
	2012	2013	2012	2013	2012	2013	2012	2013
Ambrosia	19060	14096	31088	23376	62676	50836	2128	2562
Claire	14984	10744	26440	24952	55288	44340	1921	2270
Rialto	12808	9644	21124	22276	45444	40252	2680	2584
Frontana	8640	7128	15576	11264	33192	23244	2450	3110
Sumai-3	9172	9208	14632	14628	32624	32876	2431	3443
L8	12368	9472	20220	17948	42364	36236	2268	2589
L14	10480	8472	14684	13368	35948	30464	1723	2323
L15	11784	10408	22584	16448	43272	36560	1753	1461
L16	9404	8808	13320	15664	31804	32760	2414	2564
L18	9424	6064	15944	8796	34424	21228	2295	2553
L21	11356	7568	17096	15608	37404	31012	2114	2222
L24	14236	7864	22756	16368	47700	30148	2161	2568
L28	13100	8900	19760	14828	44276	31420	1965	2378
L38	10332	9184	20032	15756	38828	31904	2526	2152
L39	13192	10004	20304	15104	44652	35344	2082	2073
L43	12388	8400	18516	11876	41100	28720	2116	2710
L48	12200	7480	14332	13280	39192	28604	2282	2509
L51	14216	9124	21508	17804	47220	34856	2034	2324
L61	9320	8288	17388	16096	34120	31784	2355	2843
L69	8780	8404	13040	13632	30000	29064	2022	2793
L77	9268	7672	17080	15560	33764	30480	2398	3217
Mean	11739	8902	18925	15935	40728	32959	2196	2536
Year	<.001		<.001		<.001		<.001	
Genotype	<.001		<.001		<.001		<.001	
Year*Genotype	0.028		0.017		0.026		<.001	
LSD (5%)	2,649		4,152		7,372		425	
CV (%)	21.40		19.86		16.69		15.02	

5.4.4 Principal component analysis of GS39 physiological traits, AUDPC and pathogen DNA

Principal component analysis was used to visualise relationships between AUDPC, GS39 physiological traits and grain pathogen DNA from the 2012 and 2013 field experiments, which accounted for 46.74 and 49.54% of the observed variation in each year, respectively.

Two predominant groups were present at GS39 in the 2012 experiment (Fig. 5.6). The first group consisted of AUDPC, flag width, fertile shoots per plant and multiple leaf area and fresh weight assessments, of which flag leaf length was negatively associated with this group. The second group consisted of all *Fusarium* and *Microdochium* DNA, excluding *F. langsethiae*, of which flag leaf height was negatively associated with this group.

Three predominant groups were present at GS39 in the 2013 experiment (Fig. 5.7). The first group consisted of all *Fusarium* and *Microdochium* spp. DNA, plus flag leaf length, flag leaf width and fertile shoots per plant. The second group consisted of all leaf area and fresh weight assessments, however this group showed considerable spread. The third group was showed a general negative relationship to the first group and consisted of AUDPC, fertile shoots per m^2, plant number per m^2 and flag height.

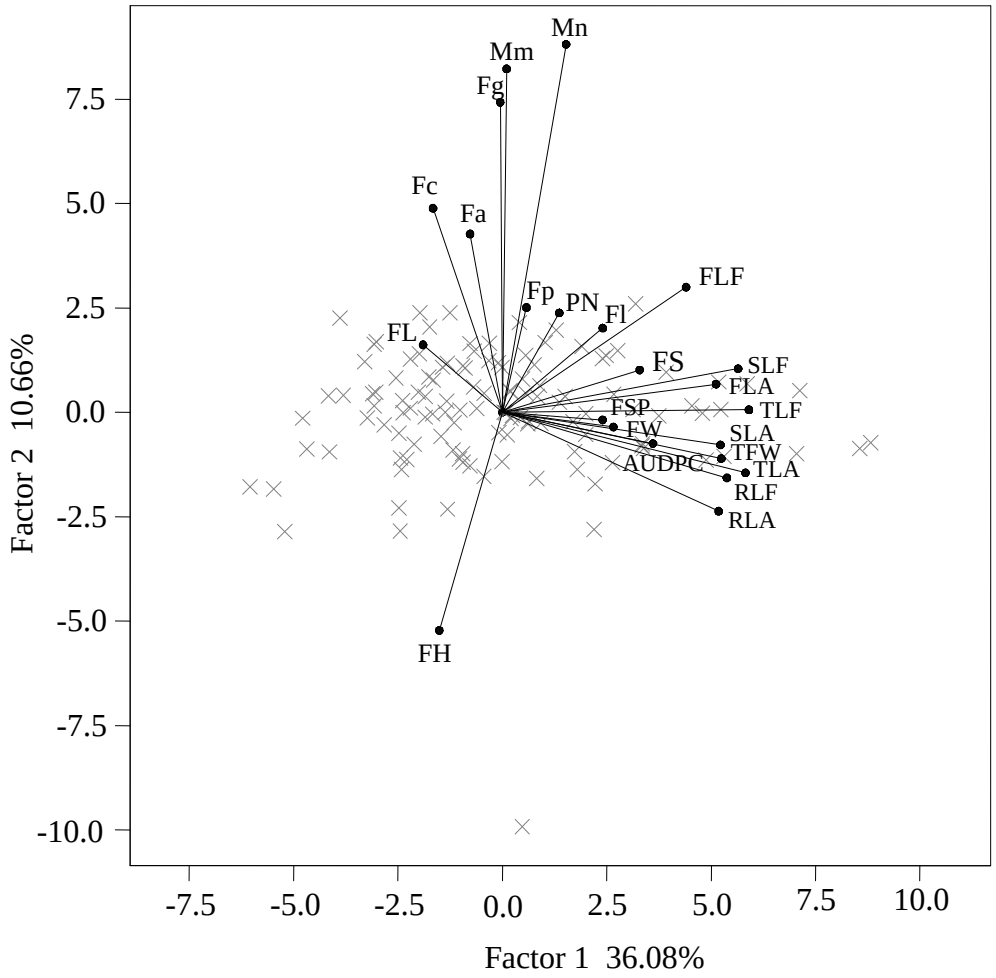

Figure 5.6. Biplot of the principal component analysis of the AUDPC, GS39 physiological traits and quantified fungal DNA from the 2012 field experiment. Key: AUDPC (Area Under Disease Progress Curve); Fa (*F. avenaceum* DNA); Fc (*F. culmorum* DNA); Fg (*F. graminearum* DNA); Fl (*F. langsethiae* DNA); FL (flag leaf length, cm); FLA (flag leaf area cm^2/m^2); FLF (flag leaf fresh weight, g/m^2); FH (flag leaf height, cm); Fp (*F. poae* DNA); FS (fertile shoots, m^2); FSP (fertile shoots per plant); FW (flag leaf width, cm); Mm (*M. majus DNA*); Mn (*M. nivale* DNA); PN (plant number, m^2); RLA (remaining leaf area, cm^2/m^2); RLF (remaining leaf fresh weight, g/m^2); SLA (second leaf area, cm^2/m^2); SLF (second leaf fresh weight, g/m^2); TFW (total fresh weight, g/m^2); TLA (total leaf area, cm^{-2}); TLF (total leaf fresh weight, cm^2).

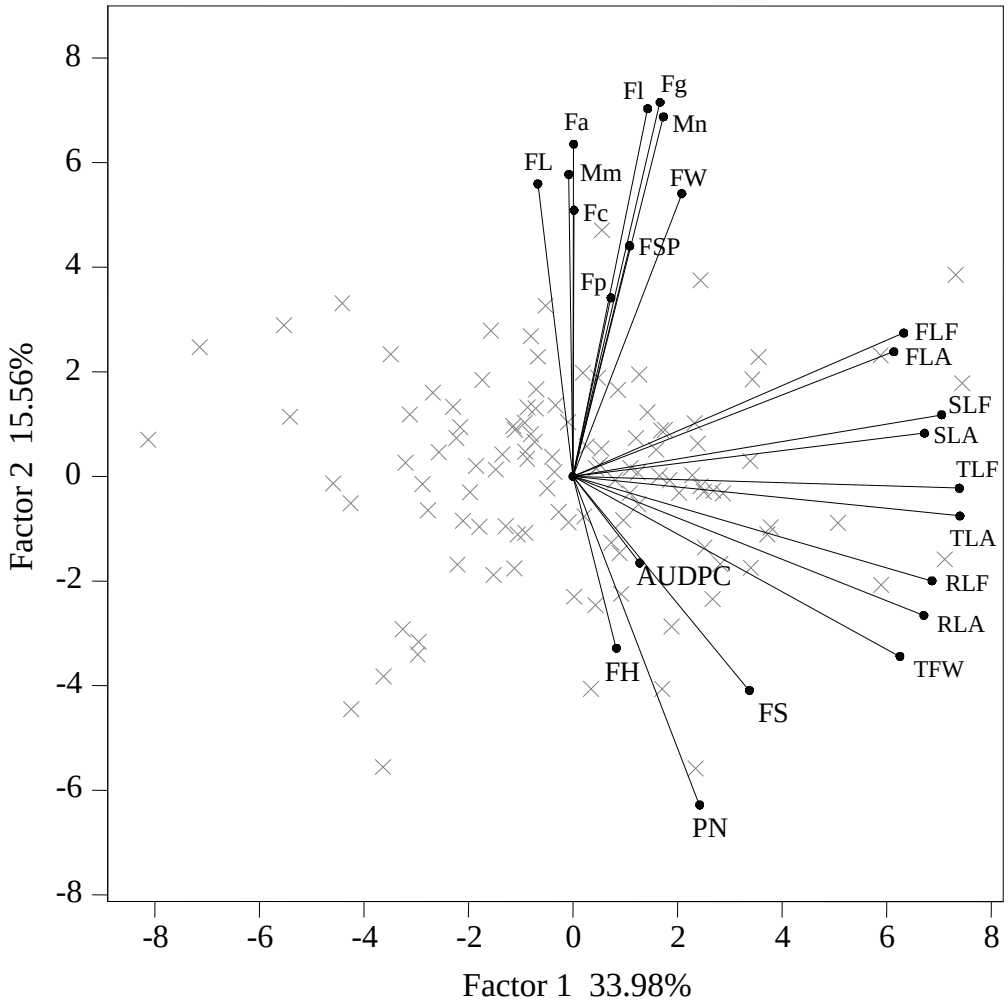

Figure 5.7. Biplot of the principal component analysis of the AUDPC, measured GS39 physiological traits and quantified fungal DNA from the 2013 field experiment. Key: AUDPC (Area Under Disease Progress Curve); Fa (*F. avenaceum* DNA); Fc (*F. culmorum* DNA); Fg (*F. graminearum* DNA); Fl (*F. langsethiae* DNA); FL (flag leaf length, cm); FLA (flag leaf area cm^2/m^2); FLF (flag leaf fresh weight, g/m^2); FH (flag leaf height, cm); Fp (*F. poae* DNA); FS (fertile shoots, m^2); FSP (fertile shoots per plant); FW (flag leaf width, cm); Mm (*M. majus DNA*); Mn (*M. nivale* DNA); PN (plant number, m^2); RLA (remaining leaf area, cm^2/m^2); RLF (remaining leaf fresh weight, g/m^2); SLA (second leaf area, cm^2/m^2); SLF (second leaf fresh weight, g/m^2); TFW (total fresh weight, g/m^2); TLA (total leaf area, cm^2); TLF (total leaf fresh weight, cm^2).

5.4.5 Multiple linear regression of AUDPC with GS39 physiological traits and grain pathogen DNA

The 2012 and 2013 data were analysed separately due to the large variation in the AUDPC between the years. In 2012, MLR revealed that total plot fresh weight had a positive, and flag leaf length a negative (P<0.001) relationship with AUDPC accounting for 36.9% of the variation (Table 5.9). In 2013, flag leaf height was the only trait that was significantly (P=0.012) related to AUDPC, accounting for 4.8% of the variation. Pathogen DNA did not significantly account for any additional variation in AUDPC in either year.

Table 5.9. Multiple linear regression models of the 2012 and 2013 field experiments accounting for variation in the AUDPC between genotypes using GS39 physiological traits and pathogen species DNA.

Model	Equation	R^2	P-value
AUDPC 2012	0.233 (TFW) - 15.25 (FL) - 171	36.9	<0.001
AUDPC 2013	4.29 – 0.0417 (FH)	4.8	0.012

Key: TFW = Total fresh weight (g/m^2); FL = Flag leaf length (cm); FH = Flag leaf height

5.4.6 Physiological traits at GS65

Differences between genotypes in the date that GS65 was reached, was spread across a 10 day period and resulted in assessment dates varying for each genotype.

Interactions (P<0.001) were detected between year*genotype for all canopy traits studied (Table 5.10). Plant populations at GS65 ranged between 198 – 377 plants/m^2 (Line 51 and Line 38, respectively) in 2012, and between 96 – 418 plants/m^2 (Line 61 and Sumai-3, respectively) in 2013, with 2013 exhibiting much greater variation than in 2012.

Flag leaf length and width were greater (P<0.001) in 2012 than 2013, but this difference was not reflected in the total leaf fresh weight or total leaf area which were non-significant between years. Plant height was greater (P<0.001) in 2012 than in 2013 (Table 5.10), with DH genotypes exhibiting far greater height variation (43.6 – 118.8 cm) than the UK elite genotypes (71.5 – 87.8 cm).

Mean fresh weight was greater (P<0.001) in 2013 than 2012 (3703 and 4219 g/m^2, respectively) although fresh weight variation in the 2013 experiment was very high (1888 – 6584 g/m^2). UK elite lines generally exhibited a greater total fresh weight than DH and control genotypes, but this was not always consistent, resulting in a year*genotype interaction (P<0.001).

Fractional interception, assessed only in 2012, showed differences between genotypes (P<0.001) ranging from 0.934 – 0.985 for Line 18 and Line 15, respectively.

A year*genotype interaction was present between all ear traits including ear length, spikelets per ear, spikelet density and awn length (Table 5.11). Wide variation was present between genotypes for all ear traits, with much wider variation in spikelet density (spikelets/ear) occurring within DH (20.1 – 26.0) and control genotypes (16.3 – 21.3) than

for the UK elite cultivars (22.1 – 26.8). Awn length differed greatly, with most genotypes exhibiting either an awnless (<1 cm) or awned (>3 cm) phenotype.

Table 5.10. Genotypic and year differences in canopy traits measured at GS65 in the 2012 and 2013 field experiments *(continued)*

	Plant number (m^2)		Fertile shoots (m^2)		Fertile shoots per plant		Flag leaf height (cm)		Flag leaf length (cm)		Flag leaf width (cm)	
	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
Ambrosia	324.2	200.0	418.9	449.6	2.67	4.50	71.12	64.70	21.72	18.55	1.90	1.95
Claire	247.0	404.2	406.9	539.1	3.35	2.70	72.81	69.66	22.80	14.63	1.93	1.55
Rialto	305.2	288.2	380.2	434.3	2.48	3.04	77.76	67.12	22.61	15.33	1.95	1.73
Frontana	316.2	386.8	393.0	454.8	2.50	2.40	91.95	80.82	23.93	16.78	1.50	1.28
Sumai-3	322.2	417.6	321.3	501.7	2.07	2.50	76.94	66.94	26.60	18.90	1.61	1.35
L8	317.8	142.2	407.5	238.1	2.58	3.13	76.59	64.95	23.85	16.63	1.95	1.55
L14	268.2	261.6	377.3	394.8	2.84	3.04	76.59	55.04	22.92	16.83	1.71	1.65
L15	198.8	175.8	210.8	451.3	1.90	5.07	99.21	78.95	25.93	19.25	1.96	1.86
L16	303.6	256.2	356.0	406.8	2.37	3.24	72.24	61.92	22.74	18.78	1.92	1.64
L18	284.2	161.6	352.3	252.7	2.50	3.34	52.33	37.51	23.03	17.36	1.74	1.54
L21	337.6	257.6	368.3	384.3	2.17	3.04	100.79	76.47	24.00	18.80	1.89	1.53
L24	288.2	268.2	334.5	370.0	2.34	2.77	80.75	57.84	20.30	11.56	1.71	1.38
L28	318.2	257.6	376.9	329.1	2.40	2.54	76.68	61.39	23.56	15.75	1.79	1.45
L38	376.8	233.6	461.2	518.9	2.44	4.54	84.23	71.45	21.41	14.16	1.54	1.25
L39	305.6	143.8	328.1	226.9	2.20	3.07	63.08	53.47	23.61	17.31	1.96	1.64
L43	307.6	248.2	366.5	385.6	2.40	3.10	90.17	65.09	24.14	19.36	1.83	1.65
L48	226.8	142.8	289.0	247.9	2.60	3.34	53.29	64.34	21.93	13.98	2.01	1.35
L51	354.2	286.8	381.3	357.6	2.14	2.54	76.07	62.54	23.84	16.85	2.13	1.79
L61	301.6	96.0	310.3	144.0	2.07	3.00	106.86	81.10	25.21	18.60	1.79	1.60
L69	314.0	290.8	345.2	410.4	2.20	2.84	78.78	63.49	24.27	16.00	1.59	1.43
L77	291.0	234.8	280.1	335.7	1.99	3.14	105.48	79.94	23.30	18.33	1.97	1.65
Mean	300.4	245.4	355.5	373.0	2.39	3.18	80.18	65.94	23.41	16.84	1.83	1.56
Year	<.001		0.017		<.001		<.001		<.001		<.001	
Genotype	<.001		<.001		<.001		<.001		<.001		<.001	
Year*Genotype	<.001		<.001		<.001		<.001		0.021		<.001	
LSD (5%)	76.3		104.9		0.67		6.91		2.16		0.13	
CV (%)	21.94		22.84		19.81		7.63		8.61		6.36	

Table 5.10. Genotypic and year differences in canopy traits measured at GS65 in the 2012 and 2013 field experiments *(continued)*

	Plant height (cm)		Total fresh weight (g/m^2)		Flag leaf fresh weight (g/m^2)		Second leaf fresh weight (g/m^2)		Remaining leaf fresh weight (g/m^2)		Total leaf fresh weight (g/m^2)	
	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
Ambrosia	78.67	71.45	4081	6584	195.6	320.0	217.6	303.2	412.8	445.5	826.0	1068.8
Claire	86.98	78.92	3419	6039	169.8	213.2	166.4	225.2	183.6	428.2	520.0	866.8
Rialto	87.78	78.20	3627	5548	186.1	202.2	189.6	207.4	287.4	500.5	663.2	910.0
Frontana	120.29	100.88	2989	4579	153.4	142.4	137.4	168.8	134.7	296.4	425.6	607.6
Sumai-3	93.89	83.23	3367	4810	173.2	175.7	171.0	188.2	177.9	350.6	522.0	714.4
L8	86.07	78.07	4286	2406	225.2	93.6	244.3	97.2	309.8	208.8	779.6	399.6
L14	82.68	63.62	3696	3468	194.8	202.0	202.1	170.0	243.4	296.0	640.0	668.0
L15	106.69	89.50	4368	5861	223.0	252.6	251.4	236.4	402.3	346.7	876.8	835.6
L16	83.87	69.55	3244	4144	198.2	223.0	206.0	195.5	237.3	346.5	641.6	764.8
L18	57.13	43.62	3192	2093	225.1	120.6	200.0	113.2	179.4	137.8	604.4	371.6
L21	111.72	89.75	4256	4790	200.1	205.4	230.9	196.2	359.3	388.8	790.4	790.4
L24	91.00	74.63	4171	3969	189.2	113.0	262.4	147.4	388.6	335.6	840.4	596.0
L28	92.20	73.32	3893	3627	217.6	135.6	224.1	160.4	286.1	350.4	727.6	646.4
L38	93.95	86.50	4491	4920	192.6	156.0	206.1	155.6	348.3	336.2	747.2	648.0
L39	74.49	63.82	3751	2597	209.5	125.8	243.5	115.5	371.1	281.1	824.0	522.4
L43	104.75	78.52	4138	4515	198.8	214.0	236.5	206.6	309.3	279.4	744.4	700.0
L48	56.55	76.12	2757	2469	207.7	96.0	188.4	92.2	164.4	200.8	560.4	388.8
L51	86.22	75.33	3571	4819	180.8	178.0	217.6	195.4	283.7	392.5	682.0	766.0
L61	118.75	92.60	3661	1888	163.6	70.4	160.7	68.8	307.6	150.4	632.0	289.6
L69	93.97	75.72	2972	4479	122.7	163.0	123.6	184.2	99.0	333.8	345.2	681.2
L77	115.02	93.12	3838	4999	146.4	185.9	167.5	181.5	342.6	415.0	656.4	782.4
Mean	91.56	77.93	3703	4219	189.2	170.9	202.3	171.8	277.6	324.8	669.0	667.5
Year	<.001		<.001		<.001		<.001		<.001		0.879	
Genotype	<.001		<.001		<.001		<.001		<.001		<.001	
Year*Genotype	<.001		<.001		<.001		<.001		<.001		<.001	
LSD (5%)	7.11		732		35.2		37.8		70.7		191.8	
CV (%)	6.76		14.8		22.76		23.40		27.16		22.95	

Table 5.10. Genotypic and year differences in canopy traits measured at GS65 in the 2012 and 2013 field experiments *(continued)*

	Flag leaf area (cm^2/m^2)		Second leaf area (cm^2/m^2)		All remaining leaf area (cm^2/m^2)		Total leaf area (cm^2/m^2)		Stem fresh weight (g/m^2)		Ears fresh weight (g/m^2)	
	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
Ambrosia	10660	15000	11580	14096	20376	19880	42616	48976	2404	3446	125.8	344.8
Claire	7540	10564	6332	11316	10408	20336	24280	42216	1927	3450	177.7	397.8
Rialto	9140	9568	9576	9656	14856	21676	33572	40896	2160	3527	133.2	255.6
Frontana	8580	8408	7336	9392	6948	14688	22864	32484	2067	3322	99.7	155.3
Sumai-3	9360	10208	8724	10104	9164	16500	27244	36808	2045	3222	135.9	184.3
L8	10564	4692	11572	4728	14988	9076	37124	18496	2549	1497	133.7	123.1
L14	10564	9736	9604	7928	11904	12884	32072	30548	2150	2092	129.6	153.1
L15	11104	11512	11592	10728	21300	14444	44000	36680	2774	3548	120.7	336.0
L16	9812	10716	9820	9696	12152	14988	31784	35400	1951	2608	91.4	168.4
L18	10088	5628	8772	4888	8416	6556	27276	17068	1684	1146	165.6	132.1
L21	9604	10016	10880	9356	15796	16608	36280	35980	3168	3149	136.7	188.7
L24	10816	6240	13484	7816	19400	15680	43696	29732	2560	2595	136.3	172.0
L28	10696	7232	11528	8024	13736	15796	35960	31048	2268	2360	123.9	135.5
L38	8300	7080	9564	7320	13996	15100	31860	29500	2774	3120	127.9	224.5
L39	9916	5904	11232	7344	15744	8880	36892	22132	2023	1500	150.7	151.3
L43	10096	10524	12096	10000	17728	14944	39916	35468	2698	2928	130.1	189.9
L48	9096	4464	8608	4380	8136	8576	25840	17424	1472	1581	122.7	96.5
L51	9156	8960	10724	9684	14144	17308	34024	35952	2021	3034	114.7	237.5
L61	7436	3424	8028	3248	17552	6624	33016	13296	2348	1312	89.3	99.2
L69	6196	8556	6248	9284	4836	15200	17276	33040	1882	2908	136.1	194.3
L77	6636	9360	8220	8784	15464	16084	30320	34224	2615	3426	94.0	175.8
Mean	9303	8466	9787	8465	13669	14373	32758	31303	2264	2656	127.4	196.0
Year	0.003		<.001		0.086		0.248		<.001		<.001	
Genotype	<.001		<.001		<.001		<.001		<.001		<.001	
Year*Genotype	<.001		<.001		<.001		<.001		<.001		<.001	
LSD (5%)	2,549		1,875		5,232		9,448		486		31.9	
CV (%)	22.96		23.77		29.71		23.54		15.74		15.9	

Table 5.10. Genotypic and year differences in canopy traits measured at GS65 in the 2012 field experiment.

