ECOLOGICAL INTERACTIONS BETWEEN GRAIN APHIDS (*Sitobion avenae*) AND FUSARIUM HEAD BLIGHT PATHOGENS (*Fusarium graminearum* & *F. langsethiae*) ON WHEAT AND THEIR IMPACT ON DISEASE

JASSY DRAKULIC, MA (Cantab.)

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

JUNE 2016

Division of Plant and Crop Sciences, University of Nottingham, Sutton Bonington, LE12 5RD, UK

Acknowledgments

I would like to thank my lead supervisors, Toby Bruce and Rumiana Ray for their support and commitment, as well as Matt Dickinson for his support as a supervisor in the early stages of this project. I would like to acknowledge Mark Meacham, Fiona Wilkinson, Lesley Smart and Janet Martin for their technical support. Thanks go also to the co-authors of the publications included in this thesis: John Caulfield, Christine Woodcock, Stephen Jones, Robert Linforth, and Bukky Ajiboye. To Sarah Khursheed, Brian Ó Loinsigh and Haziq Kahar, I thank you for your assistance in the project work and for your enthusiasm and willingness to learn.

My thanks also go to Howard Griffiths, for igniting in me a passion for plant sciences and, for motivating me to pursue further study. I owe a great deal of thanks to Linda Nielsen, for her guidance and friendship and for the excellent quality of training I received from her. To Matthew Brown, I offer my thanks and admiration for his humour, geniality and determination. I also thank Sarah Plummer for the years of friendship and companionship she has given me, and my family for providing me the freedom to follow my own path.

Finally, my eternal thanks go to Sabri Al-Safi: my partner and inspiration.

Table of Contents

List of Abbreviations

bp	Base pairs
ANOVA	Analysis of variance
AUDPC	Area under the disease progress curve
CTAB	Cetyltrimethyammonium bromide
cv.	Cultivar
DAI	Days after inoculation
DI	Deionised
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
EAG	Electroantennography
GC	Gas chromatography
GC-MS	Gas chromatography coupled mass spectrometry
ITS	Internal transcribed spacer region
ko	Knock-out
LC-MS	Liquid chromatography coupled mass spectrometry
NIV	Nivalenol
NTC	Non-treated control
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
QPCR	Quantitative real-time polymerase chain reaction
rpm	Rotations per minute
SDW	Sterile distilled water
SNA	Synthetic nutrient deficient agar
TE	Tris-EDTA
TWA	Tap water agar
VOC	Volatile organic chemicals
wt	Wild type

List of Tables

List of Figures

Conferences Presentations

Drakulic J., Bruce T., & Ray R. V. 2014. How *Fusarium graminearum* influences insect-plant interactions. 47th Annual Meeting of the Society for Invertebrate Pathology. Mainz, Germany. August 5th 2014.

Drakulic J., Bruce T., Ray R. V. 2015. Interactions between *Fusarium graminearum* and grain aphids (*Sitobion avenae*) on their shared host plant, wheat (*Triticum aestivum*). European Fusarium Seminar. Martina Franca Italy, May 11th 2015.

Publications

Drakulic J., Caulfield J., Woodcock C., Jones S. P. T., Linforth R., Bruce T., Ray R. V. 2015. Sharing a Host Plant (Wheat [*Triticum aestivum*]) Increases the Fitness of *Fusarium graminearum* and the Severity of *Fusarium* Head Blight but Reduces the Fitness of Grain Aphids (*Sitobion avenae*). *Applied and Environmental Microbiology* **81**, 3492-3501.

Drakulic, J., Bruce, T, Ray, R. V. 2016. Direct and host-mediated interactions between *Fusarium* pathogens and herbivorous insects in cereals. *Plant Pathology* (In press)

Drakulic, J., Ajiboye, O., Bruce, T., Ray, R. V. 2015 Aphid infestation increases *Fusarium langsethiae* and HT2 and T2 mycotoxins in wheat (Under review) *Applied and Environmental Microbiology*

Abstract

Fusarium head blight pathogens contribute to ear disease in cereals globally, causing yield losses, decreased grain quality, and mycotoxin accumulation in infected grain. Wheat is susceptible to head blight disease after ear emergence, during which time ears can also act as hosts for other biological agents, including insects. Grain aphids, *Sitobion avenae*, colonise ears preferentially over other tissues of wheat plants, and mutual attack of wheat by grain aphids and *Fusarium* pathogens is likely to occur in the field. Experiments were conducted to elucidate the nature of the interactions that occur between the plant host, aphid pests and *Fusarium* pathogens.

When aphids had also attacked the host plant, exacerbated disease severity was observed for both *Fusarium graminearum* and *F. langsethiae*. Of particular interest was the finding of an increase in mycotoxin accumulation in infected grain when there was aphid herbivory of host plants. Transmission of either species by aphids was not consistently observed, although the apparent compatibility of *F. langsethiae* with aphid vectors was greater than for *F. graminearum. F. langsethiae* was transmitted by aphids when moved manually from infected host ears to susceptible healthy ears, but transmission by natural movement of both winged and wingless aphids failed to produce disease in new hosts. In contrast, deoxynivalenol-producing *F. graminearum* induced the production of volatile host chemicals that repelled aphids, and population dynamics of aphids that colonised wheat ears infected with *F. graminearum* were impacted negatively, as observed by increased mortality and decreased fecundity of aphids from diseased hosts.

The role of mycotoxins in the production of host volatiles was assessed by comparing *F. graminearum* isolates of different chemotypes, including mycotoxin deficient mutants. In contrast to hosts infected with DON-producing *F. graminearum,* which produced VOCs that repelled aphids, hosts infected with a nivalenol (NIV)-producing *F. graminearum* isolate produced VOCs that were attractive to aphids. This response was shown to be due to the

mycotoxin production of the pathogen, as a transgenic line of the same isolate deficient impaired in trichothecene production produced no such effect.

The importance of timing and location of aphid herbivory relative to the infection site on the development and spread of disease was examined. It was observed that the longer the time of aphid colonisation on hosts prior to the arrival of fungal inoculum, the more severe the consequences of disease for host plants in terms of fungal biomass and visual disease severity. Aphid herbivory on host ears led to a greater increase in disease severity and spread than infestation of leaves. Systemically treated plants also became more vulnerable to elevated pathogen colonisation; however local effects were more influential on disease outcome.

The current research findings show the value of considering interactions between plant pathogens and insects. These are usually studied separately but occur together in nature and in this case the insect had a major impact on disease development. This opens questions about how aphids improve host suitability which could be answered by transcriptomic approaches. The study has important practical implications because it suggests that by incorporating traits for resistance to aphids, breeding for FHB resistance could be improved in wheat. Furthermore, control of aphid populations prior to and during heading could reduce the risk of mycotoxin contamination of wheat crops.

Chapter 1: Direct and indirect interactions between Fusarium pathogens and herbivorous insects in cereals

This chapter presents a review of the literature surrounding insect interactions in FHB epidemiology, alongside the traditional knowledge base for FHB epidemiology. This work has been accepted by *Plant Pathology* for publication, and is presented in ‘paper format’.

Author contribution

This paper was composed and researched by J. Drakulic. Supervision and editorial guidance were provided by T. J. Bruce and R. V. Ray.

REVIEW

Direct and host-mediated interactions between Fusarium pathogens and herbivorous arthropods in cereals

Running title: The role of insects in FHB and FER

Jassy Drakulic[1], Toby J.A. Bruce[2], Rumiana V. Ray[1#].

1: University of Nottingham, Sutton Bonington Campus, College Road, Sutton Bonington, LE12 5RD, UK.

2: Rothamsted Research, West Common, Harpenden, Herts AL5 2JQ, UK.

Author for correspondence:
Rumiana Ray
Tel: +44 115 951 6049
E-mail: Rumiana.ray@nottingham.ac.uk

Abstract

Fusarium Head Blight (FHB) and Fusarium Ear Rot (FER) diseases of cereal crops are significant global problems, which cause yield and grain quality losses and accumulation of harmful mycotoxins. Safety limits have been set by the European Commission for several *Fusarium*-produced mycotoxins, and mitigating the risk of breaching these limits is of great importance to crop producers as part of an integrated approach to disease management. Here we review current knowledge regarding the role of arthropods in disease epidemiology. In the field, diseased host plants are likely to interact with arthropods which may substantially impact the disease by influencing spread or condition of the shared host. For example, disease progress by *Fusarium graminearum* can be doubled if wheat plants are aphid-infested. Arthropods have been implicated in disease epidemiology in several cases and the evidence ranges from observed correlations between arthropod infestation and increased disease severity and mycotoxin accumulation, to actual experimental evidence for insect infestation causing heightened pathogen prevalence in hosts. *Fusarium* pathogens differ in spore production and impact on host volatile chemistry, which influences their suitability for arthropod dispersal. Herbivores may allow secondary fungal infection after wounding a plant or they may alter host susceptibility by inducing changes in plant defence pathways. Post-harvest, during storage, arthropods may also interact with *Fusarium* pathogens, with instances of fungivory and altered behaviour by arthropods towards volatile chemicals from infected grain. Host-mediated, indirect pathogen-arthropod interactions are discussed alongside comprehensively reviewing the evidence for direct interactions where arthropods act as a vector for inoculum.

Keywords: Fusarium Head Blight, Fusarium Ear Rot, host-pathogen-herbivore interactions, epidemiology, volatiles, cereals

Introduction

Fusarium Head Blight (FHB) is a disease that affects small-grained cereals and is caused by a complex of up to 17 *Fusarium* and 2 *Microdochium* fungal species (Parry *et al.*, 1995; Glynn & Edwards, 2010). FHB is a relatively well studied plant disease in terms of virulence (Goswami & Kistler, 2004), management and crop resistance (Bai & Shaner, 2004 , Rudd *et al.*, 2001 , Buerstmayr *et al.*, 2009), mycotoxin accumulation (Logrieco *et al.*, 2003) and other aspects of its biology, ecology and epidemiology, but information on the interaction of FHB with arthropods is lacking. Here we provide a review of what is known about how insects and mites may directly or indirectly facilitate *Fusarium* disease. Although scientists are often confined to narrow disciplines focussing on insects, plants or pathogens, this separation does not occur in nature where insects and pathogens are exposed to each other and can influence each other either directly or indirectly by altering the condition of a shared host plant. FHB, and Fusarium ear rot (FER) which affects maize, are significant global problems. By understanding and establishing control over arthropod interactions that exacerbate disease or enhance host susceptibility, the impact of FER has been demonstrably reduced, and this could be utilised in FHB control also.