	Fractional interception
	2012
Ambrosia	0.980
Claire	0.966
Rialto	0.971
Frontana	0.948
Sumai-3	0.962
L8	0.970
L14	0.955
L15	0.985
L16	0.944
L18	0.934
L21	0.963
L24	0.966
L28	0.959
L38	0.957
L39	0.962
L43	0.981
L48	0.943
L51	0.982
L61	0.974
L69	0.963
L77	0.976
Mean	0.964
Genotype	<0.001
LSD (5%)	0.015
CV (%)	1.27

Table 5.11. Genotypic and year differences in ear traits measured at GS65 in the 2012 and 2013 field experiments

	Ear length (cm)		Spikelets per ear		Spikelet density (spikelets / cm)		Awn length (cm)	
	2012	2013	2012	2013	2012	2013	2012	2013
Ambrosia	10.66	10.80	22.87	22.10	2.15	2.05	0.93	0.55
Claire	12.31	11.23	26.80	25.50	2.18	2.27	0.60	0.40
Rialto	11.46	11.27	26.44	24.97	2.30	2.22	0.62	0.38
Frontana	10.44	9.60	16.53	16.13	1.59	1.68	3.55	5.38
Sumai-3	11.00	11.00	20.63	21.27	1.88	1.94	4.00	6.05
L8	11.20	10.69	22.30	21.39	1.99	2.01	2.65	5.57
L14	11.26	10.48	23.10	21.77	2.05	2.08	0.55	0.28
L15	13.24	12.96	25.87	24.52	1.96	1.89	0.63	0.61
L16	10.28	10.22	21.60	20.30	2.10	1.99	1.10	0.53
L18	10.73	9.63	20.07	21.10	1.87	2.19	3.70	5.15
L21	13.14	12.73	24.87	22.67	1.89	1.78	0.84	0.42
L24	10.56	9.43	25.13	21.90	2.39	2.32	1.32	0.40
L28	10.94	9.83	22.20	21.37	2.05	2.17	3.26	4.68
L38	11.46	11.02	25.80	23.30	2.25	2.12	3.51	5.55
L39	14.11	12.48	25.44	22.40	1.80	1.79	4.77	6.80
L43	12.47	12.12	24.63	21.97	1.99	1.81	3.68	7.70
L48	11.67	11.30	23.12	23.40	1.98	2.08	3.30	5.53
L51	12.89	11.73	23.10	21.07	1.79	1.80	3.65	5.82
L61	12.85	13.70	23.10	23.80	1.80	1.74	2.20	6.90
L69	10.79	10.17	22.56	21.43	2.10	2.11	2.85	4.75
L77	12.38	12.08	26.00	23.13	2.10	1.92	1.58	0.50
Mean	11.70	11.20	23.40	22.17	2.01	2.00	2.35	3.52
Year	<.001		<.001		0.625		<.001	
Genotype	<.001		<.001		<.001		<.001	
Year*Genotype	0.022		<.001		<.001		<.001	
LSD (5%)	0.75		1.28		0.13		0.78	
CV (%)	5.28		4.53		5.04		21.75	

5.4.7 Principal component analysis of GS65 physiological traits, AUDPC and pathogen DNA

Principal component analysis was used to quantify relationships between AUDPC, GS65 physiological traits and grain pathogen DNA from the 2012 and 2013 field experiments, which accounted for 40.26 and 49.49% of the observed variation in each year, respectively. Four predominant groups were present in the 2012 biplot (Fig. 5.8). The first and largest grouping was between flag leaf, second leaf, remaining leaf and total leaf traits, in which each leaf assessment for fresh weight and area were tightly grouped together. The second grouping was between AUDPC and flag leaf width, which were associated with *F. langsethiae* DNA. The third group contained spikelets per ear, fractional interception, ear length, flag height, plant height and was associated with *M. majus* DNA. A fourth group consisted on awn length, which was associated with *F. culmorum* and *F. poae* DNA. A fifth group contained fertile shoots/m^2, fertile shoots per plant, *F. avenaceum* and *M. nivale* DNA, of which flag leaf length was negatively associated.

Four predominant groups were formed within the 2013 biplot (Fig. 5.9). The first group contained AUDPC and ear length, flag leaf height, flag leaf width, plant height and flag leaf length, and these traits were associated with *F. graminearum, F. avenaceum, M. majus* and *M. nivale* DNA and negatively associated with *F. poae* DNA. A second group contained spikelets per ear and plant number per m^2. A third group contained all leaf fresh weight and area traits, as well as fertile shoots per m^2. Group four contained spikelet density and fertile shoots per plant, and these traits were additionally associated with *F. langsethiae* and *F. poae* DNA. Traits within the fourth group were negatively associated with *F. culmorum* DNA. Awn length was negatively associated with traits in the second group.

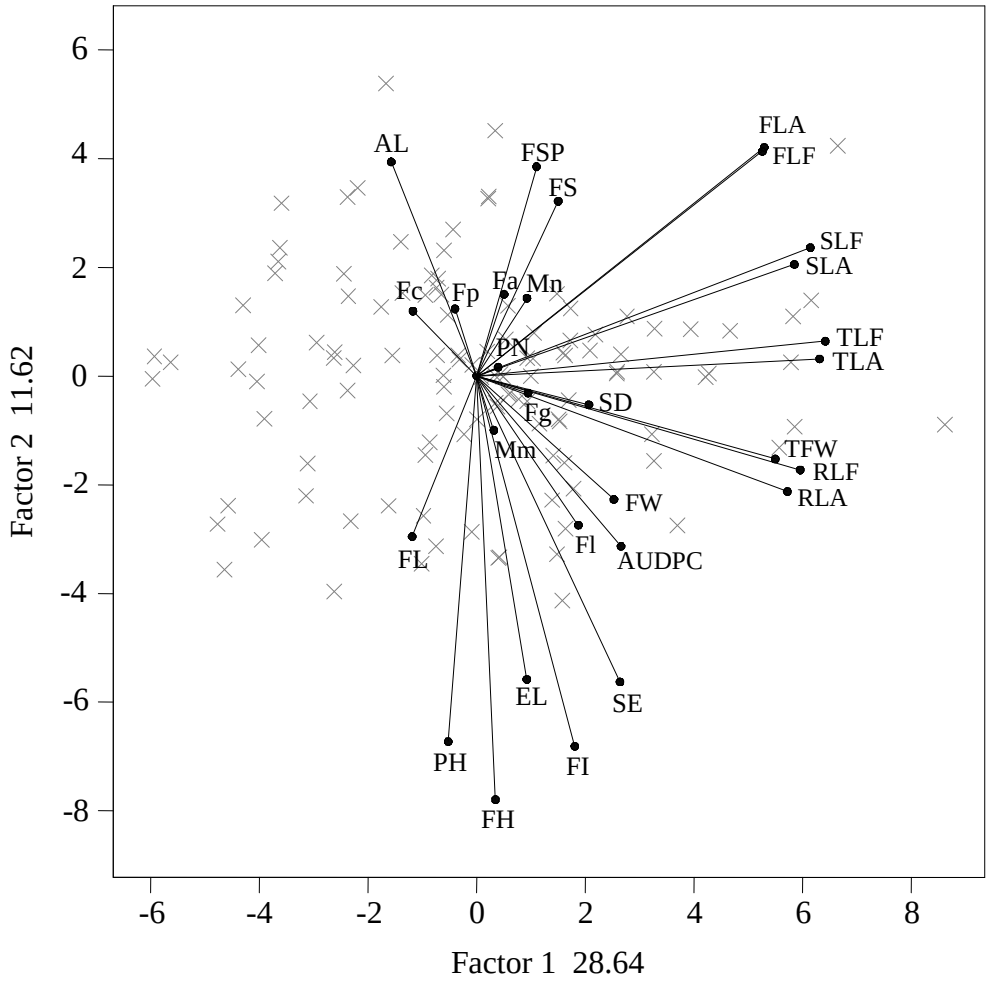

Figure 5.8. Biplot of the principal component analysis of the AUDPC, measured GS65 physiological traits and quantified fungal DNA from the 2012 field experiments. Key: AUDPC (Area Under Disease Progress Curve); AL (awn length, cm); EL (ear length, cm); Fa (*F. avenaceum* DNA); Fc (*F. culmorum* DNA), Fg (*F. graminearum* DNA); FH (Flag leaf height, cm); FI (Fractional interception); Fl (*F. langsethiae* DNA); FL (flag leaf length, cm); FLA (flag leaf area cm^2/m^2); FLF (flag leaf fresh weight, g/m^2); Fp (*F. poae* DNA); FS (fertile shoots, m^2); FSP (fertile shoots per plant); FW (flag leaf width, cm); Mm (*M. majus* DNA); Mn (*M. nivale* DNA); PH (plant height, cm); PN (plant number, m^2); SE (spikelets per ear); SD (spikelet density); RLA (remaining leaf area, cm^2/m^2); RLF (remaining leaf fresh weight, g/m^2); SLA (second leaf area, cm^2/m^2); SLF (second leaf fresh weight, g/m^2); TFW (total fresh weight, g/m^2); TLA (total leaf area, cm^2); TLF (total fresh weight, g/m^2).

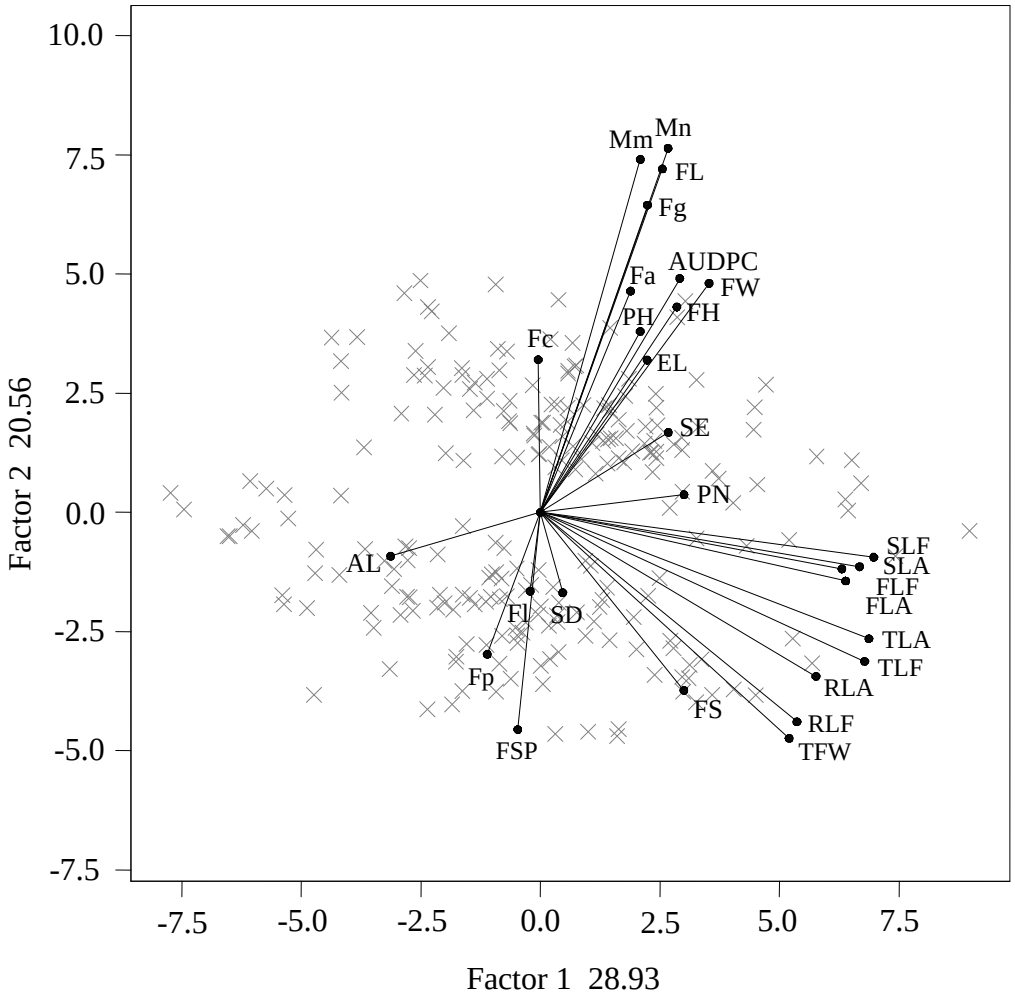

Figure 5.9. Biplot of the principal component analysis of the AUDPC, measured GS65 physiological traits and quantified fungal DNA from the 2013 field experiments. Key: AUDPC (Area Under Disease Progress Curve); AL (awn length, cm); EL (ear length, cm); Fa (*F. avenaceum* DNA); Fc (*F. culmorum* DNA), Fg (*F. graminearum* DNA); FH (Flag leaf height, cm); Fl (*F. langsethiae* DNA); FL (flag leaf length, cm); FLA (flag leaf area cm^2/m^2); FLF (flag leaf fresh weight, g/m^2); Fp (*F. poae* DNA); FS (fertile shoots, m^2); FSP (fertile shoots per plant); FW (flag leaf width, cm); Mm (*M. majus DNA*); Mn (*M. nivale* DNA); PH (plant height, cm); PN (plant number, m^2); SE (spikelets per ear); SD (spikelet density); RLA (remaining leaf area, cm^2/m^2); RLF (remaining leaf fresh weight, g/m^2); SLA (second leaf area, cm^2/m^2); SLF (second leaf fresh weight, g/m^2); TFW (total fresh weight, g/m^2); TLA (total leaf area, cm^2); TLF (total fresh weight, g/m^2).

5.4.8 Multiple linear regression of AUDPC with GS65 physiological traits and grain pathogen DNA

The two years' results were analysed separately due to the large variation in AUDPC. MLR accounted for 38.1% ($P<0.001$) of the variation in the AUDPC in 2012 being positively related to remaining leaf fresh weight and spikelets per ear, and negatively related to awn length (Table 5.12). MLR accounted for 29.3% ($P<0.001$) of the AUDPC variation in the 2013 field experiment, being negatively related to both plant height and awn length. Pathogen DNA was unable to significantly account for any additional variation in AUDPC in either year.

Table 5.12. Multiple linear regression models of the 2012 and 2013 field experiments accounting for variation in the AUDPC between genotypes using GS65 physiological traits and pathogen species DNA.

Model	Equation	R^2	P-value
AUDPC 2012	1.261 (RLF) + 22.46 (SE) - 55.9 (AL) - 227	38.1	<0.001
AUDPC 2013	6.124 - 0.0437 (PH) - 0.3146 (AL)	29.3	<0.001

Key: RLF = Remaining leaf fresh weight (g); SE = Spikelet number per ear; AL = Awn length (cm); PH = Plant height (cm).

5.4.9 Correlation between GS39 & GS65 physiological traits

Several physiological traits were measured at both GS39 and at GS65 to enable comparisons between the effects of each growth stage on FHB resistance. Correlation between each growth stage showed traits such as flag leaf length, width and plant height to be highly correlated ($P < 0.001$). The majority of remaining traits were poorly related between growth stages ($r < 0.70$) although most correlations were highly significant (Table 5.13).

Table 5.13. Correlation between physiological traits measured at both GS39 and at GS65 in the 2012 and 2013 field experiment

Plant number (m^2)	0.35^{***}
Fertile shoots (m^2)	0.14^{*}
Fertile shoots (per plant)	0.34^{***}
Flag leaf length (cm)	0.84^{***}
Flag leaf width (cm)	0.82^{***}
Flag leaf height (cm)	0.85^{***}
Total fresh weight (g/m^2)	0.45^{***}
Fresh weight of flag leaves on fertile shoots (g/m^2)	0.34^{***}
Fresh weight of second leaves on fertile shoots (g/m^2)	0.40^{***}
Fresh weight of all remaining leaf on fertile shoots (g/m^2)	0.37^{***}
Total fresh weight of all leaf on fertile shoots (g/m^2)	0.29^{***}
Flag leaf area of fertile shoots (cm^2/m^2)	0.23^{***}
Second leaf area of fertile shoots (cm^2/m^2)	0.39^{***}
All remaining leaf area of fertile shoots (cm^2/m^2)	0.37^{***}
Total leaf area (cm^2/m^2)	0.32^{***}
Fresh weight of all stems (g/m^2)	0.52^{***}

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

5.5 FIELD EXPERIMENT 2012 AND 2013 WEATHER DATA

Weather data from the University of Nottingham meteorological station, located adjacent to the trial site, was used to compare climatic conditions between the 2012 and 2013 field experiments. Due to the wide range of anthesis and maturity dates between the genotypes, weather data was taken from the date in which the first genotype reached anthesis (GS61), to the date which the last genotype reached maturity (GS91). The start of anthesis occurred on the 30 May and 10 June in 2012 and 2013 respectively, and the final harvest dates were 20 August and 23 of August in 2012 and 2013 respectively.

Rainfall patterns between years showed that the 2012 experiment received large quantities of rain over extended periods of the experiment, whereas the 2013 trial received significantly less rain, with a long dry period during mid to late grain filling (29 June – 22 July) (Fig. 5.10). In total over the period, the experiments received 260.4 mm and 161 mm of rain in 2012 and 2013, respectively. The 2012 season was exceptionally cool and wet. Cumulative relative humidity showed a linear progression in both 2012 and 2013 with humidity accumulating faster in 2012 than in 2013 (Fig. 5.11). Mean relative humidity was 83.6% and 76.1% in 2012 and 2013 respectively, with a percentage difference of 9.39%.

Clear differences in temperature between years were evident (Fig. 5.12), in which warmer temperatures were recorded in 2013 than in 2012. The cumulative graph shows the lines for 2012 and 2013 begin to converge, showing differences in temperature between years. Overall, mean temperatures were 15.2^{o}C and 16.9^{o}C in 2012 and 2013 respectively, with a percentage difference of 10.53%.

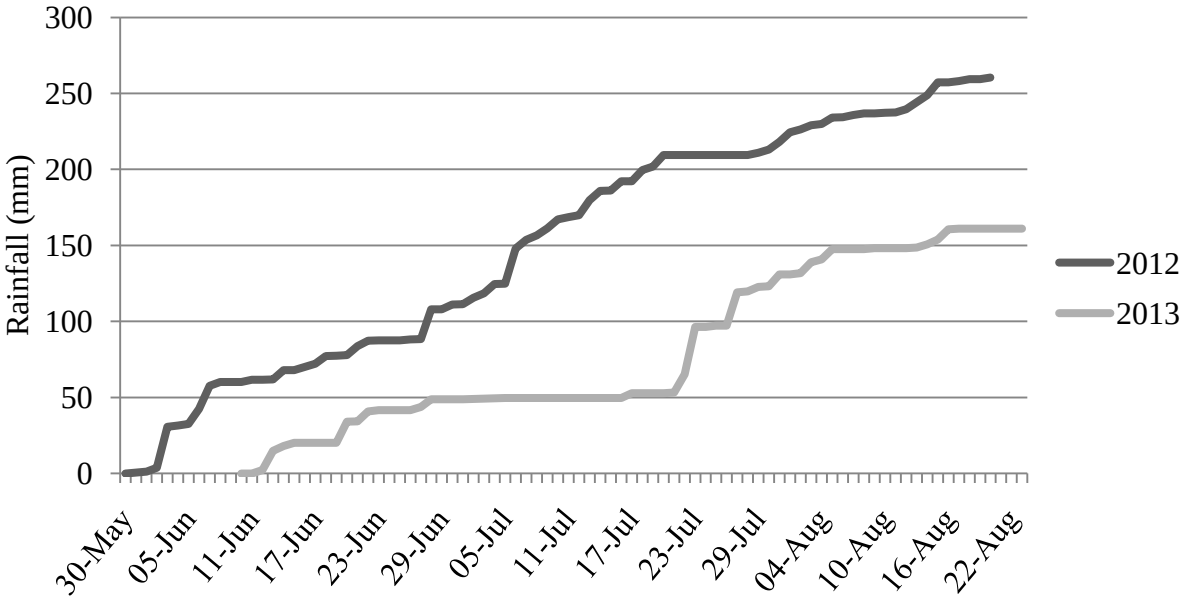

Figure 5.10. Cumulative rainfall between the start of anthesis and the end of harvest in the 2012 and 2013 field experiments.

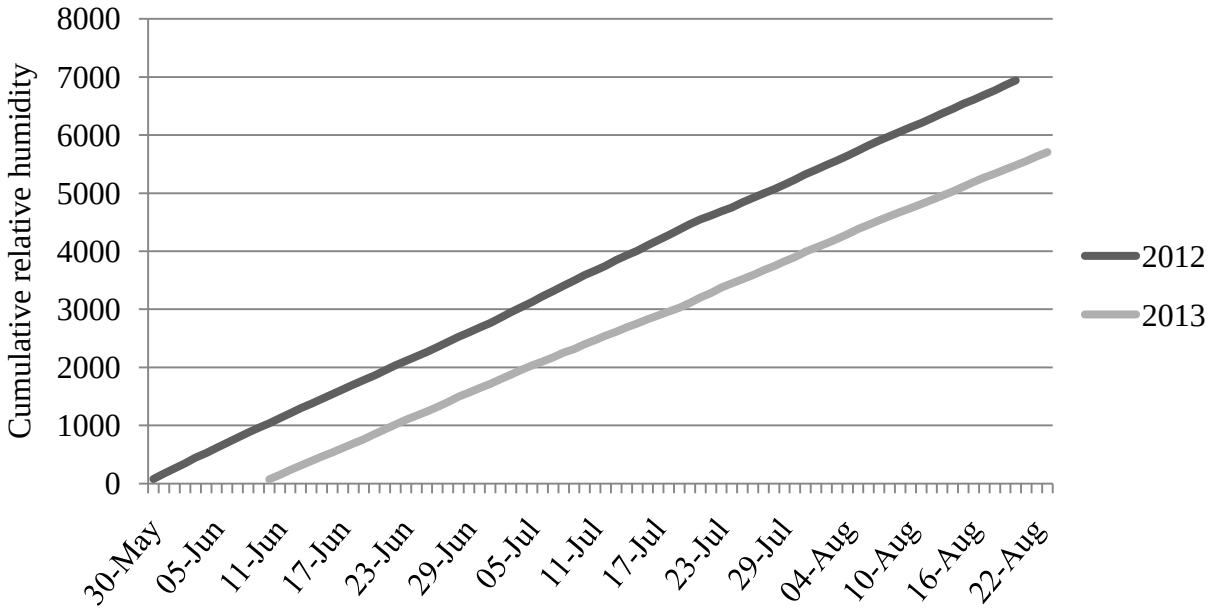

Figure 5.11. Cumulative relative humidity between the start of anthesis and the end of harvest in the 2012 and 2013 field experiments.

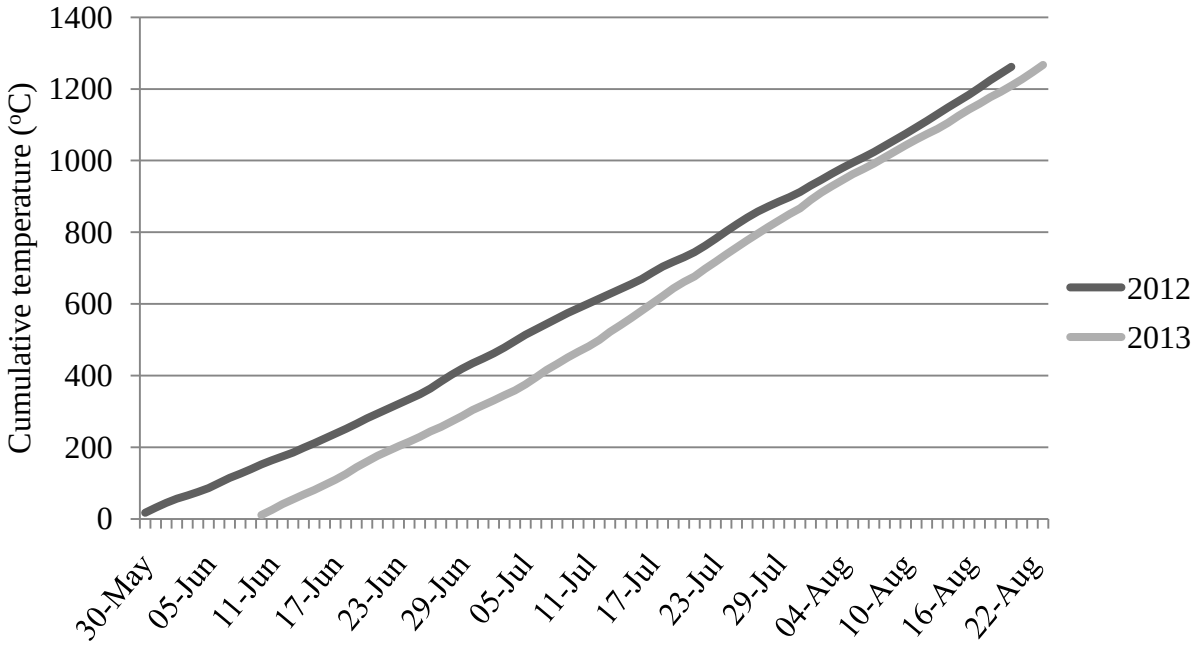

Figure 5.12. Cumulative temperature between the start of anthesis and the end of harvest in the 2012 and 2013 field experiments.

5.6 DISCUSSION

A selection of DH lines were used in these experiments to minimise the variation in height and flowering date between genotypes. This enhances the experiments sensitivity to detect physiological traits conferring passive resistance to FHB, by minimising the confounding effects of specific physiological traits that may have linkage effects. It is likely however, that elements of active resistance were still responsible for a proportion of the observed differences, partially due to the segregation of *Rht-B*1 and *Rht-D*1 genes that are present within the DH lines used during the experiments (Foulkes, pers comm), which have QTL overlapping with FHB susceptibility. Therefore, caution is required in the interpretation of results in this Chapter, as the elucidation of true passive resistance trait can only be made once each trait has been comparatively tested under spray inoculated glasshouse conditions.

Large differences in AUDPC scores were observed between the 2012 and 2013 field experiments, caused primarily by differences in environmental conditions, large amounts of summer rainfall and high relative humidity in the 2012 season compared to the 2013 season (Fig. 5.8 and 5.9). This rainfall led to lush plant growth and pre-flowering lodging in many of the taller DH genotypes, which was mitigated by staking up several plots to reduce further lodging, however in some instances this caused ears to bunch together and dry out slowly after rain. The effects of lodging on FHB epidemics in 2012 could potentially have been accounted for within the MLR if lodging scores had been recorded. The weather in the 2013 season was characterised by a dry period at the start of anthesis which limited the development of FHB. During disease scoring in 2013, it was noted that visual symptoms were few in number, the opposite situation to the previous year.

F. graminearum was the predominant pathogen quantified in both the 2012 and 2013 field experiments. However in 2012, large amounts of *M. nivale* DNA were also quantified (Fig. 5.7), with the presence of *M. nivale* most likely due to the cool temperatures and frequent rainfall experienced during anthesis (Xu *et al.* 2008). The presence of both *F. graminearum* and *M. majus* as the predominant pathogen species in 2012 is important, since the relative importance of splash or wind dispersal depends upon the species present within the pathogenic complex, and their capacity to produce ascospores (Champeil *et al.* 2004). The production of *F. graminearum* ascospores is dependent upon rainfall for sufficient soil moisture, although it is not clear if rainfall is critical for ascospores release (Xu, 2003). Due to the wet season experienced in 2012, the production of wind dispersed ascospores (Trail *et al.* 2005) by *F. graminearum* and *M. nivale* is likely to have occurred, and may have contributed to a significant proportion of the FHB inoculum in that year, however it is not known if ascospores release would have been triggered by this wet weather. Therefore conidia and ascospores produced by both *F. graminearum* and *M. nivale*, as the predominant FHB pathogen species, need to be considered as likely forms of inoculum present during the 2012 field experiment. In the 2013 field experiment, due to the dry summer season, the production of ascospores by the predominant pathogen *F. graminearum* is unlikely (Xu, 2003), as is the production of conidia, which are critically dependent on moisture as well as temperature (Xu, 2003). This perhaps partially suggests that the development of inoculum in 2013 was limited, and that FHB would generally have been low even if environmental conditions were conducive for FHB development during flowering. However these conditions would have favoured pathogens which prefer dry weather, indicating why *F. langsethiae* was the second most predominant pathogen in 2013. Interestingly, within the MLR and PCA, there was no consistent relationship between pathogen DNA and AUDPC.

Significant differences in AUDPC were not present between misted and non-misted plots within the field experiments. This was potentially due to the frequent rainfall in 2012 negating the effects of the misting treatment, although the misting treatment also failed to create differences in AUDPC in the 2013 experiment when dry weather prevailed during anthesis, despite being an excellent year for such differences to be expressed. There are a number of reasons why misting may have had no effect on FHB. Firstly, due to the absence of automation of the misting system, the experiment was thoroughly misted once a day every afternoon. This may have been insufficient when compared to He *et al.* (2014) which reported applying frequent misting for ten minutes every hour between 9am and 8pm each day to stimulate infection. Furthermore, since splash dispersal of FHB inoculum provides an important route to the ear (Jenkinson and Parry, 1994), it seems plausible that the overall dry season in 2013 resulted in fewer *Fusarium* spp. and *Microdochium* spp. spores being splash dispersed up through the canopy to the ear, therefore inoculum was not present to cause FHB, regardless of the presence of misting. Due to the lack of significant differences in AUDPC between misting treatments and absence of an interaction between misting*genotype, it was decided that analysis of the data using PCA and MLR would not separate between misted and non-misted treatments.

F. langsethiae DNA, detected at low concentration in both the 2012 and 2013 field experiments, was the only pathogen species for which misting created significant differences (P=0.026). These differences showed *F. langsethiae* DNA to increase within non-misted plots during each experimental year. This may have been due to *F. langsethiae* having a preference for dry conditions (Imathiu *et al.* 2013), therefore the misting treatment may have reduced *F. langsethiae* by creating an unfavourable environment for infection. It would have been interesting to have analysed the grain mycotoxin content of the 2012 experiment to assess the relationship between *F. langsethiae* and mycotoxin

accumulation in relation to misting, as it has been suggested that damp weather favourable to the development of fast-growing *Fusarium* spp. may result in reduced levels of HT2 + T2 toxin by out competing *F. langsethiae* and discouraging its growth (Imathiu *et al.* 2013).

Environmental conditions within each year can have a significant effect on the predominance and population dynamics of *Fusarium* spp. and *Microdochium* spp. For example, the development of *Microdochium* species is favoured by low temperatures and high rainfall during flowering, whereas the development of *Fusarium* species are generally favoured by high temperatures (Champeil *et al.* 2004). *Fusarium* and *Microdochium* species often form distinct groups, for example, Nielsen *et al.* (2014) found discrete grouping occurring between *F. graminearum* and *F. avenaceum, F. culmorum* and *F. poae, F.langsethiae* and *F.tricinctum,* and *M. majus* and *M. nivale,* in naturally infected barley samples grown in different regions of the UK. These groupings were suggested to be due to regional variation in environmental conditions in which *Microdochium* species were found in significantly higher concentrations than *Fusarium* species in the cool, wet environment of Scotland. The strongest grouping between species in the 2012 and 2013 field experiments was between *M. majus* and *M. nivale*, confirming the strong positive relationship between these species as previously described by Nielsen *et al.* (2014) and Xu *et al.* (2008). *F.graminearum* additionally co-occurred with *M. majus*, *M. nivale* and *F. avenaceum*. Negative correlations were detected between *F. poae,* and *M. majus* or *M. nivale*, confirming that these species are likely to be favoured by different environmental conditions, with *F. poae* preferring dry and warm conditions, and *Microdochium* spp. preferring wet and cool conditions (Parry *et al.* 1995; Xu *et al.* 2008).

Several canopy traits were related to FHB in the PCA and MLR analysis. The 2012 field experiments in 2012 should form the basis of this discussion due to the low AUDPC in the

2013 field experiment. In 2012, the physiological traits most consistently related to AUDPC across both growth stages was total fresh weight at GS39 and remaining leaf fresh weight at GS65, each having a significant positive relationship with AUDPC. This is the first study to report a relationship between crop fresh weight and FHB, although it is perhaps unlikely that this relationship is genuinely based upon fresh weight *per se*, being more likely due to other correlated canopy traits such as leaf area, which showed strong correlations with canopy fresh weight assessments in 2012 (Fig. 5.5 and 5.6). This positive relationship between canopy fresh weight and leaf area assessments was consistent in the 2013 experiment also, although no relationship between either trait and FHB is implied in 2013. Conversely however, both canopy fresh weight and leaf area assessments were included within the MLR, and despite the theorised relationship to leaf area, it was infact the fresh weight traits that were significantly related to AUDPC in the MLR, suggesting that the implied relationship with leaf area may not be clearly defined. The influence of leaf area ad canopy traits was further investigated in 2012 by assessing the development of canopy fractional interception between GS39 and GS65. Despite not accounting for AUDPC variation in the MLR, fractional interception was weakly positively related to AUDPC, although fractional interception itself was unrelated to leaf area traits, which may have been due to the arrangement of leaves within the canopy. Splash dispersal is the predominant route for conidia to reach the wheat ear (Jenkinson and Parry, 1994), therefore positive relationships between leaf area and AUDPC in 2012 is hypothesised to be caused by the additional canopy area providing an enhanced bridge for splash dispersed inoculum to be spread vertically between successive canopy layers. In 2012 this will have likely consisted of a mix of ascospores and conidia, as discussed previously. Despite the presence of ascospores, which can be forcibly ejected into the air (Trail *et al.* 2005), it is

likely that these canopy traits will still have acted as a ladder to the ear, since ascospores are still liable to splash dispersal.

Flag leaf height at GS39 and plant height at GS65, both accounted for significant variation ($P=0.012$ and $P<0.001$, respectively) in AUDPC in the 2013 field experiment, in which a negative relationship was observed. Flag leaf height at GS39 however accounted for only 4.8% of the AUDPC variation. Despite the low AUDPC in 2013, it seems that there was sufficient disease for this a negative relationship to be detected, concurring with many other studies which also reported negative relationships between plant height and FHB. From a passive disease resistance perspective, taller plants should help reduce FHB by increasing the distance inoculum has to be transported up the plant to the ear, in a similar manner as that shown by Shaw and Royle (1993) in which the dispersal of *Mycosphaerella graminicola* spores from the crop base reduced rapidly with height. However this negative relationship is now known to be caused by a genetic linkage between *Rht* dwarfing genes and FHB susceptibility (Srinivasachary *et al.* 2008). This negative relationship between plant height and FHB was however not detected in the 2012 experiment, perhaps due to the presence of lodging which may have negated any potential passive resistance influence of plant height, or led to an inaccurate assessment of each genotypes height, masking the true relationship. This is a plausible solution, as a significant year*genotype interaction ($P<0.001$) was present for plant height. This lack of relationship with plant height in 2012 may also have been partially due to the successful standardisation of DH lines used within the experiments, which aimed to limit the inclusion of linkage effects with plant height, which is a confounding factor in the identification of novel traits.

Flag leaf length at GS39 in the 2012 field experiment was negatively related to AUDPC. Longer flag leaves may be a passive resistance trait, however due to flag leaf length being unrelated to AUDPC in all other MLR, it seems that this relationship may be an artefact of

flag leaf lengths strong negative association with leaf fresh weight and area at GS39 in 2012, which were themselves positively related to AUDPC. It is interesting to note that flag leaf length was strongly grouped together with pathogen DNA in the 2013 experiment (Fig. 5.5).

Multiple ear traits were significantly related to AUDPC in both 2012 and 2013 field experiments, although relationships present in the 2012 experiment should carry more weight than the 2013 field experiment, due to the low AUDPC pressure in 2013. Awn length at GS65 was the most consistently significant ear trait between years, accounting for variation in AUDPC during both 2012 and 2013 field experiments, in which a negative relationship was identified. Similar negative relationships between awn length and FHB have been reported previously (Buerstmayr *et al.* 2000; Ban and Suenaga. 2000), although conflicting reports have also shown awn length to be both positively (Somers *et al.* 2003; Lui *et al.* 2013) and non-related (Buerstmayr *et al.* 2000; Buerstmayr *et al.* 2002; Hori *et al.* 2005; Liu *et al.* 2007) to FHB. From a passive resistance perspective, a negative relationship between AUDPC and awn length indicates that longer awns are able to create a barrier from the deposition of spores on the ear. It is perhaps surprising that awn length was related to FHB in 2013, considering that the limited rainfall during anthesis would not have enabled the awns to exert a large influence on the movement of spores to the ear, either by acting as a physical barrier or otherwise. The negative relationship between awn length and AUDPC may however not be due to effects of passive resistance to FHB, but due to QTL on chromosome 5A have also been identified to overlap between both FHB resistance and the B1 phenotypic marker of awnedness (Gervais *et al.* 2003; Lui *et al.* 2013). It is however not known if these genes controlling awn length are present within the genotypes used during this study.

Other ear traits that significantly accounted for variation in the AUDPC, but were identified in only one of the experiment years, included number of spikelets per ear, of which relationships with FHB have not previously been reported. This trait may be genuinely related to AUDPC in 2012 on its own merits, however due to its significance in only one of two years experiments, and because of its strong negative relationship with awn length in the PCA (Fig. 5.5) of which awn length was itself consistently related to AUDPC, suggests spikelets per ear to be unlikely as a potential FHB passive resistance trait.

Interestingly, in the 2012 and 2013 field experiments, the highly resistant control genotypes Sumai-3 and Frontana both produced consistently low AUDPC values. However, pathogen DNA concentrations for these genotypes in harvested grain showed infection levels to be broadly similar to that of other the other UK elite and DH genotypes. This shows that despite the low number of visual symptoms produced, the *Fusarium* and *Microdochium* pathogens still successfully colonised these highly resistant genotypes. This suggests the presence of Type IV resistance (tolerance to FHB) (Mesterhazy, 1995), which for this purpose can be defined as 'the ability of a plant to prevent detrimental effects of infection, despite successful colonisation by the pathogen' (adapted from: Mussell, 1980). The presence of mycotoxin data would have enabled further confirmation of the presence of FHB tolerance traits in these genotypes, although both Sumai-3 (Buerstmayr *et al.* 1996) and Frontana (Pandeya, 2008) have already been described as having at least a small element of tolerance to FHB, although clarification is not given as to how the term tolerance was defined in either case.

It was hypothesised that a reduction in UK elite genotypes and inclusion of additional DH lines into the 2012 and 2013 field experiments, would lead to increased variation in

physiological traits, above that achieved in the 2011 field experiment. This would provide increased statistical power to detect relationships between physiological traits and FHB. To achieve this comparison, percentage differences between maximum and minimum values were calculated for each physiological trait common to the 2011, 2012 and 2013 field experiments (Appendix 5D), allowing for the scale of variation to be compared. The inclusion of additional DH lines into the 2012 and 2013 field experiments failed to produce consistent increases in variation for physiological traits at either GS39 or GS65, over the variation observed in the 2011 field experiment. Furthermore, the inclusion of the additional DH lines did not increase the amount of AUDPC accounted for in the 2012 and 2013 experiments MLR compared to the 2011 experiment, despite all traits significantly accounting for variation in the 2011 AUDPC being carried forward into the 2012 and 2013 experiments.

5.6 CONCLUSIONS

The results within this Chapter enable the following conclusions to be made, based on the hypotheses in Section 5.2:

i) The inclusion of additional DH lines into the 2012 and 2013 field experiment did not lead to increased variation between physiological traits, compared to the 2011 field experiment.

ii) The highly resistant genotypes Sumai-3 and Frontana had consistently lower AUDPC values than either UK elite or DH genotypes, although pathogen DNA concentrations were broadly similar.

iii) Canopy traits including total fresh weight and remaining leaf fresh weight were positively, and plant height negatively, related to the AUDPC.

iv) Awn length was the most consistent ear trait to be significantly related to the AUDPC, having a negative relationship, as found in previous field experiments.

v) Comparisons between the physiological traits accounting for variation in AUDPC under misted and rain fed conditions could not be determined, due to a lack of significant differences between misting treatments.

vi) Physiological traits assessed at GS65 consistently account for greater amounts of variation in the AUDPC than physiological traits assessed at GS39, although the size of these differences differs greatly between years.

Chapter 6.

Glasshouse experiment 2011

& 2012

6.1 INTRODUCTION

Two glasshouse experiments were designed to identify relationships between physiological traits and FHB resistance in wheat, in an experiment where the potential role of passive disease resistance on FHB had been negated. These experiments utilised the same range of UK elite, DH and control genotypes as in the field experiments, however only non-destructive physiological assessments were made as assessments based upon m^{-2} were not applicable in a glasshouse situation. Passive resistance was negated by using a spray inoculation method, therefore by-passing the role of any potential disease escape mechanism on disease development. The use of spray inoculation would therefore ensure that resistance to FHB would be based solely on the genetic resistance of each genotype. This was a crucial part of the studys overall experimental design, as the role of passive resistance can be elucidated from genetic resistance by comparing relationships between physiological traits and FHB resistance in the field experiments, which used ground inoculum and therefore allowed passive resistance to be expressed, with the relationship between physiological traits and FHB resistance in the glasshouse experiments, which used spray inoculation to negate the potential role of passive disease resistance. Therefore, significant relationships between physiological traits and FHB in both field and glasshouse experiments were likely to have an underlying genetic basis, whereas relationships between physiological traits and FHB in the field experiments only, were likely to be caused by true passive disease resistance mechanisms.

The use of spray inoculated glasshouse experiments is a common method for the resistance screening of multiple genotypes in a commercial setting. The glasshouse experiments utilised the same mixed species inoculum as the field experiments. This was done to enable comparison between the pathogen dynamics of both field and glasshouse experiments.

Furthermore, the used of a mixed species inoculum closer mimics a real disease pressure scenario, in which *Fusarium* or *Microdochium* species rarely occur in isolation (Parry *et al.* 1995), but are more often comprised of multiple species. This is important as interactions between pathogen species can occur, with certain species predominating in years in which climatic conditions favour their development. Spray inoculation enables both Type I resistance (resistance to initial infection) and Type II resistance (resistance to spread within the ear) to be quantified, allowing comparisons between the types of resistance in each genotype to be made. The inclusion of both Frontana and Sumai-3 into the glasshouse experiment is important since Frontana has Type I resistance to FHB, conferred predominantly through a major resistance QTL on chromosome 3A (Steiner *et al.* 2004), while Sumai-3 has Type II resistance, conferred predominantly through a major resistance QTL on chromosome 6BS (Cuthbert *et al.* 2007).

Field experiments have identified significant relationships between a number of canopy traits and FHB resistance, including relationships with flag leaf length, plant height, leaf fresh weight and leaf area. Canopy architecture plays a potentially important role on the epidemiology of FHB due to the significance of splash dispersal mechanisms (Gregory *et al.* 1959; Paul *et al.* 2004), of which various canopy architecture traits may influence pathogen inoculum by providing a ladder from the plant base to the ear (Jenkinson and Parry, 1995), diluting inoculum within an expanding crop canopy (Ferrandino, 1998), and reducing splash dispersal by intercepting rain droplets (Madden, 1997).

Flag leaf length was positively related to AUDPC in the 2010 and 2011 field experiments, with this relationship theorised to be due to longer flag leaves providing more overlap between canopy layers for the upwards movement of splash dispersed spores, or due to increased contact between flag leaves and the ears of neighbouring plants. This positive

relationship was however not consistently found within the 2012 and 2013 field experiments, although it was strongly positively related to AUDPC and pathogen DNA in the 2013 GS65 PCA.

Negative relationships between AUDPC and plant height were found at GS39 and GS65 in the 2011 and 2013 field experiments, concurring with the findings of multiple other studies (Hilton *et al*. 1999; Buerstmayr *et al.* 2000; Gervais *et al.* 2003; Somers *et al.* 2003, Schmolke *et al.* 2005; Klahr *et al.* 2007; Srinivasachary *et al.* 2008; Srinivasachary *et al.* 2009; Yan *et al.* 2011; Chu *et al.* 2011; Nemati and Hokmalipor 2012; Suzuki *et al.* 2012; Lu *et al.* 2013; Liu *et al.* 2013; He *et al.* 2014). This relationship however is now known to be caused by a genetic linkage between Rht dwarfing genes and FHB susceptibility (Srinivasachary *et al.*, 2008). Despite this, it seems plausible that plant height will still possibly play a role in the spread of FHB inoculum to the ear, albeit minor, based upon the reports of vertical and horizontal splash dispersal (Gregory *et al.* 1959; Jenkinson and Parry, 1994; Paul *et al.* 2004), as well as reports that splash dispersal of other wheat pathogen inoculum decreases rapidly with height (Shaw and Royal, 1993).

Additionally, canopy traits such as total fresh weight and remaining leaf area have been found to be positively related to FHB in the field experiments, although these relationships were not always consistent. Such canopy traits may be related to FHB by providing a greater area of above ground biomass for inoculum to use as a ladder for vertical movement, as Jenkinson and Parry (1995) suggested that splash dispersed inoculum seldom reaches the ear in one go, but rather achieves this is a series of smaller jumps.

The influence of canopy traits will be negated within the glasshouse experiment by applying *Fusarium* and *Microdochium* spores directly to the ear, enabling the effects of ear morphology and genetic resistance to be studied.

Field experiments have identified significant relationships between a number of ear traits and FHB resistance, including relationships with awn length and anther extrusion. Awn length had a significant negative relationship with AUDPC in the 2011, 2012 and 2013 field experiments, in agreement with Buerstmayr *et al.* (2000) and Ban and Suenaga (2000) in which a positive relationship with FHB was also shown, although this relationship conflicts with the findings of other studies (Buerstmayr *et al.* 2000; Buerstmayr *et al.* 2002; Gervais *et al.* 2003; Somers *et al.* 2003; Hori *et al.* 2005; Liu *et al.* 2007; Lui *et al.* 2013). Anther extrusion traits were found to be related to DON in the 2011 field experiment, however since similar relationships have already been consistently reported (Skinnes *et al.* 2005; Skinnes *et al.* 2008; Graham and Brown. 2009; Skinnes *et al.* 2010; Buerstmayr *et al.* 2012; Lu *et al.* 2013; He *et al.* 2014), this trait was no longer assessed to enable the study to focus on identifying novel relationships to traits. Ear traits were theorised, in the case of awn length, to act as a barrier to spore deposition on the ear, or in the case of trapped anthers, to provide a route to entering the floret so that the susceptible inner floral tissues could be accessed.

This chapter builds upon previous work by assisting to elucidate which physiological traits, identified within previous field experiments (Chapters 4 and 5), truly confer passive disease resistance to FHB. The glasshouse experiments performed within this chapter help to achieve this by using a spray inoculation method to identifying relationships between genetic FHB resistance and physiological traits in wheat.

6.2 OBJECTIVES AND HYPOTHESES

The aim of this study was to examine relationships between FHB and physiological traits in wheat, using spray inoculation techniques to evaluate genotype resistance while negating the potential role of passive disease resistance. This was undertaken to create contrast with the field experiments, enabling genuine passive resistance traits to be identified. The objectives of these glasshouse studies were to:

1) Quantify a range of physiological traits in-situ under glasshouse conditions within a selection of UK elite and DH wheat genotypes.

2) Quantify the level of genetic FHB resistance in each wheat genotype using a spray inoculation method to negate any disease escape mechanisms from canopy traits.

3) Identify significant relationships between physiological traits and variation in the AUDPC and pathogen DNA data between genotypes.

The specific experimental hypotheses tested within this chapter are:

i) The highly resistant genotypes Sumai-3 and Frontana will have lower AUDPC and pathogen DNA content than either UK elite or DH lines.

ii) There will be no relationship present between AUDPC and canopy traits, when using a spray inoculation method.

iii) A number of ear traits will be significantly related to AUDPC, when using a spray inoculation method.