FHB infections result in decreased grain yield and quality plus production of mycotoxins in grain by *Fusarium* spp. which are harmful to the health of animal consumers (Marin *et al*., 2013). FHB is present in most cereal growing regions in the world (Parry *et al.*, 1995; Gilbert & Haber, 2013) and no variety of cereals is completely resistant to FHB (Wegulo *et al*., 2015). Visible symptoms of FHB appear as water-soaked lesions on glumes, then pink-orange sporodochia and mycelia and black fruiting bodies may develop, followed by discolouration and premature bleaching of spikelets (Bushnell *et al*., 2003; McMullen *et al*., 2012). Harvested grains may have a reduced protein content (Eggert *et al*., 2011) and can appear chalky-white and shrivelled or without outward symptoms (Goswami & Kistler, 2004). Several different species can contribute to disease on any one host, and different causal species induce a varying severity of visible symptoms, ranging from aggressive disease onset by *F. culmorum* and *F. graminearum* to infrequent symptom development by *F. poae* and mostly cryptic infection by *F. langsethiae* (Imathiu *et al*., 2013).

The largest group of mycotoxins produced by *Fusarium* are trichothecenes, which act on ribosomes to inhibit protein synthesis and cause direct damage to intestines upon ingestion (D'Mello *et al*., 1999). Trichothecenes are sesquiterpenoid secondary metabolites (Lattanzio *et al.*, 2009) which can be divided into different classes based on chemistry and mode of action. Type A trichothecenes are the most toxic to animals, including T-2 and HT-2 (Paciolla *et al.*, 2004). Type B trichothecenes include nivalenol (NIV) and deoxynivalenol (DON), of which the concentration in grain is limited by European law (Anon, 2006). Reports indicate that while mycotoxin levels do not commonly breach legal limits (Streit *et al*., 2012; Marin *et al*., 2013; Belakova *et al*., 2014), different geographic areas are at a higher risk of approaching these limits than others under favourable weather conditions (Placinta *et al*., 1999; Cui *et al*., 2013). Different acetylated forms of DON and NIV are produced by different pathogen strains, and these different forms of the toxins vary in their toxicity and phytotoxicity (Suzuki & Iwahashi, 2014) and geographical distribution (Gale *et al*., 2011). FER of maize is typically caused by *Fusarium verticillioides* (formerly *F. moniliforme*). FER infection leads to the production of fumonisin mycotoxins, which are associated with toxicity syndromes in animals (Yazar & Omurtag, 2008). Control of FHB and FER diseases is important not only to prevent physical degradation of the crop and the associated losses in yield, but to prevent mycotoxins from accumulating in the grain and the economic losses associated with breaching safety limits.

Overview of *Fusarium* disease epidemiology

The most dominant species to cause FHB across temperate regions is *F. graminearum* sensu stricto which, along with *F. culmorum* and other species from the *F. graminearum* species complex, causes the accumulation of the mycotoxins deoxynivalenol (DON) and nivalenol (NIV) in infected grain. These species prevail in climates with mild to warm summer temperatures, alongside *F. avenaceum* and *F. poae*. Both pink and red ear rot diseases of maize (which will both be referred to as FER in this review), which are primarily caused by *F. verticillioides* and *F. graminearum* respectively, also prevail in warm climates (Munkvold, 2003). Colder maritime climates, such as in Northern Europe, are more greatly affected by the non-toxigenic *Microdochium nivale* and *M. majus* (Xu *et al*., 2008; Nielsen *et al*., 2011), and the T-2 and HT-2 toxin producers *F. sporotrichioides* and *F. langsethiae* (Fredlund *et al*., 2013). *F. poae* and *F. langsethiae* have been described as early season colonisers (Sturz & Johnston, 1985; Parikka *et al*., 2012), that are capable of infecting hosts prior to anthesis (GS59, Zadoks *et al*., 1974), possibly facilitating the later colonisation of cereal heads by other species such as *F. graminearum* and *F. culmorum.* In Asia and southern USA other species from the wider *F. graminearum* species complex, particularly *F. asiaticum,* are of more prevalent (Suga *et al*., 2008; Qu *et al*., 2008; van der Lee *et al*., 2015).

In addition to FHB and FER, *Fusarium* spp. can also cause seedling blight and foot rot as part of the Fusarium disease complex on cereals, with different species prevailing in different geographical locations and on different host crops. Wheat is most susceptible to infections of FHB during mid-anthesis, GS65 (Miller, 1994) via ascospores or conidia conveyed directly onto the heads. *F. graminearum* infects wheat plants via the anthers, through stomata or at the base of the glume, then grows through the caryopsis, floral bracts to the rachis and into neighbouring spikelets (Bushnell *et al*., 2003) with the mycotoxin DON produced early in the colonisation process (Boenisch & Schaefer, 2011) which acts as a virulence factor, facilitating fungal progression through the spike (Jansen *et al*., 2005). Inoculation experiments using *F. langsethiae* on oats also found that direct panicle-applied conidia produced FHB symptoms (Divon *et al.,* 2012), but it was also found that injection of spores into the boot achieved greater levels of pathogen DNA at harvest than flowering ears (Opoku *et al*., 2013). The latter method of inoculation is unlikely to represent an infection mechanism that can be achieved in the field, although it supports the potential importance of wound sites for example through insect feeding on hosts tissues as a risk factor for increased success of these pathogens. Further support for the role of wound sites in infection of wheat with *F. langsethiae* comes from detached leaf assay experiments which showed that artificial wounds were necessary for *F. langsethiae* to cause lesions on leaf samples (Imathiu *et al.*, 2010). Damage caused by arthropod feeding on host plants could potentially provide the wound sites required for colonisation by this otherwise weak pathogen of wheat; however this interaction has not been explored in the current literature.

Upon infection, *Fusarium* pathogens carry out a phase of biotrophy upon their host plants prior to switching to necrotrophy on tissues and crop residues (Goswami & Kistler, 2004) where infected material becomes a potential source of inoculum for the next crop in rotation. Figure 1.1 shows the cycling processes of fungal inoculum types in small-grain cereals, and how each disease in the Fusarium disease complex provides inoculum for the next. *Fusarium* infected heads produce infected seed and can result in Fusarium seedling blight, conidia arising from Fusarium seedling blight can give rise to Fusarium foot rot, and conidia at the stem bases can be moved via rain splash up to the ears via the canopy layers to initiate FHB. While FHB is considered a monocyclic disease (Fernando *et al.*, 1997; Kohl *et al.*, 2007; Landschoot *et al.,* 2011) evidence suggests that multiple and potentially distant inoculum sources may contribute to the level of starting inoculum, as the population structure of *Fusarium* spp. in mature ears does not always reflect that in crop residues or soil (Landschoot *et al.*, 2011). In some cases in a Belgian survey of wheat fields, the *Fusarium* spp. population structure on ears was more similar to that on weeds, indicating that weeds are likely to be an important source of primary inoculum for some FHB outbreaks. Furthermore, this study also revealed that in one season (2008-9), in 50% of the locations *F. poae* was isolated wheat heads, but not found at the start of the season on any of the primary inoculum sources tested from the site. The increase in species diversity over the growing season indicates that inoculum can arrive throughout the season from distant sources either by wind or perhaps through insect dispersal.

The involvement of arthropods in the dispersal of inoculum in small grained cereal crops (Parry *et al.*, 1995) and maize (Munkvold, 2003) has been proposed previously although the exact role played by arthropods in Fusarium disease epidemiology is not well understood and only studied in a limited number of species-specific situations. For example, there are documented cases where insects and mites have been observed to transmit *Fusarium* inoculum between host plants (Kemp *et al*., 1996; Sobek & Munkvold, 1999), and where the activity of pests is correlated with infection by *Fusarium* species (Mongrain *et al.*, 1997; Saladini *et al*., 2008). A recent review (Gagkaeva *et al*., 2014) focussed on the potential positive or negative interferences between arthropods and specific *Fusarium* species, with more aggressive pathogens being described as antagonists to arthropods and weaker pathogens offering potential symbiotic or commensal relationships, however the significance of these interactions on host susceptibility, FHB disease progress and/or mycotoxin accumulation were not explored. There are differences in the size and shape of conidia produced by *Fusarium* species; *F. verticillioides, F. poae* and *F. langsethiae* produce small, almost spherical microconidia, whereas *F. graminearum*, *F. culmorum*, *F. avenaceum* among others produce larger boat-shaped septate macroconidia (Leslie & Summerell, 2006). The reduced size of the conidia produced by *F. verticillioides, F. poae* and *F. langsethiae* may make them more compatible for transportation by wind or arthropods to move greater distances between sources and host sites.

This review aims to discuss arthropod interactions in the epidemiology of Fusarium disease in cereal crops, which encompasses both FHB in small grained cereals and FER in maize. The interactions studied include those with insects and mites acting as vectors of inoculum, causing damage and weakening of the host so as to increase the severity and infection opportunities of *Fusarium* species, and as potential feeders on both fungi and grains during post-harvest storage. The chemical ecology that governs host-arthropod interactions has also been studied in a number of these cases, and gives an indication for the role of the pathogen in altering insect-host relationships.

Observational studies show correlation between FHB or FEB and arthropods

The majority of studies that link arthropod activity to FHB or FER diseases have documented observed correlations in their incidence, and those described are summarised in Table 1.1. The orange wheat blossom midge (OWBM), *Sitodiplosis mosellana*, has been investigated as a putative vector of FHB pathogens. Contaminated wheat crops in Canada were found to have midge infestations (Couture *et al*., 1995), and a more extensive study of 14 field sites of different districts in Quebec showed a positive correlation (R = 0.67, P = 0.001) between the number of OWBM larvae per wheat spike and per spikelet with infection by *F. graminearum*, but not by other species of *Fusarium* (Mongrain *et al*., 1997). However, the number of *F. graminearum* damaged grain was low, with mean values for each site ranging from 0 – 4%, despite midge incidence in spikes ranging from 2 – 98%. Study of OWBM physiology revealed the presence of structures that could carry conidia on adult females (Mongrain *et al*., 2000). These are features common to other groups within the Cecidomyiidae which can also feed on fungi (Borkent & Bissett, 1985). Despite the original hypothesis of vector activity by OWBM, there is no formal description in the literature of whether the correlation between OWBM and *Fusarium* spp. is due to transmission of the pathogen by the insects, increased host disease following damage from larval feeding, recruitment of insects to infected hosts so as to feed on the fungal material, or in fact whether there was any causation associated with the correlation at all.