6.3 MATERIALS AND METHODS

6.3.1 Experimental design

Two glasshouse experiments were undertaken in 2011 and 2012 at The University of Nottingham, Sutton Bonington Campus. Each experiment consisted of four randomized blocks as replicates. The 2011 glasshouse experiment used the same fifteen wheat genotypes as in the 2010 and 2011 field experiments, however, the 2012 glasshouse experiment used the same genotypes as the 2012 and 2013 field experiments, in which additional DH lines were included to increase variation (Table 6.1). An increased number of DH lines were used in the 2012 field experiment to create more variability in the experimental material and this was therefore reflected in the 2012 glasshouse experiment. Seeds were sown into trays and kept in a growth chamber at 8^{o}c for 6 weeks to complete vernalisation. One plant of each variety was then transplanted into a 3L pot filled with John Innes No.3 compost, before being placed into their individual blocks within the glasshouse. The glasshouse temperature was set to be maintained between 5-12^{o}C via a combination of automatic vents and heating system, although this maximum temperature was often exceeded during the summer. An automatic watering system was used to water pots twice a day.

6.3.2 Disease management

Powdery mildew was controlled via the use of a sulphur burner at weekly intervals until GS51. Following GS51 sulphur was not applied to wheat heads to minimise any potential negative effect on the *Fusarium* or *Microdochium* spores applied during spray inoculation.

Table 6.1. Wheat lines used within the 2011 and 2012 glasshouse experiments

2011	2012
Rialto	Rialto
Claire	Claire
Solstice	-
Ambrosia	Ambrosia
Grafton	-
-	Line 8
Line 14	Line 14
-	Line 15
Line 16	Line 16
Line 18	Line 18
Line 21	Line 21
Line 24	Line 24
-	Line 28
Line 38	Line 38
Line 39	Line 39
-	Line 43
-	Line 48
Line 51	Line 51
Line 61	Line 61
-	Line 69
Line 77	Line 77
Sumai-3	Sumai-3
Frontana	Frontana

6.3.3 Spray inoculum production

Spray inoculum production utilised the same isolates of *Fusarium* and *Microdochium* as the ground inoculum (Appendix 2A). Each isolate was grown on 5 plates of Tap Water Agar (TWA) under a 12 hour regime of near-UV light and darkness for 10 days before harvesting spores. Spore harvest utilised distilled water and a sterile loop to dislodge spores from the plate before filtering through muslin cloth to remove large pieces of

mycelia. Spore solutions were quantified using haemocytometer and were then diluted in distilled water to create mixed inoculum solutions of equal ratios of each species, totalling 100,000 spores/ml. Mixed inoculum solutions were stored at -20^{o}C until required, when they were gently defrosted in a cold water bath. The physiological traits of each genotype were assessed *in-situ* at GS65, followed by spray inoculation. Spray inoculation involved tagging each main stem ear with coloured wire so that the inoculated ear could be easily identified. Defrosted spore solutions were placed into a clean spray bottle and applied to each main stem ear as a fine mist until run off. Inoculated ears were covered with a breathable plastic bag for 24 hours to stimulate infection.

6.3.4. Trait assessments

Trait assessments involved either individually counting or measuring plant organs using a fine ruler. Traits measured in both the 2011 and 2012 glasshouse experiments are shown in Table 6.2. Further details on some specific assessments can be found in section 2.3.

Table 6.2. Physiological traits assessed in the 2011 and 2012 glasshouse experiments

Trait name	2011 GS65	2012 GS65
Flag leaf length (cm)	×	×
Flag leaf width (cm)	×	×
No of tillers (per plant)	×	×
Ear height (cm)	×	×
Peduncle length	×	×
Ear length (cm)	×	×
Number of spikelets per ear	×	×
Spikelet density (spiklets/cm)	×	×
Awn length (cm)	×	×
Hair on ear surface (present/absent)	×	×

6.3.5 Visual disease assessment

Glasshouse visual disease assessments were carried out from mid-anthesis (GS65) onwards at 4 day intervals over a 22 day period. The inoculated main stem ear of each treatment was assessed for both lesion and bleaching symptoms. Each assessment of FHB incidence was calculated as the percentage of spikelets per ear showing symptoms of FHB and this was used to create an Area Under Disease Progress Curve (AUDPC) from mid-anthesis for each genotype. Methods used for AUDPC calculations are shown in section 2.4.

6.3.6 Grain harvest

Each genotype began to senesce naturally and, once nearly complete, the automatic pot watering system was turned off to speed up the process. Once each genotype was fully mature (GS93), inoculated heads were harvested and stored in paper bags until ready for threshing. Each ear was threshed using a stationary thresher with great care exercised to retain as much grain as possible so that Fusarium damaged grains were not removed from the sample. Threshed grain was milled to a fine flour using a Krups F203 grinder (Krups, Windsor). Cross contamination was avoided by thoroughly cleaning the grinding chamber using a combination of brushes and damp tissue between samples.

6.3.7 Pathogen DNA quantification

Refer to section 2.6 for full method details.

6.3.8 Statistical analysis

All data was analysed using Genstat® Version 12.1 for Windows (VSN International Ltd, UK). Where required, DNA data was Log_{10} transformed to normalise residuals, using the addition of 0.001 to each DNA value to enable the transformation of zero values to be made. Analysis of variance was carried out on the visual disease symptoms, pathogen DNA and physiological trait data to determine significant differences between genotypes. The visualisation of groupings between physiological traits and DNA was explored using Principal Component Analysis (PCA) to create biplots. Stepwise Multiple Linear Regression (MLR), undertaken as per Section 4.3.15, was used to account for variation in the AUDPC using assessed physiological traits and pathogen DNA.

6.4 RESULTS

Due to different genotypes being used within each glasshouse experiment, ANOVA was carried out separately without grouping for AUDPC, physiological traits and DNA data for each year. Data was however grouped together by year for PCA and MLR analysis.

6.4.1 Visual disease symptoms

Significant differences in AUDPC were present between genotypes in both the 2011 ($P=0.003$) and 2012 ($P<0.001$) glasshouse experiments. Mean AUDPC values were greater in 2012 than in 2011 (184.4 and 91.7, respectively), with much more variation occurring in the 2012 experiment (Table 6.3).

In 2011, the FHB resistant control genotypes Frontana and Sumai-3 recorded low AUDPC scores (0.2 and 14.7, respectively), however both Line 14 and 51 showed lower AUDPC than that of Sumai-3. The UK elite lines Claire, Grafton and Rialto all showed fairly high AUDPC values in comparison to Ambrosia and Solstice, and in comparison with most of the DH lines. The highest overall AUDPC score was shown by Line 18 (266.7).

In 2012, the control genotypes Frontana and Sumai-3 did not have the lowest scoring AUDPC values which were instead seen in Lines 18 and 28, which both failed to produce any disease symptoms. Several other DH genotypes including Lines 51 and 69 also produced very low AUDPC scores. The highest AUDPC of 562.2 was produced by Line 43, which was significantly different ($P=0.05$) to multiple other genotypes. The UK elite genotypes had moderate to high AUDPC scores and showed poor FHB resistance compared to most of the DH genotypes.

Table 6.3. Genotype differences in AUDPC within the 2011 and 2012 glasshouse experiment.

	2011	2012
Ambrosia	20.1	501.7
Claire	138.8	409.4
Grafton	142.0	-
Rialto	182.8	377.5
Solstice	36.0	-
Frontana	0.2	109.3
Sumai-3	14.7	29.9
Line 8	-	409.5
Line 14	12.2	54.0
Line 15	-	122.4
Line 16	55.9	62.1
Line 18	266.7	0.0
Line 21	114.4	260
Line 24	69.8	258.5
Line 28	-	0.0
Line 38	204.3	225.4
Line 39	66.9	113.6
Line 43	-	562.2
Line 48	-	20.0
Line 51	13.8	8.0
Line 61	102.4	184.9
Line 69	-	1.9
Line 77	117.2	162.3
Mean	91.7	184.4
P-value	0.003	<.001
LSD	131.6	282.8
CV%	100.65	93.72

6.4.2 Pathogen DNA

In general, concentrations of pathogen DNA were greater in the 2012 experiment than in the 2011 experiment, however exceptions to this included *F. graminearum*, *F. poae* and *F. langsethiae* DNA, with the latter two species only detected in very small quantities.

In the 2011 experiment, only *F. graminearum*, *F. culmorum*, *F. poae* and *F. langsethiae* DNA was detected within the harvested grain (Table 6.4). *F. graminearum* and *F. culmorum* were by far the most prevalent pathogen species, with their grain DNA concentration accounting for 98.2% of the total quantified DNA in that year, however significant differences between genotypes were only present for *F. culmorum* DNA (P = 0.003). Rialto had the highest *F. culmorum* DNA content (2.45 pg/ng) of the assessed genotypes, with both Ambrosia and Claire also having greater *F. culmorum* DNA content than that of the DH lines, excluding Line 16. Frontana had the lowest *F. culmorum* DNA content (0.0005 pg/ng), followed closely by Sumai-3 and several DH genotypes.

In the 2012 experiment, all *Fusarium* and *Microdochium* spp. used during inoculation were detected within the harvested grain samples, although *F. graminearum*, *F. culmorum* and *F. avenaceum* were the predominant pathogen species and with significant differences present only for *F.culmorum, F. avenaceum* and *F. langsethiae* DNA (Table 6.4). *F. culmorum* DNA was found in greatest quantities within harvested grain samples, contributing 94.38% of the total pathogen DNA concentration. Line 16 had the highest *F. culmorum* DNA content (7.94 pg/ng), followed by Line 24 and Rialto (5.88 and 4.32 pg/ng, respectively). Line 69 had the lowest *F.culmorum* DNA concentration (0.49 pg/ng), while the highly resistant control lines Frontana and Sumai-3 had similar *F. culmorum* DNA contents to the DH genotypes.

Simple linear regression showed that no relationship was present between AUDPC and total mean FHB pathogen DNA in the harvested grain samples (Fig. 6.1 and 6.2).

Table 6.4. Differences between genotypes in the amount of *Fusarium spp*. and *Microdochium spp*. DNA present within harvested grain samples collected from the 2011 and 2012 glasshouse experiments, expressed as Log_{10} of total extracted DNA (pg/ng) (*continued*)

	F.graminearum		*F.culmorum*		*F. poae*		*F. avenaceum*	
	2011	2012	2011	2012	2011	2012	2011	2012
Ambrosia	-1.427 (0.037)	-0.094 (0.805)	0.015 (1.0351)	0.161 (1.449)	-4.000 (0.0001)	-4.000 (0.0001)	-	-0.971 (0.107)
Claire	-0.835 (0.146)	-2.544 (0.003)	0.194 (1.5631)	-0.208 (0.619)	-4.000 (0.0001)	-4.000 (0.0001)	-	-1.362 (0.043)
Grafton	-1.859 (0.014)	-	-2.779 (0.0017)	-	-4.000 (0.0001)	-	-	-
Rialto	-0.217 (0.607)	-1.054 (0.088)	0.389 (2.4491)	0.635 (4.315)	-4.000 (0.0001)	-3.367 (0.0004)	-	-1.752 (0.018)
Solstice	0.138 (1.374)	-	-1.195 (0.0638)	-	-3.192 (0.0006)	-	-	-
Frontana	-1.757 (0.017)	-0.871 (0.135)	-3.331 (0.0005)	-0.210 (0.617)	-4.000 (0.0001)	-3.273 (0.0005)	-	-1.746 (0.018)
Sumai-3	-2.684 (0.002)	-2.241 (0.006)	-2.425 (0.0038)	0.204 (1.600)	-4.000 (0.0001)	-4.000 (0.0001)	-	-1.336 (0.046)
Line 8	-	-1.391 (0.041)	-	0.579 (3.793)	-	-3.494 (0.0003)	-	-1.534 (0.029)
Line 14	0.119 (1.315)	-2.374 (0.004)	-0.394 (0.4036)	-0.131 (0.740)	-3.265 (0.0005)	-2.755 (0.0018)	-	-1.995 (0.010)
Line 15	-	-2.735 (0.002)	-	0.585 (3.846)	-	-3.998 (0.0001)	-	-2.149 (0.007)
Line 16	-2.171 (0.007)	-0.213 (0.612)	0.089 (1.2274)	0.900 (7.943)	-3.335 (0.0005)	-4.000 (0.0001)	-	-1.532 (0.029)
Line 18	-2.358 (0.004)	-1.323 (0.048)	-2.247 (0.0057)	0.152 (1.419)	-3.029 (0.0009)	-3.559 (0.0003)	-	-0.940 (0.115)
Line 21	-1.129 (0.074)	-2.250 (0.006)	-0.537 (0.2904)	0.409 (2.564)	-4.000 (0.0001)	-4.000 (0.0001)	-	-1.614 (0.024)
Line 24	0.273 (1.875)	-1.320 (0.048)	-1.445 (0.0359)	0.769 (5.875)	-2.577 (0.0026)	-3.344 (0.0005)	-	-0.993 (0.102)
Line 28	-	-1.406 (0.039)	-	0.525 (3.350)	-	-4.000 (0.0001)	-	-2.981 (0.001)
Line 38	-2.860 (0.001)	-1.546 (0.028)	-2.271 (0.0054)	-0.152 (0.705)	-3.050 (0.0009)	-4.000 (0.0001)	-	-2.007 (0.010)
Line 39	0.186 (1.535)	-0.664 (0.217)	-0.978 (0.1052)	0.008 (1.019)	-3.033 (0.0009)	-3.246 (0.0006)	-	-3.111 (0.001)
Line 43	-	-1.654 (0.022)	-	-0.011 (0.975)	-	-3.998 (0.0001)	-	-1.935 (0.012)
Line 48	-	-1.564 (0.027)	-	0.588 (3.873)	-	-3.483 (0.0003)	-	-1.787 (0.016)
Line 51	-1.880 (0.013)	-1.577 (0.026)	-1.524 (0.0299)	0.132 (1.355)	-4.000 (0.0001)	-3.350 (0.0004)	-	-1.752 (0.018)
Line 61	-0.815 (0.153)	-1.419 (0.038)	-2.838 (0.0015)	0.170 (1.479)	-4.000 (0.0001)	-4.000 (0.0001)	-	-1.811 (0.015)
Line 69	-	-1.282 (0.052)	-	-0.303 (0.498)	-	-3.111 (0.0008)	-	-3.178 (0.001)
Line 77	-2.134 (0.007)	-1.284 (0.052)	-2.262 (0.0055)	0.307 (2.028)	-3.244 (0.0006)	-3.558 (0.0003)	-	-1.501 (0.032)
Mean	-1.259 (0.423)	-1.467 (0.110)	-1.385 (0.425)	0.243 (2.384)	-3.572 (0.001)	-3.646 (0.0001)	-	-1.809 (0.031)
P-value	0.067	0.228	0.003	0.020	0.689	0.229	-	0.004
LSD	2.2843	1.7325	2.0312	0.6972	1.5815	0.9627	-	1.1148
CV%	127.4	83.1	103.1	201.8	31.1	18.6	-	43.3

Back transformed means in parenthesis.

Table 6.4. Differences between genotypes in the amount of *Fusarium spp*. and *Microdochium spp*. DNA present within harvested grain samples collected from the 2011 and 2012 glasshouse experiments, expressed as Log_{10} of total extracted DNA (pg/ng).

	F. langsethiae		*M.majus*		*M.nivale*	
	2011	2012	2011	2012	2011	2012
Ambrosia	-2.010 (0.0098)	-3.221 (0.0006)	-	-4.000 (0.0001)	-	-3.069 (0.0009)
Claire	-2.478 (0.0033)	-3.616 (0.0002)	-	-4.000 (0.0001)	-	-4.000 (0.0001)
Grafton	-2.327 (0.0047)	-	-	-	-	-
Rialto	-4.000 (0.0001)	-4.000 (0.0001)	-	-4.000 (0.0001)	-	-3.600 (0.0003)
Solstice	-2.293 (0.0051)	-	-	-	-	-
Frontana	-2.709 (0.0020)	-2.561 (0.0027)	-	-4.000 (0.0001)	-	-3.569 (0.0003)
Sumai-3	-2.512 (0.0031)	-4.000 (0.0001)	-	-4.000 (0.0001)	-	-4.000 (0.0001)
Line 8	-	-4.000 (0.0001)	-	-3.675 (0.0002)	-	-3.485 (0.0003)
Line 14	-2.484 (0.0033)	-4.000 (0.0001)	-	-3.982 (0.0001)	-	-3.837 (0.0001)
Line 15	-	-3.973 (0.0001)	-	-4.000 (0.0001)	-	-4.000 (0.0001)
Line 16	-3.534 (0.0003)	-3.986 (0.0001)	-	-4.000 (0.0001)	-	-4.000 (0.0001)
Line 18	-2.563 (0.0027)	-4.000 (0.0001)	-	-3.981 (0.0001)	-	-3.407 (0.0004)
Line 21	-2.436 (0.0037)	-3.721 (0.0002)	-	-4.000 (0.0001)	-	-3.615 (0.0002)
Line 24	-2.373 (0.0042)	-3.721 (0.0002)	-	-4.000 (0.0001)	-	-3.547 (0.0003)
Line 28	-	-2.942 (0.0011)	-	-4.000 (0.0001)	-	-4.000 (0.0001)
Line 38	-2.313 (0.0049)	-4.000 (0.0001)	-	-4.000 (0.0001)	-	-4.000 (0.0001)
Line 39	-1.782 (0.0165)	-3.141 (0.0007)	-	-4.000 (0.0001)	-	-2.687 (0.0021)
Line 43	-	-3.973 (0.0001)	-	-4.000 (0.0001)	-	-3.585 (0.0003)
Line 48	-	-3.443 (0.0004)	-	-4.000 (0.0001)	-	-3.638 (0.0002)
Line 51	-3.509 (0.0003)	-4.000 (0.0001)	-	-3.981 (0.0001)	-	-3.342 (0.0005)
Line 61	-3.084 (0.0008)	-2.520 (0.0030)	-	-4.000 (0.0001)	-	-4.000 (0.0001)
Line 69	-	-3.800 (0.0002)	-	-4.000 (0.0001)	-	-4.000 (0.0001)
Line 77	-2.431 (0.0037)	-4.000 (0.0001)	-	-4.000 (0.0001)	-	-3.585 (0.0003)
Mean	-2.380 (0.004)	-3.648 (0.0005)		-3.982 (0.0001)		-3.665 (0.0003)
P-value	0.321	0.020		0.649		0.407
LSD	1.509	0.972		0.219		0.982
CV%	40.2	18.7		3.9		18.8

Back transformed means in parenthesis.

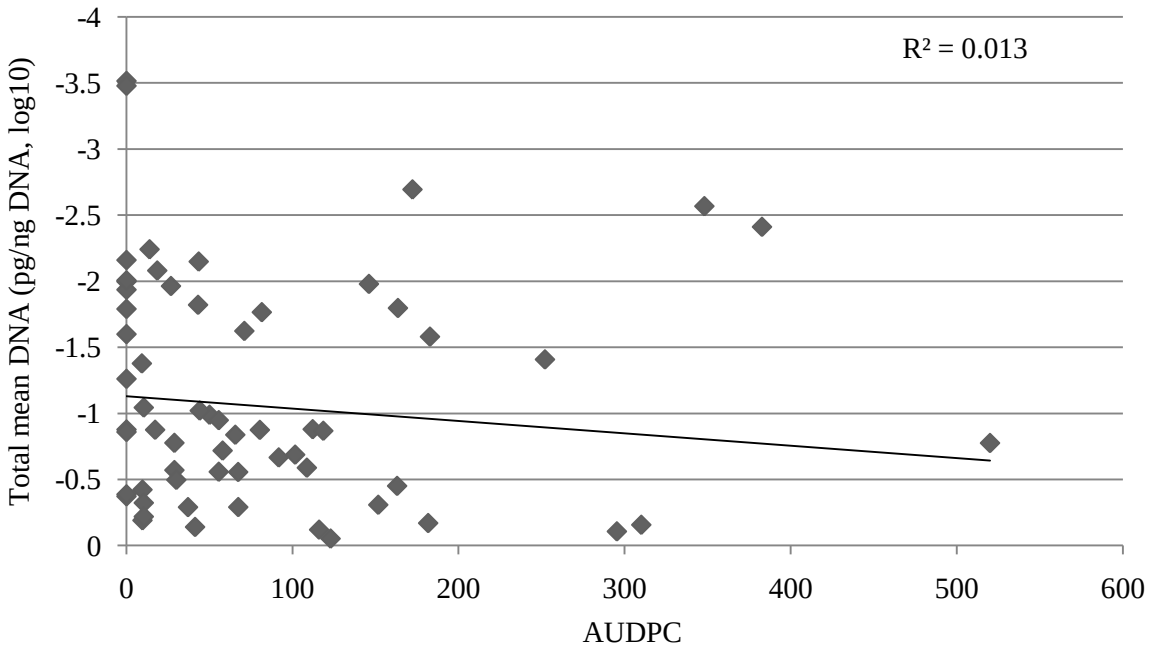

Figure 6.1. Regression between AUDPC vs total mean FHB pathogen DNA content in harvested grain from the 2011 glasshouse experiment.

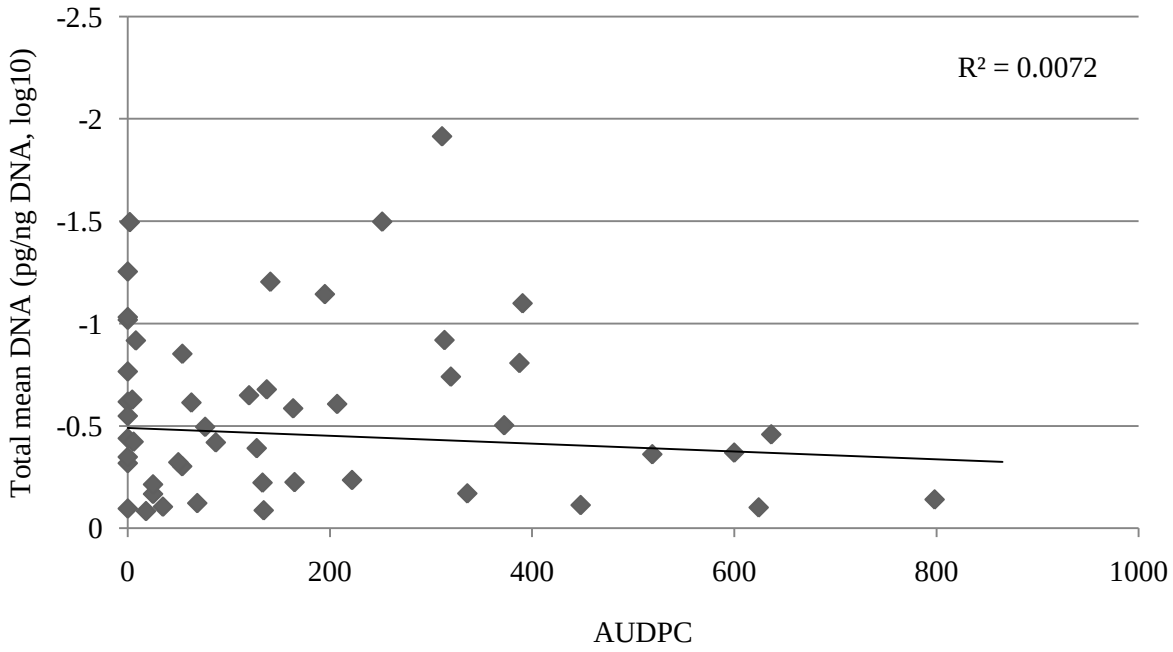

Figure 6.2. Regression between AUDPC vs total mean FHB pathogen DNA content in harvested grain from the 2012 glasshouse experiment.