In wheat, FHB severity has been associated with aphid infestation of host crops. Field trials in India measured the effect of insecticides on the incidence and severity of FHB. Insecticides were applied, targeting aphid populations; the number of aphids on treated plots was successfully reduced. In treated plots, FHB incidence and severity were also significantly reduced (Bagga, 2008) showing a correlation between aphid and FHB incidence.

A lot of attention has been paid to the interaction between Lepidoptera such as the European corn borer (ECB), *Ostrinia nubilalis*, and *F. verticillioides* infection of maize. Following chemical treatment of maize with pyrethroid insecticide lambda-cyhalothrin at 0.02 kg. ha^{-1} at 7 days after peak European corn borer (ECB) flight, a significant reduction of FER severity (29%) was observed. Following early sowing of maize in addition to insecticide treatment, severity was reduced by up to 67% (Blandino *et al*., 2008). The consequence to the host of the association between ECB and *F. verticillioides* has been measured in terms of the mycotoxin levels amassed in grain. Field experiments were conducted over a 7-year period in Italy to test the use of two different pyrethroid insecticides, deltamethrin at 0.013 kg. ha^{-1} and lambda-cyhalothrin at 0.019 kg. ha^{-1}, in their effectiveness for controlling ECB and the effect on FER in maize (Saladini *et al*., 2008). In one season where insecticide treatment failed to reduce ECB damage there was no reduction in FER either, showing that the insecticides have no direct effect on the disease. In the seasons with effective ECB control, the levels of the fumonisins B_1 and B_2 were reduced on average by 75% through the use of insecticide. The infection process of *F. verticillioides* is greatly assisted by insect activity, and insecticides have been shown to be more effective than fungicides in reducing fumonisin levels (Blandino *et al*., 2009). Furthermore, *Bt*-maize which has lower insect damage has been shown to have lower mycotoxin levels (Bakan *et al.*, 2002; Bowers *et al*., 2014). The mechanism appears to be a reduction in secondary infection when there is less insect damage because insect feeding damage can provide an entry point for disease. *Fusarium* fungi that make toxins such as fumonisin B enter through holes made by caterpillars in the cob or stem in non-GM maize.

The incidence of thrips, *Frankliniella occidentalis*, on maize ears has also been correlated with FER caused by *F. verticillioides*. Increased FER severity and fumonisin B_1 concentrations were found in field samples with increased thrips infestation in several sites in southern USA (Parsons and Munkvold, 2010; 2012). Fumonisin B_1 contamination was more strongly correlated with the number of thrips per ear ($R = 0.89$) than the amount of Lepidopteran feeding damage ($R = 0.34$). Visibly mouldy ears were also more strongly correlated with thrips frequency ($R = 0.78$) than with the frequency of Lepidopteran feeding damage ($R = 0.37$) (Parsons & Munkvold, 2012). Additionally, thrips have been implicated in the development of silk-cut symptoms in maize, and by doing so facilitate FER infection (Parsons & Munkvold, 2010). These correlative studies of thrips show that Thysanoptera pose a taxonomically diverse threat to increased FER in maize in addition to that of Lepidoptera, and as such supports the argument for the control of insects in maize FER management strategies.

Direct interactions: Arthropods as potential vectors of *Fusarium* inoculum

Direct interactions between arthropods and FHB or FER pathogens potentially involve insects or mites vectoring fungal spores. There are few documented cases of insects or mites acting as vectors of FHB and FER pathogens, and in the cases that have been studied the nature of the vector activity and the relative importance of the arthropod-pathogen association is far removed from the close-knit associations to insect vectors of pathogens such as viruses and phytoplasmas. In such cases, insect transmission is the primary dispersal mechanism, and the pathogens may benefit from propagative transmission whereby the pathogen replicates inside the vector. Although a number of studies have found increases in FER with insects (Attwater & Busch, 1983; Windells *et al*., 1976; Farrar & Davis, 1991; Darvas *et al*., 2011; Dowd 2004) they have not definitively shown that this is due to vector activity and not due to secondary infestation after insect damage or other preconditioning of the shared host plant. Incidences of transmission of *Fusarium* inoculum by arthropods reported thus far are restricted to the carriage of fungal material on the external surfaces of insects or mites, and therefore the carrying capacity of the vectors is determined by the availability of fungal material on the surfaces of host plants and the size and surface type of the arthropod bodies. This implies that the life cycle and timing of the arthropod involvement with the host plant relative to the infection process of the pathogen needs to be aligned for insect or mite transmission to be possible. That said, while control of relevant arthropod activity on high risk crops might offer only partial control of FHB or FER disease, any mitigation of the risk of breaching mycotoxin safety limits ought to be considered in FHB and FER management plans.

The association of ECB with FER in maize is well studied (Munkvold, 2003). ECB larvae are known to burrow into the stalks and ears of maize plants, causing large amounts of damage to the host tissues. The first generation of larvae make initial attacks on host plants, but the second generation are the most relevant in FER epidemiology as they emerge during ear development. Emerging larvae have been described to be able to act as vectors of *Fusarium* *verticillioides* inoculum, bringing conidia upwards from leaf surfaces to the developing ears and the site of ear infection (Sobek & Munkvold, 1999). However in glasshouse experiments in this study, larvae-free controls still became infected at a low incidence, so it could be argued that the insect attack on the host merely increased the host susceptibility to the disease, leading to the increased incidence in plants treated with ECB larvae. It has not been suggested that these insects can introduce inoculum from distant sources, and as such ECB is only described as a vector on a local scale. From field trials conducted in the same study, larvae that were artificially coated with a strain of *F. verticillioides* and placed on leaf axils were able to transmit that strain to maize ears, which supports the hypothesis that the external surfaces of larval bodies are able to carry inoculum to susceptible tissues, although the acquisition of the inoculum has not been satisfactorily demonstrated in this work.

In addition to ECB, several other insects have also been associated with the epidemiology of *F. verticillioides* in maize. These include western flower thrips, western bean cutworms (Bowers *et al*., 2014), sap beetles and corn rootworm beetles; with sap beetles and rootworm beetles having been described to commonly carry *F. verticillioides* and *F. graminearum* spores (Munkvold, 2003). Furthermore, sap beetles were shown to be attracted to the volatile chemical emissions of maize plants infected with *F. verticillioides* (Bartelt & Wicklow, 1999; Munkvold, 2003), indicating compatibility between potential insect vectors and infected hosts, thus revealing a possible mechanism for the recruitment of insects that may enhance the dispersal of *Fusarium* inoculum. Increased populations of both Lepidopteran stem borers and Coleopteran beetles were observed on maize infected with *F. verticillioides* compared to uninfected plots (Cardwell *et al*., 2000), although the authors here note that this increased level of infestation may not be due to attraction of the insects but rather due to improved survival on the infected hosts.

In small-grained cereals, there are fewer reports of arthropods acting as vectors for *Fusarium* inoculum. One such report is that of *F. poae*, which similarly to *F. verticillioides* produces mostly microconidia (Leslie & Summerell, 2006).

Mites, *Siteroptes avenae,* were shown to transmit *F. poae* inoculum. Mites were fed from cultures on agar plates placed in open petri dishes between rows of wheat plants at ear emergence. Up to 6 symptomatic spikelets per ear were observed after 3 weeks (Kemp *et al.,* 1996). Light microscopy also revealed the presence sac-like structures on female mites concluded by the authors to be sporothecae containing *F. poae* microconidia. This study is limited in that the inoculum source was not from an infected host, such as infected seedling leaves or a realistic reservois of inoculum such as crop debris. Rather the inoculum was from a fungal colony, which presumably would be a much denser source of inoculum than on living or decaying plant material as would occur in the field, so does not demonstrate a realistic infection route in nature, although it demonstrates that mites have the carrying capacity to deliver inoculum to new hosts when sufficient inoculum can be acquired. *F. poae* infection of cereals is favoured by warm and dry environmental conditions, for which insect and mite activity is also favoured.

In an attempt to demonstrate the capacity of OWBM to carry *Fusarium* spores, midge samples collected from the field were washed and the washings plated onto antibiotic amended agar (*pers. comms.*, Ray, 2010). *Fusarium* spp. were successfully grown and identified to be *F. oxysporum*, *F. langsethiae* and *F. poae*. However, the success rate of transmission of this fungal material to new hosts was not examined, and while correlations of OWBM and FHB incidences have been reported (described above), evidence of insect transmission by OWBM is lacking.

Fungivory

The capacity of arthropods to alter the disease impact caused by *Fusarium* spp. after harvest has been investigated in several species-specific studies. Studies report fungivory of *Fusarium* species by insects and mites, for example by psocids, which are able to feed on *Fusarium poae* and *F. sporotrichiodes* (Mills *et al.*, 1992). Mites, *Tyrophagus putrescentiae,* are able to feed on several species of *Fusarium* reared on oatmeal agar and *F. poae*, *F. verticillioides*, *F. culmorum* and *F. avenaceum* from inoculated barley grain, but two other mite species *Acarus siro* and *Lepidoglyphus destructor* experienced negative rates of growth on the *Fusarium* feeding substrates (Nesvorna *et al*., 2012). In low abundances, *T. putrescentiae* have been reported to be able to transmit *F. poae* inoculum from fungal cultures to stored barley grain (Hubert *et al*., 2014) as seen by the detection of *F. poae* operational taxonomic units in sampled DNA. When the pest pressure was increased, the fungus was considered to have been too heavily grazed by the mites to achieve inoculum transfer that could be detected by amplified cloning. DON levels were also raised in both pest pressure treatments, and although the authors cite this as evidence for fungal transmission, *F. poae* is not a known producer of DON (Thrane *et al*., 2004) and so this increase is likely to be due to the increased activity of other toxigenic fungi in the grain as the substrate used was not autoclaved. While further work is required to determine if the mites would be capable of transmitting the fungus from a more realistic inoculum source, i.e. from infected grain, these studies lend support for the need to control insect populations and grain residues that can act as inoculum reservoirs in grain storage sites, or risk contamination of grain with mycotoxin producing fungi such as *F. poae* and also potentially increasing the mycotoxin output by *Fusarium* spp. that infected grain prior to harvest.

Mycotoxins produced by toxigenic fungi on stored grains have been tested for toxicity on certain insect species (Magan *et al*., 2003). Arthropods that are not harmed by or are able to tolerate the toxins are considered more compatible dispersal agents for the fungi and long-term herbivores of the storage products. DON and T-2 were found not to be toxic to the confused flour beetle, *Tribolium confusum* (Wright *et al*., 1973). Mites *Tyrophagus putrescentiae* were also found to be able to feed on DON without harm (Hubert *et al*., 2014) but previous studies showed them to be sensitive to T-2 and zearalenone (Rodriguez *et al*., 1979). Screening common storage pests for toxin sensitivity may be a useful step in understanding the importance of pest pressures and mycotoxin contamination in stored grain.

Indirect interactions between FHB or FER and arthropods

Indirect interactions of arthropods and FHB or FER pathogens have consequences that are relevant to the disease process in such a way that is mediated by the host plant. In cases where dispersal of the fungus is not enhanced by arthropod activity, the effects of arthropod activity on the host can still increase the susceptibility of the host to the disease (Munkvold, 2003; Drakulic *et al*., 2015). This can include damage allowing secondary fungal infection because wounded plant tissue is easier to enter, changes in volatile emissions from disease plants that alters arthropod behaviour or pre-conditioning of the host plant by suppression of plant defence pathways.

Host weakening by arthropod activity

Synergy between the insect and fungal host attackers is thought to have a modest impact on *F. graminearum* epidemiology in maize (Munkvold, 2003). *F. graminearum* can infect maize systemically or through the silks and neither of these infection routes rely on insect involvement, but in addition to these routes the pathogen can enter the host through wound sites created by insect activity. The significance of this route in host acquisition of the pathogen varies depending on environmental and agronomic factors, but reduction in DON of up to 59% was recorded in *Bt* maize hybrids which resist insect feeding, in comparison to non-transgenic hybrid plants (Schaafsma *et al*., 2002; Munkvold, 2003). This shows that insect activity can promote *F. graminearum* infection and accumulation of DON in maize, although the circumstances under which insect involvement is most likely to impact on the disease has not been elucidated for *F. graminearum*. Insect wounding has also been linked to the increased prevalence of *F. verticillioides* in maize, with attention being drawn to *Helicoverpa zea*, the corn earworm (Dowd, 2000; Clements *et al*., 2003) in addition to the studies documenting the importance of ECB in increasing host susceptibility to FER, which were discussed previously. Populations of *H. zea* vary greatly in sensitivity to *Bacillus thuringiensis*, and populations with greater tolerance can survive on *Bt* maize, they may wound the host and lead to failure to control FER even though control of ECB herbivory is achieved.

The effect of aphid feeding on plant hosts in terms of consequences of disease has also been measured in terms of mycotoxin accumulation in the host. When aphids *Rhodopsium padi*, were fed on wheat leaves whilst ears were inoculated with *F. graminearum* an increase in DON was observed in infected grain compared to aphid-free controls (Liu *et al*., 2005). This implies that systemic changes to the host biochemistry are induced upon aphid feeding that leaves the host less able to withstand infection by the fungus. Furthermore, recent findings have examined the interaction between English grain aphids, *Sitobion avenae*, and *F. graminearum* on wheat and found that the combined effect of both plant attackers leads to increased disease severity and mycotoxin accumulation (Drakulic *et al*., 2015). The outcome of the interactions between pest and pathogen in this case also differed depending on the specific timing of the interaction, with infestation of aphids in advance of fungal infection of the hosts bringing about a rise in the level of pathogen DNA at maturity compared to when pathogen infection preceded aphid infestation.

As described earlier, several other examples of correlations between insect incidence and FHB or FER severity have also been observed. One possible mechanism to explain the increase in disease severity and mycotoxin contamination in hosts with insect infestation is suppression of plant defence by insects. Basal resistance to FHB is thought to be mediated by the salicylic acid (SA) pathway (Makandar *et al*., 2012). In contrast, if attack by insects on plant hosts upregulates the jasmonic acid (JA) pathway, which has negative crosstalk with SA-pathway (Bostock, 2005; Cipollini *et al.*, 2004) susceptibility to FHB could well be increased. A key factor that determines the outcome of the defence response by the host plant is the nature of the feeding behaviour of the arthropod attacker. ECB larvae are chewing insects that cause visible wounding to the host which upregulate JA- and wound-dependent plant defence responses, whereas aphids that feed for a prolonged time on phloem sap cause minimal cellular damage and upregulate different defence pathways including SA- and JA/ethylene-dependent processes (Walling, 2000). Furthermore, insect-produced molecules can alter the host-defence response: chewing insects transfer salivary excretions to the host in the form of foregut regurgitants; aphids and related sap-feeding insects secrete both thick gelling sheath saliva and watery saliva around and through the stylet mouthparts (Dixon, 1973). This can introduce potential elicitors to the host that can upregulate plant defences, but also present the opportunity for insect-produced signalling molecules to be injected into the host plant so as to interfere with the host defence response. Aphids and other phloem feeders in particular have been described to produce effector molecules (Bos *et al*., 2010) that deceive the host into disabling defence responses (Thompson & Goggin, 2006; Walling, 2008) leaving the host increasingly susceptible to secondary attack.

Volatile chemical interactions between infected hosts and arthropods

The frequency of host-mediated interactions between pests and pathogens can be influenced by the volatile chemistry of the host plants (Gagkaeva *et al*., 2014). Infected hosts may emit different volatile chemicals into the environment than healthy hosts, and these chemical signals may be perceptible to proximal arthropods (Drakulic *et al*., 2015). As a result of perceiving volatile chemicals, arthropods may alter their behaviour towards infected hosts and as a result alter the course of the disease (Mayer *et al*., 2008). The study of the chemical ecology of species-specific interactions is one way to identify potentially important relationships between insect herbivores and FHB or FER pathogens.

The behaviour of the cereal leaf beetle, *Oulema melanopus*, is influenced by volatile chemical emissions from maize plants inoculated with a mixture of four *Fusarium* species: *F. avenaceum*, *F. culmorum*, *F. graminearum* and *F. oxysporum* (Piesik *et al*., 2011). An array of green leaf volatiles, terpenes and shikimic acid pathway-derived volatiles were identified as being raised in infected maize emissions above that of controls. Four chemicals, ((Z)-3-hexenyl acetate, (Z)-3-hexenal, linalool and β-caryophyllene) were bioassayed individually for cereal leaf beetle behavioural responses, and significant attraction of *O. melanopus* towards all tested chemicals was observed at specific doses. Similar experiments that used wheat and barley instead of maize, a reduced inoculum mix that omitted *F. oxysporum*, and the related cereal beetle, *O. cyanella*, showed that the beetles were attracted to certain volatile chemicals ((Z)-3-hexnyl acetate and (Z)-3-hexenal) at lower doses but repelled by those chemicals and others ((Z)-β-ocimene and linalool) at high doses (Piesik *et al*., 2013). This work is limited in that the nature of the leaf beetle responses to volatiles induced by different pathogens is not compared to the disease development in infected hosts with and without, and therefore it cannot be concluded as to the impact that altered herbivore attraction would have on disease progression. However this work does show that the severity of infection and the corresponding changes in the level of volatile chemical emissions could have different influences over herbivore behaviour.

The chemical ecology of the tripartite interactions between *F. graminearum*, wheat and grain aphids *Sitobion avenae* was studied alongside analysis of the impacts of aphid activity on the disease and vice versa (Drakulic *et al*., 2015). It was shown that grain aphids were repelled by the volatile chemical emissions of *F. graminearum* infected wheat ears, and that aphids fed on infected hosts had an elevated rate of mortality. It was concluded that avoidance of volatiles indicative of *F. graminearum* infection was likely to be a behavioural adaptation by aphids to evade an inhospitable environment. Prior aphid colonisation of the host was shown to increase pathogen DNA and mycotoxin accumulation, so this work revealed that insects relevant to disease processes do not necessarily need to be attracted to the infected host to impact upon the disease, as appears to occur in FER (Cardwell *et al*., 2000; Schulthess *et al*., 2002). Moreover, the work of Drakulic *et al*. (2015) demonstrates that timing is critical in determining the outcomes of volatile organic chemical (VOC) interactions with insect pests.

The behaviour of the meal beetle *Tenebrio molitor* towards grain infected with different *Fusarium* species has been assessed on wheat grain, in addition to beetle survival when feeding on the infected grain (Guo *et al*., 2014). Beetles were attracted to grain infected with *F. culmorum*, *F. poae* or *F. proliferatum*, but repelled by grain infected with *F. avenaceum*. In accordance, survival rates were similar to controls for *F. proliferatum* or *F. poae*-infected grain, but infection by *F. avenaceum* or *F. culmorum* lead to increased mortality. This study revealed three different relationships between a single insect species and several related fungi. Meal beetles were not harmed by *F. poae* and *F. proliferatum* and were attracted to infected hosts, potentially increasing dispersal of the fungus or increasing the mechanical and biological damage to grains infected with those species thus facilitating further infection of the hosts or changes in fungal metabolism as a response. Conversely, the beetles avoided grain infected with *F. avenaceum*, so the insects are observed to avoid the damaging environment by interpreting volatile chemical cues produced by the infected grain. Finally, the beetles were attracted to *F. culmorum* infected grains, despite this environment being detrimental to the survivorship of the insects, which could be interpreted as manipulation of the insect by the pathogen: while the pathogen could benefit from the insect activity, feeding on hosts infected with this pathogen would negatively impact on the meal beetle population. Why this relatively aggressive pathogen bucks the trend is not addressed in this work, although as *F. avenaceum* produces beauvericin, whereas *F. culmorum* does not, it could be the case that the different mycotoxin contributions of the pathogen species is one factor that plays a role in differentiating the response of insects to infected host volatiles.

Species-specific interactions between *Fusarium* pathogens and the rice weevil, *Sitophilus oryzae*, have been observed (Selitskaya *et al*., 2014). Interestingly, weevils responded differently to the VOC produced by fungal colonies on agar plates versus infected wheat grain in some incidences, implying that there are host-dependent differences in VOC output from pathogens, so that the same pathogen could produce different volatiles on different host species infected. Moreover, this work highlighted different responses of weevils to volatiles of grain infected with species differing in pathogenicity to the host plant. Weaker pathogens *F. poae* and *F. langsethiae* were attractive to the weevils whereas volatiles from grain infected with *F. graminearum* and *F. culmorum* were repellent. A study has identified the VOC produced by *F. poae* on inoculated wheat grain, showing differences in abundances of chemical groups between two and five days after inoculation (Precisse *et al*., 2006). Some chemicals identified were known to be associated with infections caused by other fungal pathogens, including ethyl acetate which has been associated with *F. culmorum* infection, but others, such as 2,4-Dimethylepten, were considered to be indicative specifically of *F. poae* contamination. Further to this, several carbonyl-possessing chemicals were shown to be suppressed from grain emissions following *F. poae* infection, including 2-butanone, 3-methylbutanal and 2-heptanone. This work shows that pathogen-specific changes in VOC emissions from stored grain could potentially be used to identify early infection of grain and to determine which pathogens are likely to be present, and therefore what arthropod activity would be expected to increase risk of mycotoxin contamination.