6.4.3 Physiological traits

Significant differences (P<0.0001) between genotypes were present in both experiments for all canopy traits assessed, excluding flag leaf length in the 2012 experiment (Table 6.5). Mean plant height was similar between experiments, however large differences between genotypes were present, with Line 18 being the shortest and Frontana being the tallest in both experiments, with respective mean heights between years of 42.8 and 124.8cm. In 2011, the smallest flag leaf length (15.6cm) and width (1.5cm) were shared by a single genotype (Line 38), however the largest flag leaf length and width was found in Sumai-3 (37.60cm) and Line 61 (2.57 cm) respectively. In 2012 however, it was the largest flag leaf length (39.1cm) and width (3.28cm) that were present within the same genotype, Line 77, while the smallest flag leaf length and width were found in Line 18 (36.67 cm) and Line 69 (1.85cm) respectively. Mean tiller number was marginally greater in 2011 than in 2012 with large variation between genotypes in both years. L61 had the lowest tiller number in both years, of which Line 61 also coincidentally had a tall phenotype with long and wide flag leaves. The genotypes with the greatest tiller number were different between years, being Ambrosia in 2011 (13.25 tillers) and Line 38 (20 tillers) in 2012. Frontana had the longest peduncle length in each year, being significantly (P=0.05) higher than the next longest genotype in both years. The shortest peduncle was present in differing genotypes between years, of which several exceptionally short peduncles were observed.

Significant differences (P<0.001) between genotypes were present in both experiments for all ear traits assessed within the glasshouse experiments (Table 6.6). Mean ear length was similar between years with Line 61 having the longest ears in both experiments. Sumai-3 and Frontana both had a short ear phenotype, with only Lines 28 and 69 having shorter ears than Sumai-3 in 2012. The ear length of the UK elite genotypes was fairly consistent compared to the DH genotypes. Awn length was split between awned and awnless genotypes, ranging

between 0.31 - 0.59cm and 4.37 - 7.17cm respectively for awnless and awned genotypes in 2011, and between 0.26 - 0.56 cm and 2.93 - 8.11 cm respectively for awnless and awned genotypes in 2012. The UK elite genotypes were consistently awnless. The number of spikelets per ear and spikelet density were very similar between years, with UK elite genotypes showing much less variation than the DH genotypes. Interestingly, in 2011 the highly resistant control genotypes Sumai-3 and Frontana both showed extreme values, having short ears, long awns, low spikelets per ear and low spikelet density, a pattern also seen in 2012 except more extreme values were present due to newly included DH genotypes within the experiment.

Table 6.5. Genotypic differences in canopy traits measured at GS65 in the 2011 and 2012 glasshouse experiments

	Plant height (cm)		Flag leaf length (cm)		Flag leaf width (cm)		Tiller number		Peduncle length (cm)	
	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012
Ambrosia	68.83	67.93	17.61	36.57	2.04	2.73	13.25	17.33	9.62	5.47
Claire	69.43	76.90	20.64	32.40	2.25	2.58	10.00	17.50	11.25	6.77
Grafton	58.28	-	20.81	-	2.02	-	10.25	-	8.20	-
Rialto	75.63	77.05	19.56	35.32	2.12	2.99	11.25	18.75	9.70	4.22
Solstice	75.41	-	23.44	-	2.22	-	13.00	-	11.80	-
Frontana	118.86	130.80	32.29	34.15	1.85	2.21	12.50	14.25	30.92	28.03
Sumai-3	72.73	83.45	37.61	42.07	1.75	2.04	6.00	5.50	15.72	20.30
Line 8	-	72.42	-	36.52	-	2.90	-	17.00	-	9.35
Line 14	77.68	76.20	25.54	30.20	1.89	2.30	6.75	6.00	8.25	12.30
Line 15	-	95.13	-	38.07	-	2.92	-	19.00	-	8.13
Line 16	71.93	79.50	23.56	32.80	1.91	2.58	10.75	11.33	8.00	10.73
Line 18	48.78	36.67	27.96	28.90	1.97	2.03	11.25	5.00	3.07	6.67
Line 21	105.83	94.07	29.66	38.90	2.19	2.50	7.50	8.25	14.77	16.15
Line 24	69.93	71.50	22.81	33.25	2.10	2.38	7.75	13.00	12.32	9.08
Line 28	-	73.50	-	36.60	-	2.40	-	6.33	-	18.30
Line 38	92.79	89.30	15.61	29.62	1.50	2.06	12.34	20.00	14.36	12.60
Line 39	52.02	49.82	30.5	36.27	2.32	2.86	9.00	13.00	4.54	2.90
Line 43	-	95.20	-	35.03	-	2.67	-	15.33	-	11.47
Line 48	-	38.90	-	30.22	-	2.94	-	8.25	-	3.70
Line 51	65.11	73.70	17.69	38.43	2.25	2.92	10.25	10.67	11.17	16.60
Line 61	109.26	111.85	35.64	37.85	2.57	2.83	3.00	1.75	13.72	10.48
Line 69	-	83.70	-	32.37	-	1.85	-	7.00	-	18.27
Line 77	107.56	119.5	25.11	39.10	2.25	3.28	5.75	7.75	15.97	15.32
Mean	78.8	80.8	25.1	35.0	2.1	2.6	9.4	11.6	12.0	11.8
P-value	<.001	<.001	<.001	0.093	<.001	<.001	<.001	<.001	<.001	<.001
LSD	8.109	13.33	5.457	8.28	0.2574	0.39	3.411	6.821	2.655	5.683
CV%	7.07	10.40	14.97	14.99	8.55	9.58	25.00	36.99	15.20	30.67

Table 6.6. Genotypic differences in ear traits measured at GS65 in the 2011 and 2012 glasshouse experiments

	Ear length (cm)		Awn length (cm)		Spikelets per ear		Spikelet density	
	2011	2012	2011	2012	2011	2012	2011	2012
Ambrosia	14.47	15.23	0.37	2.93	25.50	27.33	1.77	1.82
Claire	15.95	16.22	0.34	0.35	28.50	31.75	1.79	1.96
Grafton	13.62	-	0.45	-	25.25	-	1.86	
Rialto	15.57	17.05	0.31	0.56	29.50	31.50	1.90	1.85
Solstice	14.00	-	0.37	-	24.00	-	1.73	-
Frontana	12.67	14.95	6.16	5.49	17.00	20.25	1.34	1.36
Sumai-3	12.20	12.65	7.17	7.28	18.50	20.50	1.52	1.62
Line 8	-	13.32	-	3.58	-	15.60	-	1.27
Line 14	14.00	14.00	0.37	0.45	26.25	25.00	1.88	1.79
Line 15	-	18.50	-	0.53	-	32.00	-	1.73
Line 16	12.47	13.67	0.59	0.78	24.50	27.67	1.97	2.03
Line 18	14.75	13.23	4.55	7.18	25.50	20.67	1.73	1.56
Line 21	18.57	17.62	0.51	0.48	29.75	23.50	1.61	1.32
Line 24	13.62	14.15	0.40	0.26	26.75	26.00	1.97	1.86
Line 28	-	11.33	-	5.50	-	22.67	-	2.02
Line 38	15.00	14.90	4.92	5.61	29.00	28.75	1.94	1.93
Line 39	19.87	19.05	6.38	8.11	26.34	25.75	1.32	1.36
Line 43	-	17.73	-	6.43	-	29.33	-	1.65
Line 48	-	13.55	-	5.38	-	25.25	-	1.86
Line 51	16.5	17.67	5.02	6.92	26.75	25.00	1.62	1.42
Line 61	20.07	22.00	4.37	6.48	31.25	27.25	1.57	1.26
Line 69	-	12.20	-	5.35	-	21.00	-	1.72
Line 77	17.37	20.65	0.41	0.48	30.00	29.75	1.73	1.45
Mean	15.3	15.7	2.5	3.8	26.1	25.5	1.7	1.7
P-value	<.001	<0.001	<.001	<0.001	<.001	<.001	<.001	<.001
LSD	1.37	2.57	0.76	2.30	3.18	5.26	0.19	0.42
CV%	6.19	10.36	21.54	37.45	8.39	13.14	7.77	16.10

6.4.4 Principal component analysis of GS65 physiological traits, AUDPC and pathogen DNA

Data from the 2011 and 2012 glasshouse experiments were added together for PCA, accounting for 33.19% of the variation (Fig. 6.3). No distinct groups were present, with AUDPC and physiological traits being continuously distributed throughout the biplot. Despite the lack of clear groupings, *F. culmorum* DNA and tiller number were the traits most closely associated with the AUDPC. The strongest positive grouping between traits was seen between the number of spikelets per ear and tiller number, which were negatively associated with peduncle length. Positive grouping between *F. culmorum*, *F. langsethiae*, *M. majus* and *M. nivale* were shown, which were also positively associated with flag leaf width and ear length and negatively associated with *F. poae* DNA.

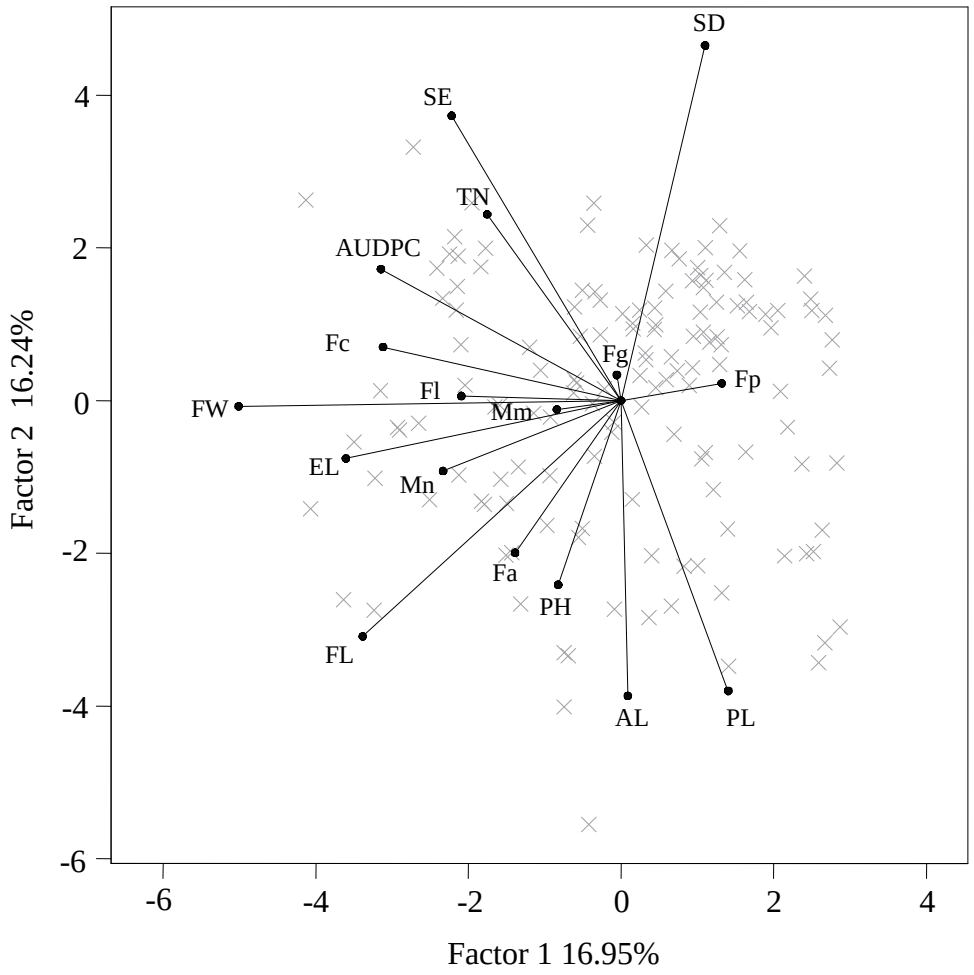

Figure 6.3. Biplot of the principal component analysis of the AUDPC, physiological traits and quantified fungal DNA from the 2011 and 2012 glasshouse experiments. Key: AL (awn length, cm); AUDPC (Area Under Disease Progress Curve); EL (ear length); Fc DNA (*F.culmorum* DNA), Fg DNA (*F.graminearum* DNA), Fl DNA (*F.langsethiae* DNA); FL (flag leaf length, cm); Fp DNA (*F.poae* DNA); FW (flag leaf width, cm); PH (plant height, cm); PL (peduncle lenth, cm); SD (spikelet density); SE (Spikelets per ear); TN (tiller number).

6.4.5. Multiple linear regression

Data from the 2011 and 2012 glasshouse experiments were grouped together by year for analysis by MLR. Physiological traits significantly (P<0.001) accounted for 29.1% of the variation in the AUDPC (Table 6.7), being positively related to flag leaf length, tiller number and plant height, and negatively related to peduncle length. Pathogen DNA was unable to account for any more of the AUDPC variation.

Table 6.7. Multiple linear regression models of the 2011 and 2012 glasshouse experiments accounting for variation in the AUDPC between genotypes using GS65 physiological traits and pathogen species DNA.

Model	Equation	R^2	P-value
AUDPC	11.61 (TN) + 6.73 (FL) - 11.56 (AL) - 4.77 (PL) - 93.4	0.24	<0.001

Key: TN = Tiller number; FL = Flag leaf length (cm); AL = Awn length; PL = Peduncle length

6.5 DISCUSSION

The inclusion of additional DH genotypes within the 2012 experiment was carried out to match genotypes included in field experiments. This successfully increased the genotypic variation of canopy and ear traits within the glasshouse experiments.

Differences in mean AUDPC between years may have been due to differences in environmental conditions, despite the experiments being undertaken within a glasshouse. Weather conditions in 2011 were generally dry and warm over the GS65 period, while conditions during 2012 were wet and humid, leading to an environment more conducive to FHB disease development (Edwards, 2007). Additionally, heavy and sharp rain showers often occurred during the 2012 experiment, in which rain frequently wetted the experiment via ingression through roof vents, which were unable to automatically close in a timely manner during these events, often leading to periods of very high humidity once vents had closed. This problem was minimised by erecting a plastic canopy above the experiment, to deflect rain from landing directly on the plants. Differences in AUDPC between years were exacerbated by the inclusion of Lines 8 and 43 into the 2012 experiment, with both genotypes developing a very high AUDPC score, accounting for part of the differences in AUDPC means between years.

AUDPC scores for genotypes were inconsistent between years. In 2011 the resistant genotypes Frontana and Sumai-3 had low AUDPC scores, with Sumai-3 being surpassed only by Lines 14 and 51, although these were not significantly different. However in 2012, the resistance of Frontana and Sumai-3 was surpassed by multiple DH genotypes, although these were not significantly different. Furthermore, in 2011, Line 18 had the largest AUDPC of all genotypes, while in 2012, Line 18 failed to produce any visual symptoms at all. The AUDPC increase in specific genotypes was reflected within the grain pathogen

DNA, in which both Frontana and Sumai-3 had increases in *F.graminearum* and *F.culmorum* DNA, reflecting the increases in AUDPC between experiments. Differences in pathogen DNA between genotypes generally reflected the differences seen within the AUDPC.

The predominant pathogen species in 2011 was *F. graminearum*, followed by *F. culmorum*, while in 2012 these species were reversed, with *F. culmorum* being predominant followed by *F. graminearum*. The predominance of *F. graminearum* and *F. culmorum* in both years experiments is likely due to the warm and humid conditions within the glasshouse during and after anthesis being suited to the development of these species (Xu *et al.* 2008), of which the upper temperature limit set for the glasshouse of 12^{o}C was often exceeded. The climatic conditions during the summer of 2012 was much cooler than that experienced in 2011, perhaps explaining the predominance of *F. culmorum* in 2012 due to its preference for cooler conditions than *F. graminearum* (Xu *et al.* 2008).

Physiological traits included within the MLR significantly accounted for 24.3% of the AUDPC variation ($P<0.001$). Tiller number was positively related to AUDPC, with Gautam *et al.* (2012) having previously reported both positive and negative relationships between tiller number and grain DON content. Mycotoxins were however unable to be quantified within this experiment due to a lack of flour from each replicate.

Flag leaf length was positively related to AUDPC, and this is the first report of a relationship between flag leaf length and FHB in a spray inoculated experiment. With spray inoculation, there is only a limited role that flag leaf length could play to influence disease development, therefore this relationship is likely due to pleiotropy or co-locating genes for flag leaf length and FHB resistance.

Awn length was negatively related to AUDPC in the glasshouse experiments. Buerstmayr *et al.* (2000) previously showed a weak negative relationship between awn length and FHB, in one of two wheat populations that were studied. Furthermore, Ban and Suenaga (2000) also showed a negative relationship between awn length and FHB, in which fully awned lines were more resistant than non-awned lines, of which genetic linkages suggested between FHB resistance and the *B1* gene for awnedness located on chromosome 5A. The evidence for a positive relationship between awn length and FHB resistance is however inconsistent, as other studies have also suggested both a positive (Somers *et al.* 2003; Lui *et al.* 2013) and no relationship (Buerstmayr *et al.* 2000; Buerstmayr *et al.* 2002; Gervais *et al.* 2003; Hori *et al.* 2005; Liu *et al.* 2007) between awn length and FHB.

Peduncle length was negatively related to AUDPC, accounting for more AUDPC variation than plant height, of which peduncle length and plant height were strongly correlated ($r = 0.71$; $P<0.001$). The resistance to *Fusarium* and *Microdochium* has previously been stated as greater for genotypes with at least 15cm of peduncle between flag leaf and ear, reducing the ear area in contact with conidia and reducing the duration of grain humidity (Mesterhazy, 1995). Negative relationships between peduncle length and FHB have also been reported by Nemati and Hokmalipoir (2012), with similar strengths of relationships with plant height supporting the theory that these traits are closely linked. Plant height, of which peduncle length is a component, has previously also been shown to be negatively related to FHB in previous experiments (Hilton *et al*. 1999; Buerstmayr *et al.* 2000; Gervais *et al.* 2003; Somers *et al.* 2003, Schmolke *et al.* 2005; Klahr *et al.* 2007; Srinivasachary *et al.* 2008; Srinivasachary *et al.* 2009; Yan *et al.* 2011; Chu *et al.* 2011; Nemati and Hokmalipor 2012; Suzuki *et al.* 2012; Lu *et al.* 2013; Liu *et al.* 2013; He *et al.* 2014), however several semi-dwarfing genes have been identified, including Rht-B1b and

Rht-D1b, which have a pleiotropic effect on increased FHB susceptibility (Lu *et al.* 2013) and this is therefore unlikely to be related to passive disease resistance.

Physiological traits such as ear length, spikelet number and spike density were unrelated to the AUDPC in the glasshouse experiments. This is generally in agreement with other studies which have also failed to identify such relationships between FHB and ear traits (Somers *et al.* 2003; Schmolke *et al.* 2005; Liu *et al.* 2007; Nemati and Hokmalipour, 2012). Exceptions to this include a weak but significant negative correlation between FHB and ear length (Suzuki *et al.* 2012) and ear density (Steiner *et al.* 2004).

Significant relationships between physiological traits and FHB were identified within this experiment, done so in an experiment where the role of passive disease resistance had been negated, therefore relationships identified between traits and FHB should be purely due to the genetic resistance of each genotype. The identified physiological traits, including flag leaf length, awn length, tiller number and plant height, are therefore likely to be related to FHB due to pleitrophy or co-segregation of genes controlling both the identified physiological trait and FHB resistance.

6.5 CONCLUSIONS

The results within this chapter enable the following conclusions to be made, based on the hypotheses in section 6.2:

i) The highly resistant genotypes Sumai-3 and Frontana had lower AUDPC values that UK elite and DH lines, with the exception of Line 14 and 51 in the 2011 experiment. Several DH lines however had lower AUDPC values that both Sumai-3 and Frontana in the 2012 experiment.

ii) Significant relationships were present between several canopy traits and AUDPC under spray inoculation conditions, being positively related to flag leaf length and negatively related to peduncle length.

iii) Awn length was the only ear trait to be significantly related to the AUDPC under spray inoculation conditions, of which a negative relationship was present.

Chapter 7.

Discussion

7.1 INTRODUCTION

Ground inoculation was utilised within the field experiments to enable passive resistance to occur by allowing the physiological traits of each genotype to interact with FHB inoculum as the season developed, thereby influencing the progression of *Fusarium* and *Microdochium* spores to the ear. Spray inoculation was used to measure the genetic resistance of genotypes in the glasshouse experiments, being applied until runoff, therefore bypassing all potential passive resistance mechanisms related to canopy traits. Significant relationships between physiological traits and FHB, occurring in both field and glasshouse experiments, were therefore likely to have an underlying genetic basis, whereas significant relationships identified in field experiments only, were likely to be caused by passive disease resistance mechanisms. These passive resistance mechanisms will potentially have been caused by physical barriers to inoculation within the field experiment, in contrast to active resistance mechanisms that are triggered by infection with a pathogen (Lewsey *et al.* 2009). An important factor for consideration is the need to separate between correlation and causation, for physiological traits relating to FHB. Therefore, before any physiological trait can be concluded to confer passive resistance to FHB, a plausible causation mechanism should be discussed.

This study has taken an important step beyond previously published experiments. It is the first study to combine both ground inoculated field experiments and spray inoculated glasshouse experiments, to elucidate which physiological traits confer a passive resistance mechanism to FHB. Previous studies have reviewed only a small number of basic physiological traits, whereas a much wider array were assessed within this study, including both canopy and ear traits.

A mixture of *Fusarium* and *Microdochium* spp. were used within the ground inoculated field experiments to reflect diversity in UK FHB pathogen populations occurring naturally and to take account of different dispersal mechanisms between species. For example, species including *F. graminearum, F. culmorum, F. poae, F. avenaceum, M. majus* and *M. nivale* produce macro- and micro- conidia (Leslie *et al.* 2006), which are splash dispersed (Jenkinson and Parry. 1994), while *F. graminearum* and *M. nivale* additionally produces ascospores that can be forcibly ejected into the air (Trail *et al.* 2005). The dispersal mechanisms of species such as *F. langsethiae* are however not yet fully understood (Imathiu *et al.* 2013). To reflect the *Fusarium* and *Microdochium* spp. range used in the field experiment, the same species were also selected for use within the glasshouse experiments.

Fungicide use was carefully considered within each field and glasshouse experiment. During the field experiments it was observed that the DH population generally had poor resistance to foliar diseases, specifically septoria leaf blotch (*Mycosphaerella graminicola*) and yellow rust (P*uccinia striiformis*). Therefore robust control of these foliar pathogens was required, while ensuring minimal deleterious effects on the *Fusarium* and *Microdochium* spores within the canopy, essential so as to avoid confounding effects within the experiment. Once ground inoculation had taken place, the fungicide strategy was based primarily around either cyproconazole or epoxiconazole, due to the limited activity of these active substances on FHB (HGCA, 2010c).

In the 2011 - 2013 field experiments, physiological trait assessments were carried out at two separate growth stages, these being GS39 and GS65. These growth stages were chosen to expand upon the 2010 field experiment and to reflect key phases in the wheat growth cycle, prior to, and during, FHB infection. Assessments at GS39 were undertaken to reflect

the emergence of the flag leaf as this was first stage in which the full crop canopy was present to influence the movement of spores. Assessments at GS65 were undertaken to reflect the state of the canopy during the critical phase for FHB infection, during which the ear is most susceptible to infection by FHB inoculum.

Variation in traits between UK elite genotypes was generally small, most likely reflecting the optimum characteristics for a high yielding UK wheat variety. In comparison, variation between DH genotypes was much greater, reflecting the large differences in parental phenotypes, as well as the absence of breeding selection. The DH genotypes were selected to provide wide variation in physiological traits. The UK elite and resistant control genotypes were included to complement the DH genotypes and enable comparison of their susceptibility.

Relationships between AUDPC and grain mycotoxin content were poor during the 2010 and 2011 field experiments. This may be due to the mixed species inoculation, of which several *Fusarium* species produce different mycotoxins (Bottalico and Perrone, 2002) and *Microdochium* spp. which are non-toxigenic but still produce visual symptoms (Logrieco *et al.* 1991). It seems plausible therefore that, since differences in visual symptoms cannot be distinguished between species (Xu *et al.* 2008), interactions between *Fusarium* and *Microdochium* spp. is likely to have caused visual symptoms that cannot be strongly related to any singular species. For grain mycotoxin content however, since each toxin is produced by a specific *Fusarium* species, it is possible to infer a relationship with an individual producer species, as shown within the multiple linear regression for each field experiment. The rationale to analyse AUDPC, pathogen DNA and grain mycotoxin content together within the PCA was based upon enabling groupings between pathogen species and mycotoxin to be visualised, as such groupings can occur when using a mixed inoculation.