Conclusions

This review explores how arthropods may interact with the processes of FHB or FER by drawing together and appraising current knowledge of *Fusarium*-arthropod interactions. In doing this we have identified some important knowledge gaps that merit attention in future studies. Firstly, there is the need for more investigation into insect dispersal of inoculum from natural sources instead of colonies. Secondly, there is a need for identification of the molecular mechanisms that mediate enhanced host susceptibility to FHB or FER disease following arthropod herbivory. Finally, the role of mycotoxins in mediating arthropod behaviour through host volatile chemistry needs to be clarified. The potential for interaction between these diseases and arthropods has generally received less attention than other aspects of the disease epidemiology, but this means it is an exciting new area of science. Little work has been done to evaluate the potential role of arthropod involvement in Fusarium disease epidemiology while host plants are growing in the vegetative stage. However, upon the production of reproductive organs and the beginning of the period of host susceptibility to FHB or FER, arthropod activity has been observed to have varying degrees of impact on the disease depending on the combination of species of host, arthropod and pathogen involved.

The most thoroughly studied system is that of maize, FER caused by *F. verticillioides* and the activity of the European corn borer (*Ostrinia nubilalis*), which has been accepted to act as a vector for the fungus despite somewhat limited direct evidence (Sobek & Munkvold, 1999; Cardwell *et al*., 2000; Darvas *et al*., 2011), and provides wound sites to the host that leads to an increase in disease symptoms and in the levels of the fumonisin mycotoxins (Munkvold 2003; Saladini *et al*., 2008; Blandino *et al*., 2009). Very few studies have investigated the interaction of arthropods on FHB epidemiology in small-grained cereals, although from the limited amount information available it appears that *F. graminearum* infection can be promoted by aphid infestation without acting as a vector for the pathogen (Bagga, 2008; Drakulic *et al*., 2015). The activity of thrips and mites have also been correlated with increased disease severity in a range of hosts and, along with sap and flour beetles, demonstrated to be capable of altering the disease process in cereal plants, with no definitive evidence to suggest their activity as vectors between infected host plants either (Parsons & Munkvold, 2010; Piesik *et al*., 2011). This small amount of research supports the hypothesis that insect and mite activity can impact the progress of FHB disease in such a way as to increase the host's susceptibility, and therefore to increase yield and grain quality losses and increased mycotoxin accumulation.

The role of host volatiles in mediating the interactions between *Fusarium* pathogens and arthropod herbivores appears to vary between systems. If pathogen species-specific compounds can be identified, screening crops early in the growth season with devices such as electronic noses might provide an early warning to allow timely application of fungicide treatments when needed. Furthermore, gaining knowledge of field and storage arthropod pest species that respond to infected host volatiles would be beneficial for informing pest monitoring and management strategies of the associated risks. Control of FHB and FER may be improved by using combinations of fungicides and insecticides at important time periods in the disease cycle, and the importance of appropriate storage environments for cereal products has been highlighted by the potentially damaging interactions that can occur between toxigenic *Fusarium* fungi and arthropods in stored grain.

Acknowledgements

This work was supported by a Lawes' Trust PhD studentship. Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council (BBSRC).

References

Anon. 2006. Commission Regulation (EC) No 1881/2006 setting maximum levels of certain contaminants in foodstuffs. Official Journal of the European Union L365, 5-24.

Attwater WA, Busch LV, 1983. Role of the sap beetle *Glischrochilus quadrisignatus* in the epidemiology of *Gibberella* corn ear rot. *Canadain Journal of Plant Pathology* **5**, 158-163.

Bagga PS, 2008. Fusarium head blight (FHB) of wheat: Role of host resistance, wheat aphids, insecticide and strobilurin fungicide in disease control in Punjab, India. *Cereal Research Communications* **36**, 667-70.

Bai GH, Shaner G, 2004. Management and resistance in wheat and barley to Fusarium head blight. *Annual Review of Phytopathology* **42**, 135-61.

Bakan B, Melcion D, Richard-Molard D, Cahagnier B, 2002. Fungal growth and *Fusarium* mycotoxin content in isogenic traditional maize and genetically modified maize grown in France and Spain. *Journal of Agricultural and Food Chemistry* **50**, 728–731.

Bartelt RJ, Wicklow DT, 1999. Volatiles from *Fusarium verticillioides* (Sacc.) Nirenb. and their attractiveness to nitidulid beetles. *Journal of Agricultural and Food Chemistry* **47**, 2447-54.

Belakova S, Benesova K, Caslavsky J, Svoboda Z, Mikulikova R, 2014. The occurrence of the selected *Fusarium* mycotoxins in Czech malting barley. *Food Control* **37**, 93-8.

Blandino M, Reyneri A, Vanara F, Pascale M, Haidukowski M, Saporiti M, 2008. Influence of sowing date and insecticide treatments *Ostrinia nubilialis* (Hubner) damage and fumonisin contamination in maize kernels. *Maydica* **52**, 199-206.

Blandino M, Reyneri A, Vanara F, Tamietti G, Pietri A, 2009. Influence of agricultural practices on *Fusarium* infection, fumonisin and deoxynivalenol contamination of maize kernels. *World Mycotoxin Journal* **2**, 409-18.

Boenisch MJ, Schaefer W, 2011. *Fusarium graminearum* forms mycotoxin producing infection structures on wheat. *BMC Plant Biology*. [http://www.biomedcentral.com] 10.1186/1471-2229-11-110.

Borkent A, Bissett J, 1985. Gall midges *Diptera cecidomyiidae* are vectors for their fungal symbionts. *Symbiosis* **1**, 185-94.

Bowers E, Hellmich R, Munkvold G, 2014. Comparison of fumonisin contamination using HPLC and ELISA methods in *Bt* and near-isogenic maize hybrids infested with European corn borer or Western bean cutworm. *Journal of Agricultural and Food Chemistry* **62**, 6463-72.

Bos JIB, Prince D, Pitino M, Maffei ME, Win J, Hogenhout SA, 2010. A functional genomics approach identifies candidate effectors from the aphid species *Myzus persicae* (Green peach aphid). *PLoS Genetics* [http://journals.plos.org/plosgenetics/] 10.1371/journal.pgen.1001216.

Bostock RM, 2005. Signal crosstalk and induced resistance: straddling the line between cost and benefit. *Annual Review of Phytopathology* **43**, 545-580.

Buerstmayr H, Ban T, Anderson JA, 2009. QTL mapping and marker-assisted selection for *Fusarium* head blight resistance in wheat: a review. *Plant Breeding* **128**, 1-26.

Bushnell WR, Hazen BE, Pritsch C, 2003. Histology and physiology of Fusarium head blight In: Leonard KJ, Bushnell WR, eds. *Fusarium Head Blight of Wheat & Barley*, St. Paul, USA: APS Press, 44-83.

Cardwell KF, Kling JG, Maziya-Dixon B, Bosque-Perez NA, 2000. Interactions between *Fusarium verticillioides*, *Aspergillus flavus*, and insect infestation in four maize genotypes in lowland Africa. *Phytopathology* **90**, 276-284.

Cipollini D, Enright S, Traw B, Bergelson J, 2004. Salicylic acid inhibits jasmonic acid-induced resistance of *Arabidopsis thaliana* to *Spodoptera exigua*. *Molecular Ecology* **13**, 1643-1653.

Clements MJ, Campbell KW, Maragos CM, Pilcher C, Headrick JM, Pataky JK, White DG, 2003. Influence of Cry1Ab protein and hybrid genotypes on Fumonisin contamination and Fusarium ear rot of corn. *Crop Science* **43**, 1283-1293.

Couture L, Dubuc JP, Comeau A, 1995. Occurrence of contamination of wheat grain by the orange wheat midge and seed microflora in Quebec. *Canadian Plant Disease Survey* **75**, 128-9.

Cui L, Selvaraj JN, Xing F, Zhao Y, Zhou L, Liu Y, 2013. A minor survey of deoxynivalenol in *Fusarium* infected wheat from Yangtze-Huaihe river basin region in China. *Food Control* **30**, 469-73.

D'Mello JPF, Placinta CM, Macdonald AMC, 1999. *Fusarium* mycotoxins: a review of global implications for animal health, welfare and productivity. *Animal Feed Science and Technology* **80**, 183-205.

Darvas B, Banati H, Takacs E, Lauber E, Szecsi A, Szekacs A, 2011. Relationships of *Helicoverpa armigera*, *Ostrinia nubilalis* and *Fusarium verticillioides* on MON 810 maize. *Insects* **2**, 1-11.

Divon HH, Razzaghian J, Udnes-Aamot H, Klemsdal SS, 2012. *Fusarium langsethiae* (Torp and Nirenberg), investigation of alternative infection routes in oats. *European Journal of Plant Pathology* **132**, 147-61.

Dixon AFG, 1973. Studies in biology No. 44 Biology of Aphids. London, UK: The Institute of Biology.

Dowd PF, 2004. Insect management to facilitate preharvest mycotoxin management. *Journal of Toxicology – Toxin Reviews* **22**, 327-350.

Dowd PF, 2000. Indirect reduction of ear molds and associated mycotoxins in *Bacillus thuringiensis* corn under controlled and controlled and open field conditions: utility and limitations. *Journal of Economic Entomology* **93**, 1669-1679.

Drakulic J, Caulfield J, Woodcock C, Jones SPT, Linforth R, Bruce TJA, Ray RV, 2015. Sharing a host plant (wheat: *Triticum aestivum*) increases the fitness of *Fusarium graminearum* and the severity of Fusarium head blight but reduces the fitness of grain aphids (*Sitobion avenae*). *Applied and Environmental Microbiology* **81**, 3492-501.

Eggert K, Rawel HM, Pawelzik E, 2011. *In vitro* degragation of wheat gluten fractions by *Fusarium graminearum* proteases. *European Food Research and Technology* **233**, 697-705.

Farrar JJ, Davis RM, 1991. Relationships among ear morphology, western flower thrips, and Fusarium ear rot of corn. *Phytopathology* **81**, 661-666.

Fernando WGD, Paulitz TC, Seaman WL, Dutilleul P, Miller JD, 1997. Head Blight gradients caused by *Gibberella zeae* from area sources of inoculum in wheat field plots. *Phytopathology* **80**, 414-421.

Fredlund F, Gidlund A, Sulyok M Borjesson T, Krska R, Olsen M, Lindblad M, 2013. Deoxynivalenol and other selected *Fusarium* mycotoxins in Swedish oats – Occurrence and correlation to specific *Fusarium* species. *International Journal of Food Microbiology* **167**, 276-283.

Gagkaeva TY, Shanmshev IV, Gavrilova OP, Selitskaya OG, 2014. Biological relationships between *Fusarium* fungi and insects (review). *Sel'skokhozyaistvennaya Biologiya* **3**, 13-23.

Gale LR, Harrison SA, Ward TJ, O'Donnell K, Milus EA, Gale SW, Kistler HC, 2011. Nivalenol-type populations of *Fusarium graminearum* and *F. asiaticum* are prevalent on wheat in southern Louisiana. *Phytopathology* **101**, 124-134.

Gilbert J, Haber S, 2013. Overview of some recent research developments in Fusarium Head Blight of wheat. *Canadian Journal of Plant Pathology* **35**, 149-74.

Gilbertson RL, Brown WM, Ruppel EG, Capinera JL, 1986. Association of corn stalk rot *Fusarium* spp. and Western corn rootworm beetles in Colorado. *Phytopathology* **76**, 1309-1314.

Glynn NC, Edwards SG, 2010. Evaluation of PCR assays for quantifying seed-borne infection by *Fusarium* and *Microdochium* seedling blight pathogens. *Journal of Applied Microbiology* **108**, 81-7.

Goswami RS, Kistler HC, 2004. Heading for disaster: *Fusarium graminearum* on cereal crops. *Molecular Plant Pathology* **5**, 515-25.

Guo ZQ, Doll K, Dastjerdi R, Karlovsky P, Dehne HW, Altincicek B, 2014. Effect of fungal colonization of wheat grains with *Fusarium* spp. on food choice, weight gain and mortality of meal beetle larvae (*Tenebrio molitor*). *Plos One* [http://journals.plos.org/] 10.1371/journal.pone.0100112.

Hubert J, Nesvorna M, Kopecky J, 2014. The effect of *Tyrophagus putrescentiae* on *Fusarium poae* transmission and fungal community in stored barley in a laboratory experiment. *Insect Science* **21**, 65-73.

Imathiu SM, Edwards SG, Ray RV, Back MA, 2013. *Fusarium langsethiae* – a HT-2 and T-2 toxins producer that needs more attention. *Journal of Phytopathology* **161**, 1-10.

Imathiu SM, Hare MC, Ray RV, Back M, Edwards SG, 2010. Evaluation of pathogenicity and aggressiveness of *F. langsethiae* on oat and wheat seedlings relative to known seedling blight pathogens. *European Journal of Plant Pathology* **126**, 203-16.

Jansen C, von Wettstain D, Schaefer W, Kogel K, Felk A, Maier FJ, 2005. Infection patterns in barley and wheat spikes inoculated with wild type and trichodiene synthase gene disrupted *Fusarium graminearum*. *PNAS* **102**, 16892-16897.

Kemp GHJ, Pretorius ZA, Wingfield MJ, 1996. Fusarium glume spot of wheat: A newly recorded mite-associated disease in South Africa. *Plant Disease* **80**, 48-51.

Kohl J, de Haas BH, Kastelein P, Burgers SLGE, Waalwijk C, 2007. Population dynamics of *Fusarium* spp. and *Microdochium nivale* in crops and crop residues of winter wheat. *Phytopathology* **97**, 971-8.

Landschoot S, Audenaert K, Waegeman W, Pycke B, Bekaert B, De Baets B, Haesaert G, 2011. Connection between primary *Fusarium* inoculum on gramineous weeds, crop residues and soil samples and the final population on wheat ears in Flanders, Belgium. *Crop Protection* **30**, 1297-305.

Lattanzio VMT, Pascale M, Visconti A, 2009. Current analytical methods for trichothecene mycotoxins in cereals. *Trends in Analytical Chemistry* **28**, 758-68.

Leslie JF, Summerell BA, 2006. The Fusarium laboratory manual. Ames, USA: Blackwell.

Liu Y, Walker F, Hoeglinger B, Buchenauer H, 2005. Solvolysis procedures for the determination of bound residues of the mycotoxin deoxynivalenol in *Fusarium* species infected grain of two winter wheat cultivars preinfected with barley yellow dwarf virus. *Journal of Agricultural and Food Chemistry* **53**, 6864-6869.

Logrieco A, Bottalico A, Mule G, Moretti A, Perrnoe G, 2003. Epidemiology of toxigenic fungi and their associated mycotoxins for some Mediterranean crops. *European Journal of Plant Pathology* **109**, 645-667.

Magan N, Hope R, Cairns V, Aldred D, 2003. Post-harvest fungal ecology: Impact of fungal growth and mycotoxin accumulation in stored grain. *European Journal of Plant Pathology* **109**, 723-30.

Makander R, Nalam VJ, Lee H, Trick HN, Dong Y, Shah J, 2012. Salicylic acid regulates basal resistance to Fusarium head blight in wheat. *Molecular Plant-Microbe Interactions* **25**, 431-439.

Mayer CJ, Vilcinkas A, Gross J, 2008. Phytopathogen lures its insect vector by altering host plant odor. *Journal of Chemical Ecology* **34**, 1045-1049.

Marin S, Ramos AJ, Cano-Sancho G, Sanchis V, 2013. Mycotoxins: Occurrence, toxicology, and exposure assessment. *Food and Chemical Toxicology* **60**, 218-37.

McMullen M, Bergstrom G, De Wolf , Dill-Macky R, Hershman D, Shaner G, Van Sanford D, 2012. A unified effort to fight an enemy of wheat and barley: Fusarium Head Blight. *Plant Disease* **96**, 1712-28.

Miller JD, 1994. Epidemiology of Fusarium ear diseases of cereals In: Miller JD, Trenholm HL, eds. *Mycotoxins in grain: Compounds other than aflatoxin*. St. Paul, USA: Eagen Press, 19-36.

Mills JT, Sinha RN, Demianyk CJ, 1992. Feeding and multiplication of a psocid, *Liposcelis-bostrychophilus* badonnel (Psocoptera, liposcelidae), on wheat, grain screenings, and fungi. *Journal of Economic Entomology* **85**, 1453-62.

Mongrain D, Couture L, Comeau A, 2000. Natural occurrence of *Fusarium graminearum* on adult wheat midge and transmission to wheat spikes. *Cereal Research Communications* **28**, 173-80.

Mongrain D, Couture L, Dubuc JP, Comeau A, 1997. Occurrence of the orange wheat blossom midge Diptera: Cecidomyiidae in Quebec and its incidence on wheat grain microflora. *Phytoprotection* **78**, 17-22.

Munkvold GP, 2003. Cultural and genetic approaches to managing mycotoxins in maize. *Annual Review of Phytopathology* **41**, 99-116.

Nesvorna M, Gabrielova L, Hubert J, 2012. Suitability of a range of *Fusarium* species to sustain populations of three stored product mite species (Acari: Astigmata). *Journal of Stored Products Research* **48**, 37-45.

Nielsen LK, Jensen JD, Nielsen GC, Jensen JE, Spliid NH, Thomsen IK, Justesen AF, Collinge DB, Jorgensen LN, 2011. Fusarium Head Blight of cereals in Denmark: Species complex and related mycotoxins. *Phytopathology* **101**, 960-969.

Opoku N, Back M, Edwards SG, 2013. Development of *Fusarium langsethiae* in commercial cereal production. *European Journal of Plant Pathology* **136**, 159-70.

Paciolla C, Dipierro N, Mule G, Logrieco A, Dipierro S, 2004. The mycotoxins beauvericin and T-2 induce cell death and alteration to the ascorbate metabolism in tomato protoplasts. *Physiological and Molecular Plant Pathology* **65**, 49-56.

Parikka P, Hakala K, Tiilikkala K, 2012. Expected shifts in *Fusarium* species' composition on cereal grain in Northern Europe due to climatic change. *Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment* **29**, 1543-55.

Parry DW, Jenkinson P, McLeod L, 1995. Fusarium ear blight (scab) in small-grain cereals - a review. *Plant Pathology* **44**, 207-38.

Parsons MW, Munkvold GP, 2010. Relationships of immature and adult thrips with silk-cut, Fusarium ear rot and fumonisin B-1 contamination of maize in California and Hawaii. *Plant Pathology* **59**, 1099-106.

Parsons MW, Munkvold GP, 2012. Effects of planting date and environmental factors on Fusarium ear rot symptoms and fumonisin B1 accumulation in maize grown in six North American locations. *Plant Pathology* **61**, 1130-42.

Piesik D, Lemnczyk G, Skoczek A, Lamparski R, Bocianowski J, Kotwica K, Delaney KJ, 2011. *Fusarium* infection in maize: Volatile induction of infected and neighboring uninfected plants has the potential to attract a pest cereal leaf beetle, *Oulema melanopus*. *Journal of Plant Physiology* **168**, 1534-42.

Piesik D, Panka D, Jeske M, Wenda-Piesik A, Delaney KJ, Weaver DK, 2013. Volatile induction of infected and neighbouring uninfected plants potentially influence attraction/repellence of a cereal herbivore. *Journal of Applied Entomology* **137**, 296-309.

Placinta CM, D'Mello JPF, Macdonald AMC, 1999. A review of worldwide contamination of cereal grains and animal feed with *Fusarium* mycotoxins. *Animal Feed Science and Technology* **78**, 21-37.

Presicce DS, Forleo A, Taurino AM, Zuppa M, Siciliano P, Laddomada B, Logreico A, Visconti A, 2006. Response evaluation of an E-nose towards contaminated wheat by *Fusarium poae* fungi. *Sensors and Actuators B-Chemical* **118**, 433-8.

Rodriguez JG, Potts M, Rodriguez LD, 1979. Survival and reproduction of 2 species of stored product beetles on selected fungi. *Journal of Invertebrate Pathology* **33**, 115-7.

Rudd JC, Horsley RD,McKendry AL, Elias EM, 2001. Host plant resistance genes for Fusarium head blight: Sources, mechanisms and utility in conventional breeding systems. *Crop Science* **41**, 620-627.

Saladini MA, Blandino M, Reyneri A, Alma A, 2008. Impact of insecticide treatments on *Ostrinia nubilalis* (Hubner) (Lepidoptera: Crambidae) and their influence on the mycotoxin contamination of maize kernels. *Pest Management Science* **64**, 1170-8.