7.2 CANOPY TRAITS

A wide range of canopy traits were assessed within the field experiments to test their influence on the movement of FHB inoculum towards the ear. This was deemed important as several studies have reported rain splash to be the primary dispersal mechanism for a wide range of fungal species, including *Phaeosphaeria nodorum* (Griffiths and Ao, 1976), *Rhynchosporium secalis* (Stedman, 1980), *Oculimacula spp* (Fitt and Bainbridge, 1983) and *Mycosphaerella graminicola* (Eriksen *et al.* 2003). *Fusarium* inoculum has also been found to be transported via rain splash, with research showing splash dispersal to potentially move *Fusarium* spores between 70 - 100 cm horizontally (Gregory *et al.* 1959; Jenkinson and Parry, 1994), and between 45 - 100 cm vertically (Jenkinson and Parry, 1994; Paul *et al.* 2004). It is noted by Jenkinson and Parry (1994) however, that it is unlikely for *Fusarium* conidia to be splash dispersed directly onto the ear, and that successful isolation of *Fusarium* species from the flag leaves of winter wheat suggests inoculum is carried towards the ear in a series of leaps. This evidence helps form the hypothesis that the canopy acts as a bridge for splash dispersal of inoculum between the ground and the ear. This hypothesis is supported by Edwards and Godley (2010), in which applications of *Fusarium* active fungicides at GS31 and GS39 reduced FHB, suggesting that fungicide applications reduced inoculum within the canopy which would have otherwise contributed to FHB infection. Additionally, it is clear that ground based inoculum is the primary source of FHB infection rather than wind transported airborne spores, since FHB is more abundant in wheat crops following maize (Maiorano *et al.* 2008), whereas as if airborne spores were the primary source of inoculum then the previous crop would have no significant effect. It should be noted, however, that the relative importance of both splash and wind dispersal depends upon the *Fusarium* and *Microdochium* species present within the pathogenic complex and their capacity to

produce ascospores (Champeil *et al.* 2004). Due to the reported predominance of splash dispersal, it seems likely that any physiological trait which increases the above ground area of plant material is likely to assist the vertical movement of spores. Alternatively, increasing ground cover and plant canopy area may negatively impact upon disease spread by reducing splash dispersal of ground level inoculum (Madden, 1997). Each physiological trait identified within this study needs to be considered in relation to their influence on the prior mentioned dispersal mechanisms.

Plant height was hypothesised to be negatively related to FHB, reflecting the genetic linkage between semi-dwarfing genes and FHB susceptibility that are well documented (Srinivasachary *et al.* 2008; Srinivasachary *et al.* 2009). In the 2011 and 2013 field experiments, a significant negative relationship between plant height and AUDPC was present at GS39 and GS65, showing that taller plants had less FHB infection, reflecting the role of either passive or genetic resistance, or both. In the glasshouse experiments, peduncle length, rather than plant height *per se*, was negatively related to AUDPC. Plant height and peduncle length can perhaps be considered as synonymous since peduncle length is a component of plant height, as shown by the strong positive relationship ($r = 0.69$; $P<0.001$) between the two traits in the glasshouse experiments. Therefore, since passive resistance was negated in the glasshouse experiments, it would appear that linkages between FHB resistance genes and height are present, as reported by Srinivasachary *et al.* (2009) with the *Rht-B*1 and *Rht-D*1 genes that are present within the DH genotypes used during the experiments (Foulkes, pers comm.), of which both of these *rht* genes confer reductions in Type I resistance. This however does not completely rule out plant height as having a passive resistance role in reducing FHB, for example Shaw and Royle (1993) reported that spores of *M.graminicola* were spread upwards from the base of the crop canopy, with the amount of inoculum transported decreasing rapidly with height. Therefore

logic dictates that taller plants should also have less FHB inoculum deposited on to the ear via rain splash, a route through which *Fusarium* spores are known to be dispersed (Gregory *et al.* 1959; Jenkinson and Parry, 1994; Paul *et al.* 2004). It is however difficult to separate between the role of genetic resistance linkage to plant height and true passive disease resistance. It is likely that genetic linkage makes up a significant proportion of the resistance, but once this link has been made within a study, it is commonplace for researchers to assume that the negative relationship between plant height and FHB is entirely due to the role of genetics, whereas the contribution that passive resistance may still make is overlooked. This study has not conclusively shown plant height to be a true FHB passive resistance trait, however evidence suggests that while linkage between plant height and FHB susceptibility plays a significant role, there is still room for plant height to confer an element of passive resistance.

Additional plant height traits such as peduncle length and distances between flag, second and third leaves were measured in the 2011 field experiment, based on the hypothesis that a shorter peduncle and smaller distances between leaf layers would increase overlap between the leaf layers, enabling spores an easier route to move up the canopy. Similar studies have reported a ladder effect on spore progression that was affected by the distances between each successive leaf layer (Bahat *et al*. 1980). However such traits within this study were all strongly correlated with plant height, therefore to avoid multi co-linearity within the MLR, only plant height was used. It is very difficult to distinguish if the distance between leaf layers had any effect on spore movement through the canopy or if the negative relationship between FHB and plant height was exclusively due to genetic linkage. Due to this, in the 2011 field experiment, pathogen spores within the canopy were quantified at GS65 using real time-PCR to detect differences between genotypes on the flag and second leaves. However, due to the low number of spores present on the leaves,

they were unable to be detected by real time-PCR. It is suggested that for this work to be repeated, an isolation technique from leaves onto potato dextrose agar should be used, enabling individual spores to produce colony forming units allowing for low spore numbers to be quantified. This experiment would enable spore quantities within the canopy to be related back to plant height, with a negative relationship indicating plant height as a true passive resistance trait. Despite being closely related to plant height, peduncle length could still provide a useful passive resistance trait by holding the ear away from spores present on the flag leaf as previously discussed by Mesterhazy (1995).

Flag leaf length and width were assessed within the field and glasshouse experiments. It was theorised within the field experiments that flag leaf length would be positively related to FHB by creating a larger area for spores to be deposited, from which to then be transferred to the ear. Additionally, longer flag leaves were expected to lead to more contact between each individual plant's flag leaf and the ears of neighbouring plants. Flag leaf width was theorised to compound the effects of flag leaf length, with a larger surface area for spores to be deposited. In the field experiments, flag leaf length had a significant positive relationship with AUDPC in 2010, at both GS39 and GS65 in 2011, although it was negatively related to HT2+T2 in 2010. Flag leaf length at GS65 in the 2013 field experiment PCA, was positively correlated with AUDPC, although this relationship was not significant in the MLR. This shows moderately consistent positive relationships between flag leaf length and FHB in the field experiments. In the glasshouse experiments, a positive significant relationship between AUDPC and flag leaf length was also present. Therefore, despite the moderately consistent positive relationships between FHB and flag leaf length in the field experiments, the presence of the same relationship in the glasshouse experiment suggests this relationship to be likely controlled by genetic linkage with FHB susceptibility, rather than due to passive resistance *per se*. The control of flag leaf length

itself is polygenic in nature (Sharma *et al.* 2003) although the specific genes controlling it have yet to be identified. Genetic linkage between flag leaf length and FHB susceptibility has not previously been reported in literature, therefore further work needs to confirm if a linkage is present. The flag leaf, however, is the most important photosynthetic driver of yield in wheat, contributing to around 41 - 43% of the total grain yield alone (Abrahim and Abo Elenein, 1977). It seems implausible therefore, for a breeding programme to select for a shorter flag leaf with the specific aim of improving FHB resistance, although it has been reported that flag leaves are gradually becoming shorter in modern cultivars (Shearman *et al.* 2005). Flag width at GS39 was significantly negatively related to AUDPC in the 2011 experiment only, indicating a lack of relationship with FHB.

Leaf area was hypothesised to be positively related to FHB by providing an increased surface area for splash dispersed spores to gain access to the ear of the plant, as this is the predominant route for inoculum to access the ear (Jenkinson and Parry, 1994). Leaf area per m^2 was assessed during the 2011, 2012 and 2013 field experiments, in which leaf areas of separate canopy layers were distinguished. These separate canopy layers (flag leaf area, second leaf area and remaining leaf area) were strongly positively correlated with each other. At GS39 in the 2011 field experiment, leaf area per m^2 was positively correlated with mycotoxins and *F. graminearum* DNA in the PCA, but this was non-significant within the MLR. At GS65 in the 2011 field experiment, leaf area per m^2 was also positively correlated with *F. graminearum* DNA in the PCA, except a significant positive and negative relationship between remaining leaf area and AUDPC and DON was present, respectively. Remaining leaf fresh weight at GS65 was significantly positively related to AUDPC in the 2012 field experiment, of which remaining leaf fresh weight was itself positively related to leaf area per m^2. Assessments of leaf area were unable to be undertaken within the glasshouse experiments, due to both the destructive nature of the

assessment and because it requires a plant population per unit area rather than a single plant. It is therefore not possible to directly conclude if the positive relationships between leaf area per m^2 and FHB in the field experiments were a passive resistance trait or not. It is interesting to note however, that the leaf areas within a unit of crop were not correlated to plant height traits in any of the field experiments, indicating that the confounding factor of genetic linkage between plant height and FHB susceptibility was not relevant here, indicating a possible basis to suggest that leaf area may be a true passive resistance trait to FHB. As with plant height, this potentially passive relationship could be further elucidated by quantifying the amount of FHB inoculum within the canopy at anthesis, and relating this back to leaf area per unit area. Fewer spores towards the flag and second leaves in a less dense canopy would indicate the ability of a less dense canopy to suppress the progression of FHB inoculum up the plant.

Fraction interception was assessed in the 2011 and 2012 field experiments to determine if the arrangement of the canopy on a population level could influence the development of FHB, reflecting specific relationships with canopy traits. In the 2011 experiment, fractional interception was measured at GS65, to assess the canopy at a critical stage in FHB infection, however only small differences between genotypes were observed at this growth phase, and no relationship with FHB was found. In 2012, a modified fractional interception technique started assessments at GS31 as there were large differences between genotypes at this stage, after which assessments continued at 7 day intervals until GS65. This was used to create a development curve, to show the development in fractional interception between genotypes over time in which larger differences between FI could be detected. This assessment was similar to that carried out by Lovell *et al.* (1997) in which sunflecks were assessed beneath the crop canopy to indirectly estimate the proportion of rain droplets passing directly through, finding that low nitrogen treatments resulted in between two to

four times more raindrops penetrating to the base without attenuation by the canopy. This is important as it shows that if canopy area were to be manipulated in order to reduce splash dispersal as a passive resistance mechanism, then this could likely be achieved via a crop management route than via breeding, although this seems an unlikely route though as nitrogen fertilisation is an important factor of crop yield.

Interestingly, it has been noted in terms of polycyclic foliar pathogens, that the introduction of fresh leaf material into the developing canopy can result in a simple dilution effect in which the host growth proportionally reduces the apparent infection rate (Ferrandino, 1998). This concept is relevant to the distribution of FHB inoculum through the canopy, despite FHB inoculum not being a polycyclic foliar disease. This system could help dilute early splash dispersal of spores on the base of the canopy, to replace it with new inoculum free material. This could be a potential passive resistance explanation as to why less FHB is observed in early heading varieties (Steiner *et al.* 2004), as a rapid growth phase between the start of stem extension and anthesis may help to keep spore number diluted within the canopy. This theory may however not be true since the relationship between leaf area and FHB in this study was generally positive, whereas if inoculum dilution within a rapidly expanding canopy were to play a significant role, then a negative relationship would have been expected.

Tiller number was hypothesised to potentially have a relationship with FHB by spreading the flowering date over a slightly longer period, thereby helping to spread risk during anthesis from weather conducive to FHB. Significant relationships between fertile shoots per plant (i.e. tiller number) and FHB were not present within any of the field experiments MLR, however positive groupings between fertile shoots per plant and mycotoxins were present in the PCA for the 2010 field experiment and at GS39 in the 2011 field experiment.

To contrast the lack of relationship between fertile shoots per plant in the 2010 and 2011 MLR, fertile shoots per plant was positively grouped together with mycotoxins in PCA of the 2010 field experiment and at GS39 in the 2011 field experiment. Mycotoxins were not quantified in the 2012 and 2013 field experiments, so relationships with fertile shoots per plant could not be determined, but the relationship with AUDPC remained weak in the PCA at both growth stages. This evidence shows an inconsistent relationship between tiller number and mycotoxin content during the field experiments and it is therefore unlikely to be a passive resistance trait.

For simplicity within this discussion, plant number and fertile shoots per m^2 have been grouped together since they are usually strongly related to each other and their hypothesised influence on passive resistance are the same. These traits were theorised to be positively related to FHB by providing a denser canopy for inoculum to be splash dispersed up the plant, or conversely, be negatively related to FHB by reducing the impact of splash dispersing rain at the base of the canopy. Plant number was positively related to DON content at GS39 in the 2011 field experiment MLR, while both plant number and fertile shoots per m^2 at GS65 were positively related to mycotoxin content in the 2011 field experiment PCA. Plant number and fertile shoots per m^2 were not assessed within the glasshouse experiment, due to the nature of the experiment, however, the lack of consistent relationship between these traits and FHB shows that it is unlikely that either plant number and fertile shoots per m^2 impart a passive resistance mechanism.

Dead/dying and potentially infertile shoots per plant and per m^2 were assessed only within the 2011 field experiment. These traits were assessed to reflect the amount of tiller death within the canopy with the hypothesis that tiller death may be caused by an underlying

gene linked to FHB resistance. This appeared not to be the case, with no relationship between either the number of dead/dying shoots or potentially infertile shoots with FHB.

Overall, further work is required to elucidate the influence of canopy morphology on the distribution of spores throughout the crop. This firstly requires for a better understanding of how *Fusarium* spores are dispersed through wind or rain dispersal, followed by being better able to visualise where spores are located within the canopy and how these are influenced by canopy morphology.

7.3 EAR TRAITS

Ear traits were assessed within field and glasshouse experiments to determine their relationship with FHB as a passive resistance trait. It was hypothesised that ear traits could passively influence the development of FHB via a number of different potential mechanisms.

Theoretically, longer ears may result in an increased level of FHB, by increasing the surface area present for spores to be deposited, or conversely, if longer ears contribute to plant height and are therefore controlled by semi-dwarfing genes, then a longer ear may have a genetic basis to resist FHB. No significant relationships were present between ear length and FHB in any field or glasshouse experiment, although a positive relationship was present in the 2013 GS65 PCA, showing a general lack of ability to confer passive resistance. This is in agreement with other studies which report an absence of relationship between ear length and FHB (Somers *et al.* 2003), with the exception of Suzuki *et al.* (2012) in which a negative relationship was reported. Interestingly, ear length and plant height were both strongly positively correlated at GS65 in the 2011 and 2012 & 2013 field experiments PCA, although this correlation was much weaker in the glasshouse experiment. A positive relationship between plant height and ear length ($r = 0.55$) was also reported by Suzuki *et al.* (2012), of which overlapping QTL on chromosomes 4BS and 5AS were identified. This shows potential for ear length to be controlled by the same genes that control plant height, which may either be plant height genes linked to FHB susceptibility such those described by Srinivasachary *et al.* (2009) or Suzuki *et al.* (2012), or plant height genes which are independent of FHB susceptibility (Gervais *et al.* 2003). It is important to note that in several studies in which relationships between plant height and FHB were assessed, plant height assessments included the length of the ear (Buerstmayr *et*

al. 2000; Gervais *et al.* 2003; Klahr *et al.* 2007; Srinivasachary *et al.* 2008), Srinivasachary *et al.* 2009; Chu *et al.* 2011). This confirms the potential for QTL attributed to plant height to also influence ear length, as initially suggested by Suzuki *et al.* (2012), meaning that any relationship identified between FHB and ear length are likely due to genetic linkage between plant height and FHB susceptibility (Srinivasachary *et al.* 2009).

Significant positive relationships between spikelets per ear and HT2+T2, and spikelets per ear and AUDPC, were present in the 2010 and 2012 field experiments, respectively. Strong negative correlations between spikelets per ear at GS65 and AUDPC were present in the 2011 field PCA also, but this was not significant within the MLR. No relationship between spikelets per ear and FHB was present within the glasshouse experiment. Relationships between spikelet number per ear and FHB were inconsistent between field experiments, although the lack of relationship within the glasshouse experiment indicates a potential passive resistance trait. Previous studies have not assessed spikelet number, therefore no relationships between spikelet number and FHB have previously been reported with which to compare these results. Due to the lack of consistent relationship with FHB, it seems unlikely that spikelet number per ear could confer any meaningful level of passive resistance. It is important to consider however, that an ear morphology optimised for grain yield has been noted as an important step in breeding progress for grain yield in future years (Foulkes *et al.* 2007), suggesting that it would be unlikely for ear morphology traits such as spikelet number to be selected for in favour of passive disease resistance. This would be especially applicable in high yielding environments in which simultaneous selection for increases in sink and source size are required to maintain the present rate of yield progress (Foulkes *et al.* 2007).

Spikelet density was hypothesised to increase type II resistance by increasing the distance within the rachis that the invading fungus has to travel to further infect more spikelets, as spread within the rachis is an important means of Type II spread (Mesterhazy 1995). A similar hypothesis was also briefly suggested by Rudd *et al.* (2001). A less dense ear was also hypothesised to allow moisture to evaporate faster, creating a less conducive environment for FHB infection. Spikelet density was not significantly related to FHB in any of the field or glasshouse experiments. Weak but significant correlations between FHB and ear density have previously been found, with both positive (Buerstmayr *et al.* 2012) and negative (Steiner *et al.* 2004) relationships reported, however the majority of studies have failed to identify any relationship at all (Schmolke *et al.* 2005; Nemati and Hokmalipour, 2012). Overlapping QTL controlling both ear density and FHB, as well as controlling plant height also, have been identified on chromosome 6AL, although no significant correlation between ear density and FHB severity being observed within the study (Schmolke *et al.* 2005). Additional studies have identified QTL controlling ear density on chromosomes 5A and 7A, and FHB severity on chromosomes 3B, 4B, 6A, 6B and 7B, which did not overlap for the two traits (Buerstmayr *et al.* 2012). Despite the identification of a single overlapping QTL with FHB resistance, the evidence consistently suggests that relationships between spikelet density and FHB are inconsistent or absent, suggesting that spikelet density does not confer passive resistance to FHB.

Awn length was theorised to be a potential passive resistance trait by acting as a barrier to *Fusarium* spores being deposited on the ear, be it via splash dispersed conidia or windblown ascospores, therefore minimising contact between the susceptible ear surfaces and the pathogen. This theory could be tested by trying to isolate *Fusarium* spores from healthy awns, to see if they are capable of capturing spores. Conversely, longer awns may themselves be capable of being infected by *Fusarium* spores, and increasing their length

could increase their surface area on which inoculum could be deposited. One aspect of this theory that requires further research is whether spores are able to directly infect the awns, before moving down their vascular tissue to infect the ear. Only awn presence/absence was measured in the 2010 experiment, which was not related to FHB. The specificity of this assessment was improved in further field experiments, by measuring awn length instead. Awn length at GS65 was found to have a consistent negative relationship with AUDPC in the 2011, 2012 and 2013 field experiments, and a significant negative relationship with AUDPC in the glasshouse experiment too. These results are in agreement with other published results which found relationships between longer awns and decreased FHB infection (Buerstmayr *et al.* 2000; Ban and Suenaga 2000; Buerstmayr *et al.* 2012) but they also conflict other studies that have shown that longer awns can increase FHB infection (Somers *et al.* 2003; Lui *et al.* 2013), and some that have shown there is no relationship between awn length and FHB at all (Buerstmayr *et al.* 2000; Buerstmayr *et al.* 2002; Hori *et al.* 2005; Liu *et al.* 2007). This shows that, due to the significant negative relationship between awn length and AUDPC in both the field and glasshouse experiments, that awn length is unlikely to be a true passive resistance trait. Furthermore, linkage between awn presence and FHB have been found on chromosomes 4B (Snijders, 1990), and on chromosome 5A in which the B1 marker for awndness is present (Gervais *et al.* 2003; Lui *et al.* 2013). However not all awn length genes are coincident with FHB resistance genes, such as that found on chromosome 6BS (Somers *et al.* 2003). Despite the reported genetic linkage between FHB and the presence of awns, it has been shown that this linkage can be easily broken and the development of awnless cultivars with improved levels of FHB possible (Gervais *et al.* 2003).

Hairs on the ear surface were hypothesised as a passive resistance trait for their ability to create a barrier between the ear surface and FHB inoculum. No significant relationships

were identified between FHB and ear hairs, potentially due to only Line 61 having ear hairs present. This suggests that the genotypes used within these experiments did not have enough variation in ear hair traits to form a suitable test to determine the relationship between ear hair and FHB. To test this trait in future experiments, it is suggested that additional wheat genotypes with ear hairs present are included within the study.

Anther extrusion was assessed in the 2011 field experiment only. Previous studies have consistently identified negative relationships between anther extrusion and FHB (Skinnes *et al*. 2005; Skinnes *et al.* 2008; Graham and Brown. 2009; Skinnes *et al.* 2010; Buerstmayr *et al.* 2012; Lu *et al.* 2013; He *et al.* 2014), theorising that, since emasculation of anthers after inoculation reduces FHB (Liang *et al.* 1981), that increased anther extrusion removes the readily colonisable anthers away from the pathogens use. In most of these studies only anther extrusion was assessed, whereas in reality the fate of anthers can fall within one of three categories, that being fully retained, trapped between the lemma and palea, or fully extruded. It was hypothesised that trapped anthers would be the most important of the three scenarios as it would act as a nutrient rich bridge between the tough outer floral tissues and the susceptible internal floral tissues. Therefore within the 2011 experiment, the proportion of anthers falling into each category was distinguished to enable the relationship between FHB and each anther scenario to be determined. Trapped anthers had a significant positive relationship with grain DON content in the 2011 field experiment, which conflicts with the results of Graham and Brown (2009) which found retained anthers to be more strongly related to FHB. Anther extrusion was not assessed within the glasshouse experiments due to the assessments destructive nature, nor in the 2012 and 2013 field experiments, as it had previously been identified as consistently related to FHB, therefore resources were used instead to identify novel traits. Within the glasshouse experiments, despite being designed to negate passive resistance, differences in

anther extrusion between genotypes could have theoretically played a passive role in the development of FHB. This is due to differences in anther extrusion potentially influencing how readily the spores could gain entry and infect the ear once applied. Most anther extrusion experiments carried out using spray inoculation techniques have shown a significant negative relationship with FHB, adding weight to the theory that anther extrusion is a true passive resistance trait. In contrast, a genetic basis for this negative relationship has also been identified, with several QTL overlapping between anther extrusion and FHB severity (Lu *et al.* 2013). Additionally, Lu *et al.* (2013) found positive correlations between anther extrusion and plant height, with two common QTL between them. This indicates that the degree of anther extrusion may be genetically linked to well documented relationship between plant height and FHB susceptibility. Regardless of whether anther extrusion is a true passive resistance trait, the low interaction between the degree of anther extrusion and year, high broad-sense heritability (0.90 – 0.91) (Skinnes *et al.* 2005; Skinnes *et al.* 2010) and consistent negative relationship with FHB (Skinnes *et al.* 2005; Skinnes *et al.* 2008; Graham and Brown. 2009; Skinnes *et al.* 2010; Buerstmayr *et al.* 2012; Lu *et al.* 2013; He *et al.* 2014) make this trait a suitable candidate in breeding programmes for improving FHB resistance. Interestingly, it has been suggested that low anther extrusion enables inoculum to penetrate floral tissue and trigger active resistance mechanisms, whereas these active resistance mechanisms contribute much less to overall FHB resistance for high anther extrusion, due to the reduced initial inoculum load entering the floret (Skinnes *et al.* 2008) and therefore indicating high anther extrusion as a Type I resistance trait. It would be useful to compare the up-regulation of FHB active resistance transcripts after inoculation, between high and low anther extrusion lines, to confirm this hypothesis. Overall, anther extrusion seems to be one of the physiological traits that could offer significant improvements to FHB resistance. This makes sense, as anther extrusion

occurs within the FHB infection site, and at a crop development phase in which the majority of FHB infection occurs, therefore logic dictates that any physiological changes within this region are the most likely to influence levels of FHB infection.

The passive influence of ear traits on FHB were perhaps somewhat confounded within the field experiments. This is because the passive influence of ear traits is subject to the interaction between pathogen inoculum and the canopy prior to reaching the ear, thereby the ears of each genotype are likely to have been subject to an uneven spore load. It stands to reason that a fair comparison between the effects of ear traits cannot be made unless equal pathogen spore loads are applied to each ear. It is recommended for future field experiments, that both separate ground inoculation and spray inoculation experiments should be used to make fair assessments between the passive influence of ear and canopy traits on FHB.

7.4 GROWTH STAGES

Assessments of physiological traits were carried out at GS39 and GS65 in the 2011, 2012 and 2013 field experiments. In general, only a small proportion of traits were strongly correlated ($r > 0.70$) between growth stages, indicating that the comparable morphological development between each growth stage is different for each genotype.

In the 2011 field experiment, GS39 and GS65 accounted for similar proportions in AUDPC (51% and 49%, respectively) but more variation in grain DON content was accounted for by GS65 (19% and 28%, respectively). In the 2012 field experiment, similar proportions of the AUDPC were accounted for between GS39 and GS65 (37% and 38%, respectively). However, in the 2013 field experiment, large differences were present between GS39 and GS65 for the variation accounted for in the AUDPC (4.8% and 29.3% respectively).

This shows that, despite the lack of correlation between growth stages for physiological traits, assessments using GS65 traits are marginally better able to account for variation in AUDPC and DON. It is therefore suggested for future work that assessments at GS39 are not necessary and offer no additional benefits over assessing GS65 alone. Additionally, on a practical note, since growth analysis is a time consuming process, the assessment of two growth stages at such close intervals leads to constraints on time and results in an overlap of growth stage assessments which is a potential source of experimental error.

7.5 BREEDING

For any identified FHB passive resistance trait to be of benefit to the wider community, it must be capable of manipulation through crop management, a heritable trait suitable for selection via plant breeding, or it must enable better prediction of FHB epidemics and hence mycotoxin contamination of grain. Plant breeding is the most likely option, as crop inputs are already optimised for high yielding wheat, and varietal resistance is already accounted for within predictive FHB models (Edwards, 2010). Plant breeding is however considered the most sustainable option for reducing the impact of FHB. Passive resistance traits would potentially provide a welcome source of resistance to FHB breeding programmes, since passive resistance predominantly prevents contact occurring between FHB inoculum and the susceptible ear, therefore it can be considered a Type I resistance mechanism by providing resistance against initial infection. Type I resistance is much more elusive than Type II resistance, which reduces pathogen spread within the ear via the rachis (Mesterhazy, 1995). However, Type II resistance has been reported to be more genetically stable than that of Type I resistance (Bai and Shaner, 2004). Ear traits such as length and density were hypothesised within this study to be passive resistance traits which could provide Type II resistance by slowing the spread of the pathogen within the ear, however these physiological traits did not show consistent relationships to FHB.

Consideration within breeding programmes needs to be given towards the trade-off between the implementation of passive resistance traits to improve FHB resistance, and the impacts on grain yield. For example, in relation to the identified positive relationship between height and FHB resistance, optimum crop height for yield is estimated to be around 70 to 80 cm (Shearman *et al.* 2005). This shows potential yield penalties, whether in the presence or absence of disease, for breeding taller crops for passive disease

resistance, especially where constrained by lodging risk (Berry *et al.* 2000). Furthermore, in relation to the positive relationship identified between flag leaf length and FHB, it has been suggested that reductions in flag leaf size in modern wheat varieties has possibly reduced supersaturating light intensities at the top of the crop canopy (Shearman *et al.* 2005), enabling improved light penetration to the lower leaf layers, with potential benefits for yield.

For breeding programmes to produce new varieties with specific desirable traits, suitable sources of parental breeding material must firstly be identified. While the DH genotypes used within this study exhibited wide variation in physiological traits, they are unlikely to be suitable for incorporation in the breeding programme for high yielding wheat due to linkage drag from the non-elite DH lines, which occurs when genes of deleterious effects are tightly linked to traits of interest (Zamir, 2001). Incorporation of such passive resistance traits would be best achieved by locating suitable variation within elite material to provide both improved FHB resistance and acceptable agronomic traits such as grain yield and quality.

7.6 OVERALL CONCLUSIONS

Addressing the specific hypotheses presented in Section 1.7 of this study, the following conclusions can be drawn:

i) Correlations between visual FHB symptoms and both pathogen DNA and grain mycotoxin contamination were poor. Therefore the use of visual FHB assessments is a poor indicator of both pathogen infection and mycotoxin contamination under mixed species inoculation.

ii) Each grain mycotoxin contaminant was successfully related back to a specific *Fusarium* producer species.

iii) Flag leaf length and remaining leaf area were the most consistent physiological traits to be positively related to FHB in ground inoculated field experiments.

iv) Plant height and awn length were the most consistent physiological traits to be negatively related to FHB in ground inoculated field experiments.

v) Tiller number and flag leaf length were the most consistent physiological traits to be positively related to FHB in spray inoculated glasshouse experiments.

vi) Awn length and peduncle length were the most consistent physiological traits to be negatively related to FHB in spray inoculated glasshouse experiments.

vii) Differences in physiological traits related to FHB under high or low disease pressure in ground inoculated field experiments could not be determined, due to lack of differences between misting treatments.

viii) (a) Since canopy leaf area showed a moderately consistent positive relationship to FHB in ground inoculated field experiments, but was not correlated to plant height and hence the genetic linkage to FHB susceptibility, there is a basis to support the role of reduced canopy leaf area as conferring passive resistance to FHB.

viii) (b) Plant height showed a negative relationship with FHB in both ground and spray inoculated experiments, confirming the likely role of genetic linkage between plant height and FHB susceptibility, although a small FHB passive resistance role for plant height remains likely.

The primary aim of this study was to identify physiological traits in wheat conferring passive resistance to FHB. This objective has been achieved by identifying plant height and remaining leaf area as potential passive resistance traits, although these relationships with FHB are not always consistent and require further work to confirm their effects.

This study could have been further improved by including a wider range of physiological traits such as anther extrusion and heading date from the 2011 field experiment, into the 2012 and 2013 field experiments, to quantify their relationship with FHB. Additionally, time constraints resulted in mycotoxin analysis on harvested grain from the 2012 and 2013 field experiments being unable to be performed, which would have otherwise provided a valuable source of data.

7.7 FUTURE WORK

Within this study, multiple relationships between physiological traits and FHB were identified. Physiological traits conferring passive resistance to FHB were elucidated by comparing ground inoculated field and spray inoculated glasshouse experiments. It was felt that the elucidating strength of these experiments would be improved by additionally quantifying the FHB inoculum during anthesis at different leaf levels within the canopy, since splash dispersal is the predominant form of disease spread and it would therefore be possible to compare the effects of physiological traits on inoculum progression towards the ear. This experiment would be useful for the assessment of canopy traits, such as canopy area, however as previously discussed, this method is perhaps not suited to assessing the relationship between ear traits and FHB, due to canopy effects resulting in each ear receiving an uneven spore load. To assess the influence of ear traits, it is suggested that spray inoculated field experiments be used as this enables the effects of the ear's microclimate to take effect, such as reduced drying time on less dense ears.

Making fair and comparable visual assessments of FHB symptoms in each DH line was difficult, due to the differences in visual appearance of the wheat ear. Many lines had ears with natural markings or blemishes on the glumes, or with small amounts of Septoria nodorum (*Phaeosphaeria nodorum*) present, which all had to be carefully distinguished from FHB symptoms. Visual FHB assessments in future experiments would benefit from a predetermined definition (or visual threshold) of a lesion or bleaching symptom, to take into account the differences in visual appearance of ears between genotypes.

Consideration needs to be given to the size of plots used in future experiments. Plot sizes in the 2012 and 2013 field experiments were reduced to $1m^2$, down from $12m^2$ in the 2010 and 2011 field experiments, and this was done to facilitate the inclusion of a larger number

of genotypes and a misting treatment into the trials area available. The perceived problem with this is that small plots may have not be able to truly reflect passive spore movement, in that the whole plot would be subject to an edge effect. For example, for the canopy to realistically interact with wind dispersed *F. graminearum* ascospores released below the canopy, then a wider area of crop would be needed, otherwise the spores can potentially migrate around the edges of the plot. Additionally with rain splash dispersed conidia, rain landing onto bare soil at the edge of the plot would not have been slowed down via the canopy, therefore creating a greater horizontal and vertical dispersal of soil inoculum, as described similarly by Madden (1997). The result of this would potentially be that the open edge of each plot becomes an easier target for these splashed spores to be deposited onto, continually working further up the plant. It is recommended that future experimental work reverts back to using $12m^2$ plots, to minimise potential edge effects.

Further consideration to the use of AUDPC (as a measurement of visual FHB symptoms over time) and mycotoxin quantification should be made for future experiments. In this study, AUDPC provided a very useful tool for standardising the assessment of visual symptoms in a range of wheat genotypes which differed widely for flowering date and visual FHB symptom expression. This method of visual assessment is a valid tool for comparisons to be made to grain mycotoxin levels in harvested grain, as mycotoxins and visual symptoms often occur simultaneously in the field, although this relationship is not exclusive. It would be interesting however to also compare mycotoxin accumulation to a score of visual symptom expression at a specific time point post mid-anthesis, for example 14 days after mid-anthesis, to see if mycotoxin content is more strongly related to AUDPC or to an assessment made at a single point in time.

A specific aspect to the dispersal of FHB inoculum that is seldom considered, but warrants further investigation, is the potential role of secondary inoculum derived from the parasitic and saprophytic infection of wheat leaves, creating additional spores within the canopy that may then be splash dispersed to the ear. Ali and Francl (2001) successfully isolated several *Fusarium* species associated with FHB, including *F. graminearum and F. avenaceum*, from both healthy (asymptomatic) and diseased leaves, concluding that these species can survive both parasitically and saprophytically on leaves and may therefore contribute to additional FHB inoculum. Wagacha *et al.* (2012) also reported the colonisation of leaves by various *Fusarium* species producing infection that was both symptomatic and asymptomatic, although senescing tissue could be induced to produce conidia under humid conditions, being suggested to be capable of spreading to the ear to increase the inoculum load. Ali and Francl (2001) suggested that the management of foliar pathogens may additionally help to reduce the intensity of FHB by decreasing FHB inoculum, a statement that is supported by Edwards and Godley (2010), in which the foliar application of *Fusarium* active fungicides at either GS31 and GS39 led to reductions in visual FHB symptoms and DON contamination, suggesting that fungicide applications prior to anthesis can reduce *Fusarium* inoculum within the canopy. However, further research is required to confirm if the production of secondary FHB inoculum on leaves under field conditions is in an order of magnitude great enough to result in increased FHB incidence, and if this is a common or sporadically produced source of inoculum.

Plant height is a trait requiring further elucidation from the genetic linkage between height and FHB susceptibility, and the contribution to passive FHB resistance that plant height could make should be further investigated. It is recommended that ground inoculated experiments using plant growth regulators to vary the heights of elite cultivars would be most suitable as this ensures that the genetic resistance of each cultivar remains constant. It

is recommended that growth analysis would also be carried out to assess the influence of plant growth regulation on other physiological traits.

Most importantly, it would be beneficial for QTL analysis to be performed on the full Rialto/Line 8 DH population comprising 88 lines, including the DH lines used with this study, to help further elucidate genetic linkages between physiological traits and FHB resistance. This would benefit future field and glasshouse experiments significantly.

Chapter 8.

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Chapter 9.

Appendices

Oral presentation: Epidemiology Canopy Architecture International Conference, French National Institute for Agricultural Research (INRA), Rennes : France, 1-5 July 2012.

IDENTIFICATION OF PHYSIOLOGICAL TRAITS IN WHEAT CONFERRING PASSIVE RESISTANCE TO FUSARIUM HEAD BLIGHT.

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Fusarium head blight (FHB) is a devastating fungal disease of wheat and other cereals worldwide, caused by a complex of *Fusarium* and *Microdochium* species. Infection of the wheat head occurs during anthesis and leads to reductions of grain yield, grain quality and the increased production of harmful mycotoxins. Methods available for the control of FHB include crop rotations, deep cultivations and fungicide applications. Whilst these methods have been shown to partially control the disease, the development of cultivars with improved FHB resistance is considered the essential step towards reducing the impact of this disease and ensuring future food security. A number of FHB resistance genes have been identified within the wheat genome, however the potential contribution of passive resistance to disease control has generally been overlooked.

This study aims to identify and quantify canopy and ear traits in wheat conferring passive resistance to FHB through disease escape strategies. A field experiment was carried out in 2010/2011 using 5 UK winter wheat varieties and 10 doubled-haploid lines derived from a cross between a spring wheat advanced line of large-ear phenotype from CIMMYT, Mexico and the UK winter wheat variety, Rialto. All 15 wheat genotypes were ground inoculated using infected oat grains with a mixture of *Fusarium graminearum, F. culmorum, F. avenaceum, F. langsethiae, F. poae, Microdochium majus* and *M. nivale* at GS30. Canopy and ear traits of the wheat genotypes were assessed at GS39 and GS65. Visual disease symptoms were scored at regular intervals from mid-anthesis and used to calculate the area under the disease progress curve (AUDPC) for each wheat genotype. Mature grain from each wheat genotype was milled to fine flour which underwent DNA extraction and quantification of individual *Fusarium, Microdochium* species and Tri5 DNA using Real-Time PCR.

Five of the CIMMYT/Rialto lines had a similar AUDPC compared to the control lines, while the other five doubled haploid lines showed a significantly higher AUDPC to that of the controls. Overall, in descending order, the predominant species found at harvest were *F. poae*, *F. culmorum* and *F. langsethiae*. Multiple linear regression ($P<0.001$) accounted for 50% of the AUDPC variance and showed that Y (AUDPC) = 19.7 – 0.0814 x1 (GS65 total plot fresh weight) – 0.2302 x2 (GS65 Average ear height) + 1.605 x3 (GS65 Flag leaf length) + 152.4 x4 (*F. poae* DNA). Tri5 DNA, quantified in grain flour was positively correlated with AUDPC ($P=0.003$) and was significantly related to the individual DNA of *F. poae* and *F. langsethiae*.

Work is continuing on the simultaneous quantification of multiple Fusarium mycotoxins in wheat flour. This additional information will assist in building a more complete picture of the resistance of the experimental lines used in this study.

Keywords

Fusarium, Passive, Resistance.

Poster presentation: 12th European Fusarium Seminar, French National Institute for Agricultural Research (INRA), Bordeaux : France, 12-16 May 2013.

PHYSIOLOGICAL TRAITS IN WHEAT CONFERING PASSIVE RESISTANCE TO FUSARIUM HEAD BLIGHT

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INTRODUCTION

Fusarium head blight (FHB) is a devastating fungal disease of wheat and other small-grain cereals. Strategies to control FHB have so far paid little attention to the contribution that passive disease resistance could play in reducing the disease. This study aims to quantify the effects that physiological traits play in the severity of FHB epidemics and mycotoxin contamination.

MATERIALS AND METHODS

Field experiments were performed at Sutton Bonington in 2009/2010 and 2010/2011 using 5 elite UK winter wheat varieties and 10 doubled-haploid (DH) lines to examine the effects of varying traits on the passive disease resistance of wheat to FHB.

Both experiments were ground inoculated at GS31 using oats infected with a mixture of *Fusarium graminearum*, *F.culmorum*, *F.poae*, *F.langsethiae* and *F.avanaceum*. Physiological traits were assessed at GS65.

Visual disease assessments were made from GS65 onwards to calculate the Area Under Disease Progress Curve (AUDPC). Real-time PCR and LC-MS was carried out on the grain to quantify fungal DNA and mycotoxin contamination respectively.

RESULTS

Multiple linear regression (MLR) was used to analyse the relationship between physiological traits, AUDPC, fungal DNA and mycotoxin content of the grain (Table 1).

Table 1. MLR of physiological traits with AUDPC and mycotoxin content

Model	Equation	R2	P-value
AUDPC 2010	5.39 + 1.06 (Fc) + 0.511 (FL) - 1.295 (TN)	0.44	<0.001
AUDPC 2011	8.19 - 0.0219 (SL) + 0.2899 (FL) + 1.099 (Fp) - 0.596 (EL)	0.44	<0.002
DON	-0.028 (FL) - 0.004 (SE) - 0.008 (TN) + 0.794 (Fg) + 4.276 (2010)	0.64	<0.001
	-0.028 (FL) - 0.004 (SE) - 0.008 (TN) + 0.794 (Fg) + 3.52 (2011)		
HT2+T2	3.322 (2010) - 0.0303 (FL) + 0.143 (FW) - 0.0117 (SE) + 0.0002 (TN) + 0.7 (FI)	0.71	<0.001
	2.622 (2011) - 0.0303 (FL) + 0.143 (FW) - 0.0117 (SE) + 0.0002 (TN) + 0.7 (FI)		

Fc = *F.culmorum* DNA (pg/ng); FL = Flag leaf length (cm); TN = Tiller number per plant; SL = Stem length (cm); Fp = *F.poae* DNA (pg/ng); EL = Ear length (cm); SE = Spikelets per ear; Fg = *F.graminearum* DNA (pg/ng); YR = Year; FW = Flag width (cm); FI = *F.langsethiae* DNA (pg/ng)

The main pathogens related to the AUDPC were *F.culmorum* and *F.poae* in 2010 and 2011, respectively (Table 1). Deoxynivalanol (DON) and HT2+T2 contamination in both years were related to *F.graminearum* and *F.langsethiae* respectively (Table 1).

ANOVA was carried out on all data to detect significant differences in the selected experimental material used in the field trial, as well as the interactions between years (Table 2).

Table 2. ANOVA of the AUDPC and main physiological traits

	AUDPC		Flag leaf length		No of tillers		Ear length		Spikelets per ear	
	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011
Rialto	3.4	4.1	12.5	16.0	6.9	1.4	8.3	10.9	19.9	25.3
Line 14	75.0	13.7	15.8	12.8	4.0	1.3	9.1	9.4	19.6	21.1
Line 16	106.6	41.0	17.2	17.4	5.2	2.0	8.7	9.8	18.3	21.1
Line 18	23.1	41.8	17.2	20.8	5.8	1.7	8.7	9.2	18.5	18.9
Line 21	57.5	16.2	17.2	21.3	4.1	1.7	11.5	13.4	19.5	24.1
Line 24	23.0	16.6	14.7	14.1	5.7	2.0	8.3	9.6	19.1	22.9
Line 38	9.3	2.6	15.0	14.1	6.7	2.1	9.6	10.9	19.6	24.2
Line 39	24.1	4.3	16.9	15.5	6.2	1.5	10.9	12.9	20.5	24.4
Line 51	30.4	4.2	17.2	17.8	5.2	2.3	9.6	11.7	17.8	21.3
Line 61	19.9	3.2	18.4	19.0	5.8	1.7	12.3	12.7	20.7	21.2
Line 77	24.7	2.0	15.4	16.6	6.7	1.6	9.8	11.2	20.0	23.7
Claire	3.8	7.3	14.3	16.7	6.8	2.0	9.7	11.3	21.5	26.3
Solstice	2.1	6.6	15.5	15.9	5.2	1.8	9.9	10.2	19.6	21.1
Ambrosia	1.8	5.0	14.7	15.7	6.6	2.0	8.6	10.0	19.2	21.3
Grafton	10.5	4.6	13.8	15.1	6.7	2.9	7.6	9.3	17.2	19.6
P-value (Year*Variety)	<0.001		0.002		<0.001		0.015		<0.002	
L.S.D. (Year*Variety)	25.55		2.262		0.9207		0.2279		1.389	
CV%	79.7		8.6		14.7		5.5		4.1	

Table 3. ANOVA of selected mycotoxins and DNA content

	DON	Fg Log10	HT2+T2	FI (Log 10)
Rialto	1.96	-1.96 (0.011)	1.25	-1.98 (0.011)
Line 14	1.77	-2.23 (0.006)	1.31	-2.15 (0.007)
Line 16	2.40	-2.24 (0.006)	0.95	-2.42 (0.004)
Line 18	1.50	-2.39 (0.004)	0.93	-2.05 (0.009)
Line 21	1.42	-2.19 (0.007)	0.99	-2.51 (0.003)
Line 24	1.60	-2.12 (0.008)	1.28	-1.94 (0.011)
Line 38	1.41	-2.43 (0.004)	0.88	-2.16 (0.007)
Line 39	1.82	-2.21 (0.006)	1.15	-1.97 (0.011)
Line 51	1.58	-2.65 (0.002)	0.60	-2.71 (0.002)
Line 61	1.69	-2.43 (0.004)	1.01	-2.50 (0.003)
Line 77	1.41	-2.67 (0.002)	0.82	-2.65 (0.002)
Claire	1.80	-2.45 (0.004)	1.43	-2.10 (0.008)
Solstice	2.12	-2.10 (0.008)	1.00	-1.94 (0.012)
Ambrosia	2.30	-1.48 (0.033)	1.46	-1.39 (0.041)
Grafton	1.75	-2.51 (0.003)	1.36	-1.86 (0.014)
P-value (Variety)	0.002	0.033	0.017	0.007
L.S.D. (Variety)	0.5156	0.5976	0.4688	0.624
L.S.D. (Year)	0.1883	0.2182	0.1712	0.2279
CV%	25.3	22.8	37.1	25.1

The effect of variety on mycotoxins and DNA concentrations was consistent for both years of experimentation (Table 3). DNA and mycotoxin concentrations were significantly higher in 2010 than in 2011.

CONCLUSIONS

- Flag leaf length, tiller number, ear length and stem length were identified as the main traits contributing significantly to the variation in AUDPC.
- There were no significant relationships between AUDPC and mycotoxin contamination, therefore under mixed inoculation, AUDPC is not a useful measure to screen for resistance to mycotoxins.
- Relationships between DON, HT2+T2 and physiological traits showed that several traits could potentially be used by breeders to increase resistance to DON accumulation.

Appendix 2A. *Fusarium* and *Microdochium* isolates used in 2010 & 2011 and 2012 and 2013 field experiments.

Species	Isolate code
F.graminearum	212, 241, 214
F.culmorum	218, 215, 236
F.poae	246, 245, 232
F.avanaceum	248, 210, 235
F.langsethiae	243, 247, 221
M.majus	213, 211, 224
M.nivale	226, 222

Appendix 2B.1. CTAB buffer recipe used within the extraction of grain DNA

Chemical	Quantity
Sodium chloride	87.7 g
D-sorbital	25 g
Sarkosyl	10 g
Cetyltrimethylammonium bromide (CTAB)	8 g
Ethylenediaminetetraacetic acid (EDTA)	8 g
Poly(vinylpolypyrrolidone) (PVPP)	10 g
(deionised water)	Made up to 1 litre

Appendix 2B.2. Potassium acetate solution recipe used within the extraction of grain DNA

Chemical	Quantity
Potassium acetate	500 g
Deionised water	Made up to 1 litre

Appendix 2C. Forward and reverse primers used in the real time-PCR of Tri5, *Fusarium* and *Microdochium* spp.