Schaafsma AW, Hooker DC, Baute TS, Illincic-Tamburic L, 2002. Effect of *Bt*-corn hybrids on deoxynivalenol content in grain at harvest. *Plant Disease* **86**, 1123-6.

Schulthess F, Cardwell KF, Gounou S, 2002. The effect of endophytic *Fusarium verticillioides* on infestation of two maize varieties by Lepidopterous stemborers and Coleopteran grain feeders. *Phytopathology* **92**, 120-128.

Selitskaya OG, Gavrilova OP, Schenikova AV, Shamshev IV, Gagkaeva TY, 2014. The effect of toxin-produced *Fusarium* fungi on behaviour of the rice weevil *Sitophilus oryzae* l. (Coleoptera, dryophthoridae). *Entomologicheskoe Obozrenie* **93**, 303-10.

Sobek EA, Munkvold GP, 1999. European corn borer (Lepidoptera: Pyralidae) larvae as vectors of *Fusarium moniliforme*, causing kernel rot and symptomless infection of maize kernels. *Journal of Economic Entomology* **92**, 503-9.

Streit E, Schatzmayr G, Tassis P, Tzika E, Marin D, Taranu I, Tabuc C, Nicolau A, Aprodu I, Puel O, Oswald IP, 2012. Current situation of mycotoxin contamination and co-occurrence in animal feed - focus on Europe. *Toxins* **4**, 788-809.

Sturz AV, Johnston HW, 1985. Characterization of *Fusarium* colonization of spring barley and wheat produced on stubble or fallow soil. *Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie* **7**, 270-6.

Suzuki T, Iwahashi Y, 2014. Phytotoxicity evaluation of Type B trichothecenes using a *Chlamydomonas reinhardtii* model system. *Toxins* **6**, 453-463.

Thompson GS, Goggin FL, 2006. Transcriptomics and functional genomics of plant defence induction by phloem-feeding insects. *Journal of Experimental Botany* **57**, 755-766.

Thrane U, Adler A, Clasen PE, Galvano F, Langseth W, Lew H, Logrieco A, Nielsen KF, Ritieni A, 2004. Diversity in metabolite production by *Fusarium langsethiae*, *Fusarium poae*, and *Fusarium sporotrichioides*. *International Journal of Food Microbiology* **95**, 257-266.

Van der Lee T, Zhang H, van Diepeningen A, Waalwijk C, 2015. Biogeography of *Fusarium graminearum* species complex and chemotypes: A review. *Food additives and contaminants Part A – Chemistry Analysis Control Exposure & Risk Assessment* **32**, 453-460.

Walling L, 2008. Avoiding effective defences: Strategies employed by phloem-feeding insects. *Plant Physiology* **146**, 859-866.

Walling L, 2000. The myriad plant responses to herbivores. *Journal of Plant Growth Regulation* **19**, 195-216.

Windels CE, Windels MB, Kommedahl T, 1976. Association of *Fusarium* species with picnic beetles on corn ears. *Phytopathology* **66**, 328-331.

Wright V, De Las Casas E, Harein PK, 1973. The development and reproduction of confused flour beetles exposed to the mycotoxin trichothecene. *Proceedings North Central Branch Entomological Society of America* **28**, 175.

Xu XM, Nicholson P, Thomsett MA, Simpson D, Edwards SG, Cooke BM, Doohan FM, Managhan S, Moretti A, Tocco G, Mule G, Hornok L, Beki E, Tatnell J, Ritieni A, 2008. Relationship between the fungal complex causing Fusarium head blight of wheat and environmental conditions. *Phytopathology* **98**, 69-78.

Yazar S, Omurtag GZ, 2008. Fumonisins, trichothecenes and zearalenone in cereals. *International Journal of Molecular Sciences* **9**, 2062-90.

Figures

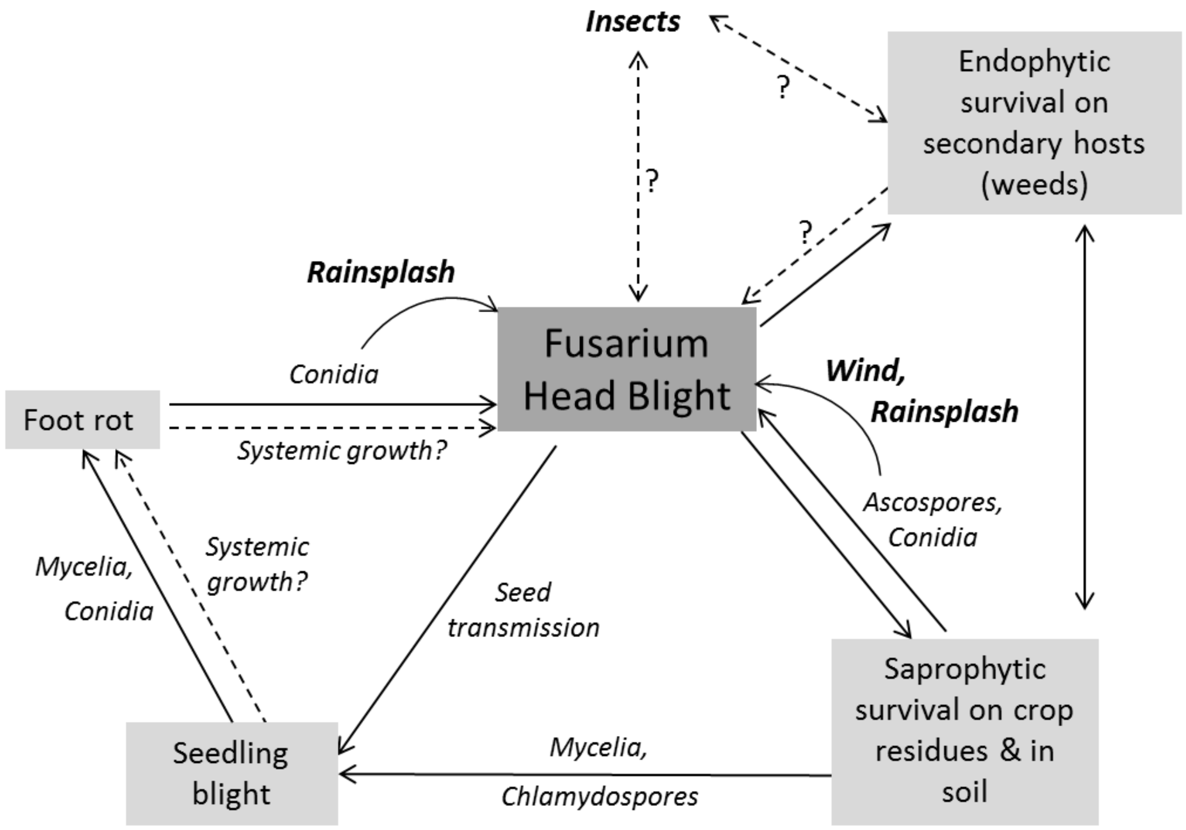

Fig. 1.1. Sources of fusarium head blight inoculum and the factors that promote dispersal of different spore types. Dashed lines indicate unconfirmed processes.

Tables

Table 1.1: Summary of studies observing correlations between insect activity and fusarium disease incidence, severity and mycotoxin levels.

Insect(s)	Crop	Pathogen(s)	Country	Finding	Citation
Sitodiplosis mosellana Orange wheat blossom midge (OWBM)	Wheat	*F. graminearum*	Canada	Number of OWBM larvae per spike and per spikelet positively correlated with *F. graminearum* seed contamination (R = 0.67).	Mongrain *et al*., 1997
Sitobion avenae English grain aphid	Wheat	*F. graminearum*	India	Monocrotophos (0.1%) insecticide at booting and heading or only at heading reduced aphid population by 80% and FHB incidence and severity by 21% and 30% respectively.	Bagga, 2008
Ostrinia nubilalis European corn borer (ECB)	Maize	*F. verticillioides*	Italy	ECB damage 23% greater in late sown maize, and early sowing reduced FER incidence and severity by 25% and 49%. Early sowing with insecticides (deltamethrin @ 0.012 kg AI ha^{-1}) which reduced ECB reduced $FB_1 + FB_2$ by 79%.	Blandino *et al*., 2008
"	"	"	Italy	Insecticides (deltamethrin @ 0.013 kg AI ha^{-1} or lambda-cyhalothrin @ 0.019 kg AI ha^{-1}) reduced ECB severity and reduced $FB_1 + FB_2$ by 75%, FER incidence by 55% and severity by 68%.	Saladini *et al*., 2008
O. nubilalis & *Sesamia inferens* Pink stem borer	*Bt*-maize	*F. verticillioides* *F. proliferatum*	France & Spain	*Bt*-maize grain had up to 18 times lower fungal biomass and up to 30 times lower FB_1 than grain from near-isogenic traditional maize hybrids.	Bakan *et al*., 2002
Frankinella occidentalis Western flower thrips	Maize	*F. verticillioides*	USA	Insecticides (lambda cyhalothrin & dimethoate @ 0.035 & 0.56 kg AI ha^{-1}) reduced thrips infestation as well as silk-cut symptoms, FER and FB_1 in field trials. Intra-ear immature thrips were more strongly correlated with FB_1 (R = 0.53) than mature thrips (R = 0.36).	Parsons & Munkvold, 2010
"	"	"	USA	Intra-ear thrips infestation correlated with mould symptoms (R = 0.78) and FB_1 (R = 0.83).	Parsons & Munkvold, 2012

FHB: fusarium head blight; FER: fusarium ear rot; $FB_1 + FB_2$: fumonisin B_1 + fumonisin B_2.

Chapter 2: Sharing a host plant (Wheat [*Triticum aestivum*]) increases the fitness of *Fusarium graminearum* and the severity of *Fusarium* Head Blight but reduces the fitness of grain aphids (*Sitobion avenae*)

Experimental work investigating the nature of the interactions between *Sitobion avenae* and *Fusarium graminearum* fungus that occur on wheat plants is presented in this chapter. The work has been published by the scientific journal *Applied and Environmental Microbiology* in 2015, and is presented in paper format.

Author contribution

The paper was composed and practical work conducted by J. Drakulic, with the exceptions of insect electroantennography performed by C. Woodcock, analysis of volatile chemicals by GC and GC-MS by John Caulfield, and analysis of mycotoxin extractions by S. P. T. Jones with consultation from R. Linforth. The project was supervised by R. V. Ray and T. Bruce.