Tri5	SETRI5/F	5'-CAGATGGAGAACTGGATGGT-3'
	SETRI5/R	5'-GCACAAGTGCCACGTGAC-3'
F.graminearum	Fg16NF	5'-ACAGATGACAAGATTCAGGCACA-3'
	Fg16NR	5'-TTCTTTGACATCTGTTCAACCCA-3'
F.culmorum	Fc01F	5'-ATGGTGAACTCGTCGTGGC-3'
	Fc01R	5'-CCCTTCTTACGCCAATCTCG-3'
F.langsethiae	FspoF1	5′-CGCACAACGCAAACTCATC-3'
	LanspoR1	5′-TACAAGAAGACGTGGCGATAT-3'
F. avenaceum	Fave574 fwd	5'-TATGTTGTCACTGTCTCACACCACC-3'
	Fave627 rev	5'-AGAGGGATGTTAGCATGATGAAG-3'
F. poae	FpoaeA51	5'-ACCGAATCTCAACTCCGCTTT-3'
	FpoaeA98 rev	5'-GTCTGTCAAGCATGTTAGCACAAGT-3'
M. majus	Mmajus1f	5'-AACCCCTCCCGGGTCAG-3'
	Mmajus1r	5'-GGATAAACGACACTTGAAGACAGAAAA-3'
M. nivale	Mniv1f	5'-TTGGCTTGCACAAACAATACTTTTT-3'
	Mniv1r	5'-AGCACAACAGGCGTGGATAAG-3'

Appendix 2D.1. RT-PCR protocol for quantification of Tri5, *F.graminearum* and *F.culmorum* DNA

Process	Temperature (°C)	Duration	Cycles
Denaturisation	95	3 minutes	1
Denaturisation,	94	30 seconds	
Annealing &	64	30 seconds	40
Extension	72	30 seconds	
Melt curve analysis	65 - 95	-	-

Appendix 2D.2. RT-PCR protocol for quantification of *F.lansethiae* DNA

Process	Temperature (°C)	Duration	Cycles
Denaturisation	95	3 minutes	1
Denaturisation, Annealing & Extension	95	15 seconds	40
	62	10 seconds	
	72	30 seconds	
	82	10 seconds	
Melt curve analysis	55 - 95	-	-

Appendix 2D.3. RT-PCR protocol for quantification of *F.avanaceum and F.poae* DNA

Process	Temperature (°C)	Duration	Cycles
Denaturisation	95	3 minutes	1
Denaturisation,	95	15 seconds	
Annealing &	62	30 seconds	40
Extension	72	30 seconds	
Melt curve analysis	60 - 95	-	-

Appendix 2D.4. RT-PCR protocol for quantification of *M.majus and M.nivale* DNA

Process	Temperature (°C)	Duration	Cycles
Denaturisation	95	3 minutes	1
Denaturisation,	95	15 seconds	
Annealing &	62	30 seconds	40
Extension	72	30 seconds	
Melt curve analysis	60 - 95	-	-

Appendix 3A. VALIDATED LC-MS METHOD

The validated LC-MS methodology using DZT MS-PREP clean-up columns has been fully documented within section 3.4. This methodology was utilised for the mycotoxin analysis of harvested grain samples from the 2010 and 2011 field trials.

Chemicals and reagents

100µg/ml standards of nivalenol (NIV), deoxynivalenol (DON), 3-acetyl-DON (3-AC-DON), HT-2 toxin (HT-2), T-2 toxin (T-2) and zearalanone (ZEAR) and 25µg/ml of internal NIV, DON, T-2 and ZEAR standards were purchased from Biopure (Romer Labs, Runcorn, UK). LCMS grade acetonitrile, gradient grade methanol, Whatman No1 filter, phosphate buffered saline (PBS) tablets were purchased from Sigma-Aldrich. Glass microfibre filter paper was purchased from Fisher Scientific. HPLC grade water was produced via a Purite Select, producing water to a quality of around 16.7MΩ. Technical nitrogen gas was supplied by BOC (Derby, UK). MycoSep® 226 clean-up columns were provided by Romer Labs (Romer Labs, Runcorn, UK) and DZT MS-PREP® clean up columns were provided by R-Biopharm (Glasgow, UK).

Sample preparation (deoxynivalanol, HT-2, T-2 and zearalanone)

50ml of methanol/deionised water (70:30 v/v) was added into a blender jar followed by 12.5g of flour sample. A positive displacement syringe was used to add 10ul of 25µg/ml 13C-DON internal standard solution to the blender jar to spike the flour sample to 20ppb 13C-DON before blending at high speed for 3 min. The supernatant was filtered through a Whatman No.1 filter, before having 2ml of the filtrate added to 48ml of PBS. The PBS and

filtrate mix was then filtered through a glass microfibre filter. 20ml of this filtered solution was passed through a DZT MS-PREP® column by gravity, before the column was washed with 20ml of deionised water and then finally eluted into a 2ml glass vial using 2ml of 100% methanol. This filtrate was then evaporated to dryness under a gentle stream of nitrogen gas before being redissolved in 500µl of methanol/deionised water (10:90 v/v)

Sample preparation (nivalenol)

10g of milled sample flour was weighed into a blender jar along with 80ml of methanol/deionised water (70:30 v/v) before being blended at high speed for 3 min. Samples were then filtered through a Whatman No.1 filter, followed by filtration through a glass microfibre filter. 5ml of filtrate was placed into a 50ml falcon tube and spiked with 2.5µl of 13C-NIV internal standard to give an equivalent spiking of 100ppb in the original flour sample. The sample was dried down under a gentle stream of nitrogen gas, before being reconstituted in 1ml of methanol/deionised water (10:90 v/v), placed into a 1.5ml eppendorf tube and centrifuged at 12,000 rpm for 5 min. The sample was then aliquoted into a 2ml sample vial, without disturbing the waste pellet at the bottom of the eppendorf tube.

Standard preparation

Standards were created to allow for the quantification of mycotoxins from various samples. Samples we created to contain 1000ppb of each standard and 20ppb of each internal standard. To achieve this, 10µl of each 100µg/ml standard was added to a 2ml vial, along

with 0.8µl of 25µg/ml 13C-DON and 13C-NIV standard. These were dried under a gentle stream of nitrogen and redissolved in 1ml of 10% methanol.

HPLC conditions

The chromatographic analysis was carried out on an Agilent 1100 HPLC system (Stockport, UK) controlled via Chemstation software. Separation was achieved using a 5µm Luna reverse phase C18 250mm x 3mm HPLC column (Phenomenex, Macclefield, UK) held at 25^{o}C, with a gradient run of bottle A (10% methanol) and bottle B (100% methanol + 0.1mM of NaCl) in which bottle B was run from 0% to 100% from 0 to 15 min, held at 100% from 15 to 22 min, 100% to 0% from 22 to 22.2 min, and held at 0% from 22.2 to 27 min for re-equilibration. The flow rate was 0.5ml/min^{-1} and the injection volume was 50µl.

MS conditions

Detection and quantification was performed via a Micromass Platform LCZ, controlled by MassLynx software. The system was equipped to run with a ESI source. The parameters used to run and setup the MS under Electrospray Ionisation (ESI) mode are shown in Tables 3A.1 and 3A.2.

Appendix 3A.1. Optimised MS parameters

Ionization mode	SIR ESI+ and ESI- modes used.
Gas flow (l/h-1)	646 litre/hr
Capillary (v)	3.5
Cone (v)	21
Extractor	5
Rf lens	0.1
Source block temp (^{o}C)	Uncontrolled
Desolvation temp (^{o}C)	450 ^{o}C
LM res	12.5
HM res	12.5
Ion energy	1.4
Multiplier	650

Appendix 3A.2. Additional MS setup details

Mycotoxin name	Retention time (min)	Major ion	Ionisation mode
NIV	9.21	347	ESI-
13C-NIV	9.21	362	ESI-
DON	11.10	331	ESI-
13C-DON	11.10	346	ESI-
HT-2	17.23	447	ESI+
T-2	17.91	489	ESI+
ZEAR	18.75	317	ESI-

Running samples

Samples were run using an auto sampler. Two mycotoxin standards were run prior to the first extracted sample to allow for the MS equipment to warm up. Extracted samples were run in sets of five, before running an external mycotoxin standard used for quantification.

Processing chromatograms

Chromatograms were processed using MassLynx (v3.2) to integrate and quantify peak areas of detected mycotoxins in both the standards and the extracted samples.

Calculating mycotoxin concentration

Peak areas were used to calculate the final mycotoxin concentration of the extracted samples in a Excel spreadsheet. The following equation was used:

$$\text{Mycotoxin concentration (ppb)} = \left(\frac{A}{B} \times \frac{C}{D}\right) \times 1000$$

Where:

A = Peak area of the mycotoxin in the sample

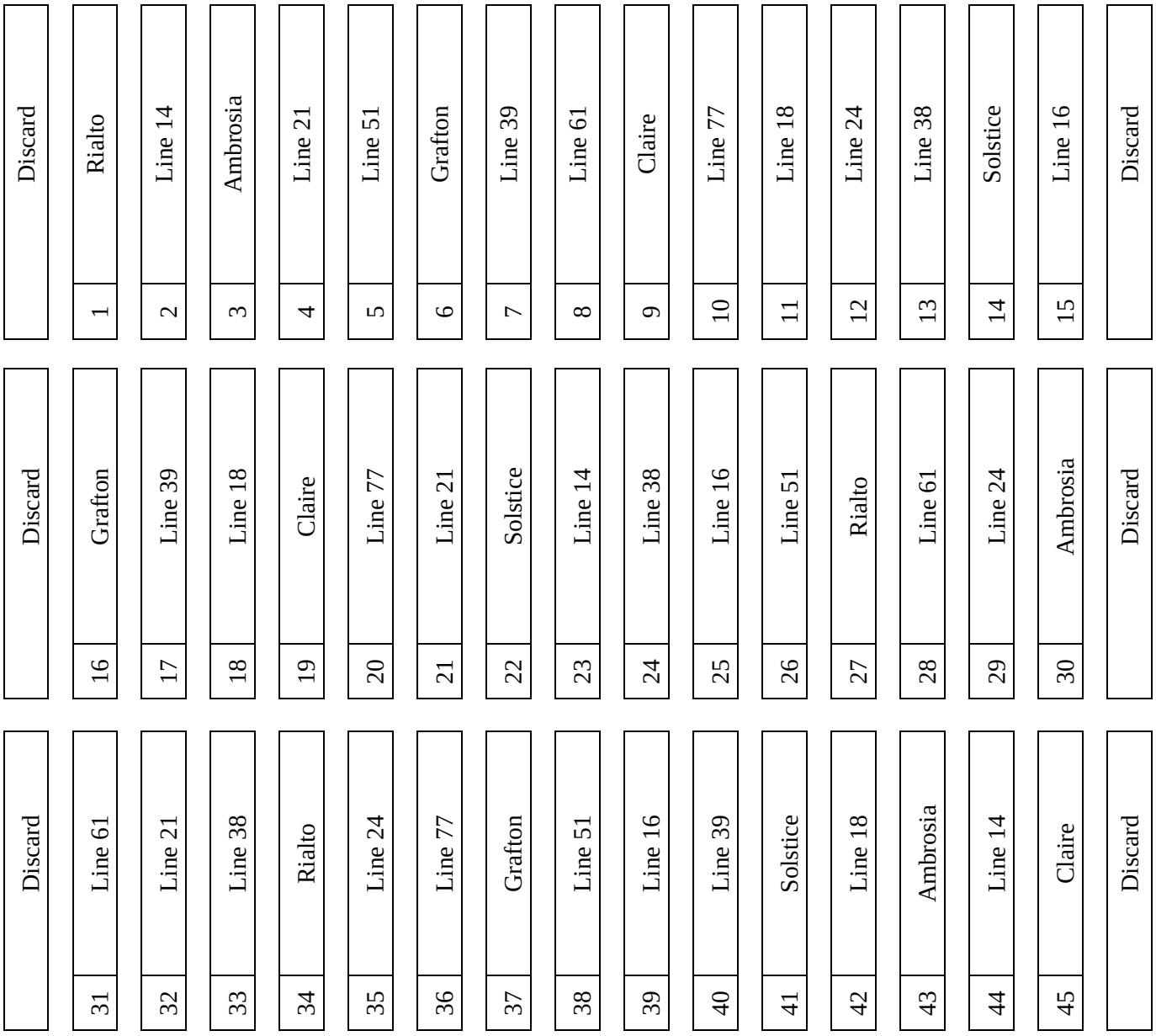
B = Peak area of the mycotoxin in the standard

C = Peak area of the 13C labelled mycotoxin in the standard

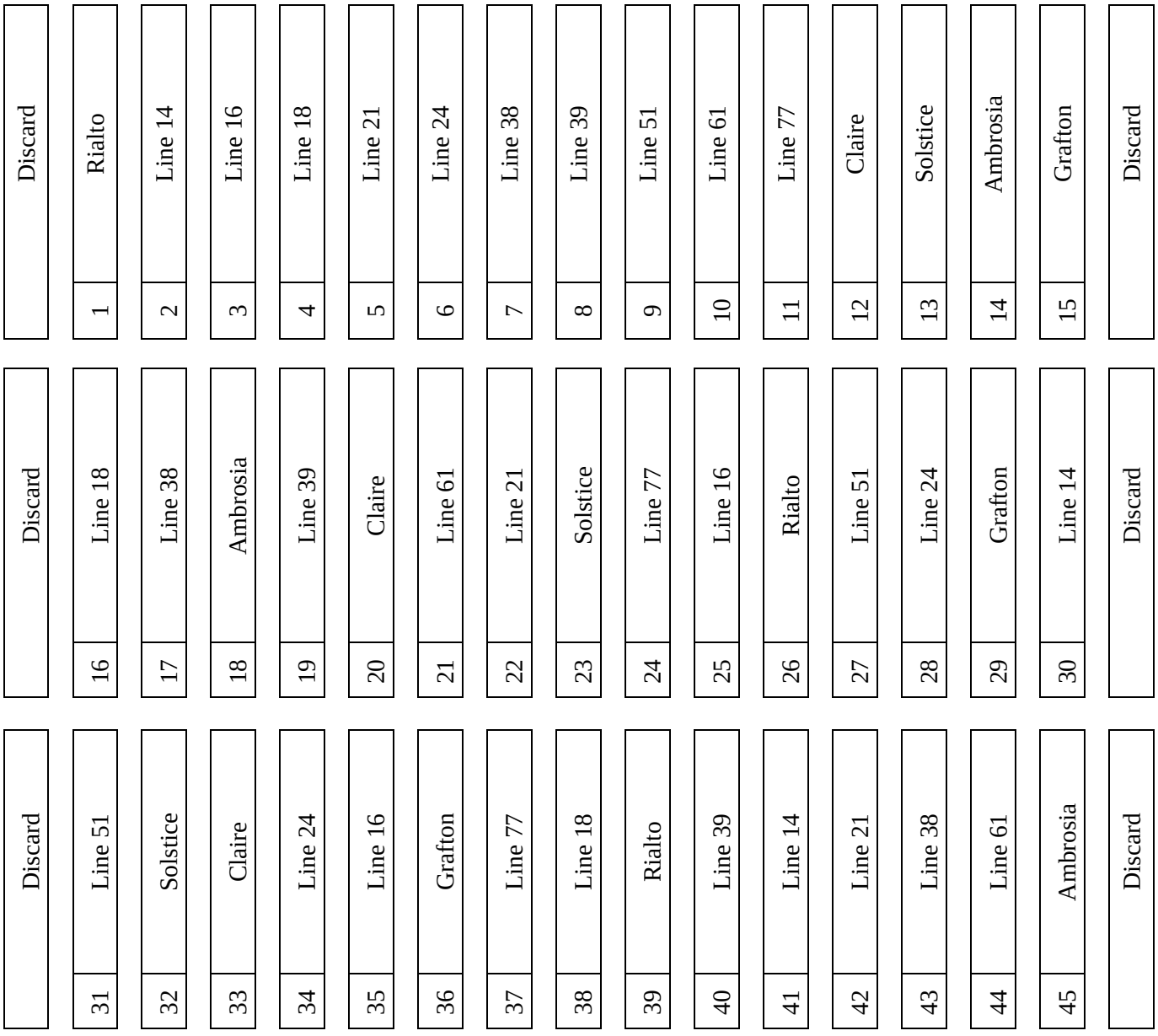
D = Peak area of the 13C labelled mycotoxin in the sample

1000 = Multiplier to give the correct units

Appendix 4A. Field experiment 2010 layout



Appendix 4B. Field experiment 2011 layout



Appendix 4C.1. Crop establishment details for the 2010 and 2011 field experiments.

	2010	2011
Soil texture	Sandy loam	Sandy loam
Previous crop	Winter oats	Winter oats
Cultivations	Plough (14/09/09)	Plough (16/09/10)
	Power harrow (15/10/09)	Power harrow (08/10/10)
	Drilling (22/10/09)	Drilling (11/10/10)
	Rolling (22/10/09)	Rolling (11/10/10)
Drill type	Ojyard	Wintersteiger
Plot size	6m x 2m	6m x 2m
Seed density	375 seeds m^2	375 seeds m^2
Seed dressing	Redigo deter	Redigo deter

Appendix 4C.2. Herbicide and insecticide regimes for the 2010 and 2011 field experiments.

	Date of application	Active substance	Rate
2010	20/11/2009	picolinafen	48 g/ha
		pendimethalin	960 g/ha
		cypermethrin	25 g/ha
	27/04/2010	mesosulfuron-methyl	12 g/ha
		iodosulfuron-methyl-sodium	2.4 g/ha
	27/05/2010	fluroxypyr	123 g/ha
		florasulam	3.1 g/ha
2011	07/11/2010	mesosulfuron-methyl	10 g/ha
		iodosulfuron-methyl-sodium	2 g/ha
		picolinafen	27.2 g/ha
		pendimethalin	544 g/ha
	07/03/2011	cypermethrin	25 g/ha
	15/06/2011	pirimicarb	125 g/ha

Appendix 4C.3. Fungicide regime for the 2010 and 2011 field experiments.

	GS30	GS31	GS 39
2010	75 g/ha azoxystrobin 187.5 g/ha tebuconazole	233g/ha boscalid 67g/ha epoxiconazole 125 g/ha metrafenone	750g/ha fenpropimorph 80 g/ha cyproconazole 750 g/ha chlorothalonil
2011	150g/ha metrafenone 150g/ha azoxystrobin 30g/ha cyproconazole	100g/ha pyraclostrobin 7.5 g/ha cyflufenamid 500g/ha chlorothalonil	750g/ha fenpropimorph 80 g/ha cyproconazole 750 g/ha chlorothalonil

Appendix 5A. Field experiment 2012 layout

Note: Top left = Block 1; Top right = Block 2; Bottom left = Block 3. Blue denotes a misted plot.

Appendix 5B. Field experiment 2013 layout

Note: Top left = Block 1; Top right = Block 2; Bottom left = Block 3. Blue denotes a misted plot.

Appendix 5C.1. Crop establishment details for the 2012 and 2013 field experiments.

	2012	2013
Soil texture	Sandy loam	Sandy loam
Previous crop	Winter oats	Winter oats
Cultivations	Plough (13/09/11)	Plough + Press (12/09/12)
	Power harrow (22/09/11)	Drilling (15/10/12)
	Drilling (26/10/11)	Rolling (15/10/12)
	Rolling (26/10/11)	
Drill type	Wintersteiger	Wintersteiger
Plot size	1m x 1m	1m x 1m
Seed density	375 seeds m^2	375 seeds m^2
Seed dressing	Redigo deter	Redigo deter

Appendix 5C.2. Herbicide and insecticide regimes for the 2012 and 2013 field experiments.

	Date of application	Product	Rate
2012	09/11/2011	diflufenican	60 g/ha
		flufenacet	240 g/ha
		cypermethrin	25 g/ha
	19/03/2012	metsulphuron-methyl	5 g/ha
	24/04/2012	fenoxaprop-P-ethyl	69 g/ha
	23/05/2012	fluroxypyr	100 g/ha
		florasulam	5g/ha
2013	02/10/2012	glyphosate (pre-drilling)	1,125 g/ha
	19/11/2012	methiocarb	0.12 g/ha
	10/04/2013	mesosulfuron-methyl	10 g/ha
		iodosulfuron-methyl-sodium	2 g/ha
		diflufenican	0.31 g/ha
		flufenacet	124 g/ha
	03/06/2013	metsulphuron-methyl	5 g/ha

Appendix 5C.3. Fungicide regime for the 2012 and 2013 field experiments.

	GS30	GS31	GS 39
2012	250 g/ha pyraclostrobin 233 g/ha boscalid 67 g/ha epoxiconazole 750 g/ha fenpropimorph	150 g/ha azoxystrobin 750 g/ha chlorothalonil 62 g/ha epoxiconazole 150 g/ha metrafenone	60 g/ha pyraclostrobin 500 g/ha chlorothalonil 62 g/ha epoxiconazole
2013	250 g/ha pyraclostrobin 233 g/ha boscalid 67 g/ha epoxiconazole 750 g/ha fenpropimorph	150 g/ha azoxystrobin 750 g/ha chlorothalonil 375 g/ha cyprodinil 94 g/ha epoxiconazole	125 g/ha epoxiconazole 100 g/ha pyraclostrobin

Appendix 5D.1. Percentage differences between maximum and minimum values for common physiological traits at GS39 between the 2011, 2012 and 2013 field experiments

	Year	Min	Max	Percentage difference
Plant number (m2)	2011	250.7	426.7	52%
	2012	206.8	293.2	35%
	2013	135.8	438.6	105%
Fertile shoots (m2)	2011	368.0	586.7	46%
	2012	273.4	484.5	56%
	2013	311.1	550.2	56%
Fertile shoots per plant	2011	1.27	1.93	41%
	2012	2.24	4.20	61%
	2013	2.50	7.20	97%
Flag leaf height (cm)	2011	28.6	71.2	85%
	2012	48.4	103.6	73%
	2013	47.5	83.4	55%
Flag leaf length (cm)	2011	13.3	21.8	48%
	2012	21.6	27.7	25%
	2013	14.5	20.4	34%
Flag leaf width	2011	1.23	1.86	41%
	2012	1.54	2.13	32%
	2013	1.22	2.00	48%
Flag leaf area (cm2 /m2)	2011	3063	8789	97%
	2012	7412	13864	61%
	2013	4856	13368	93%
Second leaf area (cm2/m2)	2011	5391	10793	67%
	2012	8640	19060	75%
	2013	6064	14096	80%
All remaining leaf area (cm2/m2)	2011	9980	29079	98%
	2012	13040	31088	82%
	2013	8796	24952	96%
Total leaf area	2011	18434	48231	89%
	2012	30000	62676	71%
	2013	21228	50836	82%

Appendix 5D.2. Percentage differences between maximum and minimum values for common physiological traits at GS65 between the 2011, 2012 and 2013 field experiments

	Year	Min	Max	Percentage difference
Plant number (m^2)	2011	202.7	357.3	55%
	2012	198.8	376.8	62%
	2013	96.0	417.6	125%
Fertile shoots (m^2)	2011	293.3	608.0	70%
	2012	210.8	461.2	75%
	2013	144.0	539.1	116%
Fertile shoots per plant	2011	1.33	2.87	73%
	2012	1.90	3.35	55%
	2013	2.40	5.07	71%
Flag leaf height (cm)	2011	35.3	99.2	95%
	2012	52.3	106.9	69%
	2013	37.5	81.1	74%
Flag leaf length (cm)	2011	12.84	21.27	49%
	2012	20.30	26.60	27%
	2013	11.56	19.36	50%
Flag leaf width (cm)	2011	1.26	1.88	39%
	2012	1.50	2.13	35%
	2013	1.25	1.95	44%
Plant height (cm)	2011	40.02	118.41	99%
	2012	56.55	120.29	72%
	2013	43.62	100.88	79%
Flag leaf area (cm2/m2)	2011	3817	9853	88%
	2012	6196	11104	57%
	2013	3424	15000	126%
Second leaf area (cm^2/m^2)	2011	5543	13630	84%
	2012	6248	13484	73%
	2013	3248	14096	125%
Remaining leaf area (cm^2/m^2)	2011	6167	27929	128%
	2012	4836	21300	126%
	2013	6556	21676	107%
Total leaf area (cm^2/m^2)	2011	19664	49009	85%
	2012	17276	44000	87%
	2013	13296	48976	115%
Ear length (cm)	2011	9.25	13.41	37%
	2012	10.28	14.11	31%
	2013	9.43	13.7	37%
Spikelets per ear	2011	18.87	26.33	33%
	2012	16.53	26.8	47%
	2013	16.13	25.5	45%
Spikelet density (spikelets / cm)	2011	1.67	2.37	35%
	2012	1.59	2.39	40%
	2013	1.68	2.32	32%
Awn length (cm)	2011	0.27	7.51	186%
	2012	0.55	4.77	159%
	2013	0.28	7.7	186%