RESEARCH ARTICLE

Sharing a Host Plant (Wheat [*Triticum aestivum*]) Increases the Fitness of *Fusarium graminearum* and the Severity of *Fusarium* Head Blight but Reduces the Fitness of Grain Aphids (*Sitobion avenae*)

Suggested running title: *Fusarium graminearum* infection renders wheat repellent to aphids

Jassy Drakulic[1,2], John Caulfield[2], Christine Woodcock[2], Stephen P.T. Jones[1], Robert Linforth[1], Toby J.A. Bruce[1,2], Rumiana Ray[1#]

1 University of Nottingham, Sutton Bonington Campus, College Rd, Sutton Bonington, LE12 5RD, UK

2 Rothamsted Research, Harpenden, Herts AL5 2JQ, UK

Author for correspondence:

Rumiana Ray

Tel: +44 115 951 6049

E-mail: Rumiana.ray@nottingham.ac.uk

Abstract

We hypothesised that interactions between Fusarium head blight-causing pathogens and herbivores are likely to occur because they share wheat as a host plant. Our aim was to investigate the interactions between the grain aphid, *Sitobion avenae*, and *Fusarium graminearum* on wheat ears and the role that host volatile chemicals play in mediating interactions. Wheat ears were treated with aphids and *F. graminearum* inoculum, together or separately, and disease progress was monitored by visual assessment, pathogen DNA and mycotoxin quantification. Plants exposed to both aphids and *F. graminearum* inoculum showed accelerated disease progression, with a two-fold increase in disease severity and five-fold increase in mycotoxin accumulation compared to plants treated only with *F. graminearum*. Furthermore, the longer the period of aphid colonisation of the host prior to inoculation with *F. graminearum* the greater the amount of pathogen DNA accrued. Headspace samples of plant volatiles were collected for use in aphid olfactometer assays and analysed by gas-chromatography mass spectrometry (GC-MS) and GC-coupled electroantennography. Disease-induced plant volatiles were repellent to aphids and 2-pentadecanone was the key semiochemical underpinning the repellent effect. We measured aphid survival and fecundity on infected wheat ears and found that both were markedly reduced on infected ears. Thus, interactions between *F. graminearum* and grain aphids on wheat ears benefit the pathogen at the expense of the pest. Our findings have important consequences for disease epidemiology because we show increased spread and development of host disease, together with greater disease severity, pathogen DNA and mycotoxin accumulation when aphids are present.

Key words: Plant-pathogen interactions, plant-insect interactions, chemical ecology, deoxynivalenol, disease epidemiology.

Introduction

Plant interactions with pathogens or insects are usually studied in isolation even though plants are exposed to both in the field. It is likely that organisms that share the same host plant can alter its condition and thereby interact with each other. Although *Fusarium* pathogens and grain aphids cause substantial damage to wheat and coincide little is known about interactions between these species.

Fusarium head blight (FHB) is a disease of small grain cereals that occurs globally and is caused by a complex of species from the genera *Fusarium* and *Microdochium* (1, 2). The most aggressive species causing FHB is *Fusarium graminearum*, a potent producer of the mycotoxin deoxynivalenol (DON) that is harmful to human and animal health if consumed (3). European legislation has set the acceptable limit of DON in grain marketed for human consumption at 1250 µg kg^{-1} (4) in unprocessed cereals. Consequently, FHB epidemics result in large economic losses from the combined effects of reductions in yield, increased mycotoxin contamination and reduced quality and marketability of the crop (5).

In the spring, ascospores and/or conidia of *Fusarium graminearum* are released from crop residues and spread by wind or rainsplash. Wheat is most susceptible to FHB during anthesis, GS61-69 (6, 1). During this time period, grain aphids are also active on wheat ears and they may influence disease development. The English grain aphid, *Sitobion avenae*, is an ear feeding species (7) and the behaviour of apterous (wingless) individuals is influenced more strongly by volatile chemical emissions from wheat ears than wheat seedlings (8). Volatile emissions from plants can be affected by abiotic or biotic stress including that caused by pathogens which can alter the attractiveness of the plant to visiting insects in turn influencing the disease where the insect may act as the vector of the pathogen (9). The interactions of phytopathogenic fungi and insect herbivores on shared host plants can have a variety of outcomes (10). It has been hypothesised that necrotrophs, such as *F. graminearum* (11), consistently reduce the fitness of insects feeding on the infected host, although there is contradictory evidence in support of this claim

(12, 13). It has also been shown that insect herbivory of hosts indirectly causes changes to plant chemistry, biochemistry, physiology and growth (14) in such a way as to alter the host's capacity to withstand secondary infection. We hypothesised that co-localisation of *F. graminearum* and aphids on wheat ears could significantly influence each other's fitness by altering the condition of the shared host plant. To test this hypothesis, we designed a series of experiments investigating FHB disease progress and aphid performance on plants exposed to them individually or in combination.

Interactions between FHB pathogens and aphid insect herbivores, occurring on shared plant hosts, are likely to be bidirectional. Pathogenesis of the plant and the resulting changes to induced plant host responses are likely to affect the behaviour and success of insects, and insect feeding on the plant may alter the disease progression and severity induced by the pathogen. Pathogen-induced changes in plant secondary metabolites, toxin accumulation and volatile chemical emissions have the potential to enhance or diminish the feeding and reproductive success of aphids on infected hosts. Aphids may facilitate weakening and injury of the host that could benefit opportunistic pathogens or prime plant defences to be more resilient against pathogen attack (14). If there is any negative crosstalk between the plant defence pathways activated by the respective organism then prior colonisation by one could facilitate the success of the other (15). Furthermore, these interactions are likely to be influenced by the changes in volatile emissions resulting from infection of wheat ears by FHB pathogens, and the effects of these chemicals on aphid populations and behaviour have not yet been explored. The impact of aphid-FHB interactions on the shared host plant needs to be better understood since they both occur together in real field situations and their interactions may influence crop protection strategies for both aphid damage and FHB disease.

The aim of this study was thus to elucidate the interactions between aphids and the pathogen *F. graminearum* on wheat ears, their shared plant host. The objectives were to i) determine the role of aphids in FHB disease progression in terms of visual symptom development as well as pathogen DNA and mycotoxin accumulation, and ii) identify the impact of pathogenesis by *Fusarium graminearum* on volatile profile of the host plant and its effect on grain aphid survival and fecundity. In addition, following the first series of experiments a further objective arose: iii) to elucidate the importance of the timing of the interaction between aphids and *F. graminearum* on the host.

Materials & Methods

Plant material

Winter wheat cv. Gallant was sown in compost (Levington F2+S) and vernalised for six weeks at 6°C. Seed was treated with 10 g of prothioconazole and 50 g clothianidin 100 kg^{-1} seed (Redigo® Deter®, Bayer CropScience). Seedlings were then grown in a glasshouse in individual 5 l pots in compost (John Innes type 2) at 18°C maximum/ 15°C minimum daytime temperature, 12°C maximum/ 10°C minimum night time temperature, under a 16 h photoperiod using supplementary lighting.

Aphid rearing

Grain aphids (*Sitobion avenae*) sourced from the Rothamsted Research insectary colony (Harpenden, UK) were reared on winter wheat cv. Tybalt in a controlled environment chamber under variable humidity at 20°C daytime temperature with 16 h daylength.

Production of Fusarium graminearum inoculum

Three single spore isolates of *Fusarium graminearum* (#212, 214 & 216, University of Nottingham) were cultured on potato dextrose agar, from which ten plugs were transferred to carboxymethyl cellulose liquid media (16) and shaken at 90 rpm at 23°C for three days. Spore suspensions were filtered through sterile muslin and concentrations were calculated using a Neubauer improved haemocytometer (Marienfeld-Superior, Germany) then adjusted to 2.5 x 10^5 spores ml^{-1} with sterile distilled water.

Production of F. graminearum infected ears for aphid transmission study and study of aphid mortality and reproductive rate

To generate an infected feedstock for aphids prior to transfer of aphids to susceptible uninfected hosts, wheat (cv. Gallant) ears at GS65 (6) were spray inoculated with spore suspension (2.5 x 10^5 spores ml^{-1}) until runoff and then bagged with polythene bags for 48 h. Following bag removal plants were subjected to misting with an automated system (Access Irrigation, UK) for one minute every hour for ten hours per day for seven days in a controlled environment room at 25°C/ 18°C 16 h/ 8 h day/night, by which time visual symptoms could be observed. Control ears were maintained under the same glasshouse conditions.

To generate infected ears for headspace sampling, ten single spikelets per ear (GS65) were point inoculated with 10 µl spore suspension (2.5 x 10^5 spores ml^{-1}) between the lemma and palea (layers of the spikelet) and allowed to develop symptoms in the same controlled environment chamber conditions as above. Control plants were treated in the same way, but with sterile distilled water used instead of spore suspension.

The role of aphids in FHB disease caused by F. graminearum

To test the ability of aphids to act as a vector of *F. graminearum* inoculum, five treatments, described in Table 2.1, were used. Aphids were applied to ears firstly following transfer from infected previous hosts and secondly after being artificially coated in *F. graminearum* spores. To test the effect of co-localisation of aphids and *F. graminearum* on FHB disease progression of wheat ears, ears were treated simultaneously with aphids transferred from a pathogen-free previous host and with *F. graminearum* point inoculation. Three treatments were included as controls: negative control ears were point inoculated only with water; positive control ears were point inoculated with *F. graminearum* spores; and aphid only control ears had no pathogen inoculum added, rather aphids were added from a pathogen-free previous host. The experiment had randomised block design with six treatments and eight replicates.

Following treatment, ears were encased in netting for 48 h. After this time netting and aphids (if present) were removed. Single ears of the main tillers of wheat plants at GS65 were used to assess the disease progression of *F. graminearum* with different treatments. Visual disease assessment was conducted at five time points between three and five days apart, from two up until 21 days after inoculation, by counting the number of spikelets with FHB symptoms (water-soaked lesions and/or bleached spikelets, 17). Disease severity was calculated as the percentage of total spikelets with symptoms per ear.

The effect of timing of aphid colonisation on F. graminearum disease progression

As a result of the previously described experiment, further exploration into the interaction between aphids and *F. graminearum* when both were added to hosts was conducted. To elucidate the importance of the timing of the interaction between grain aphids and *F. graminearum* inoculum on the shared host plant, aphids were either added prior to or following spray inoculation (1×10^5 spores ml^{-1}) of all heads of single wheat plants until runoff which were then bagged with polythene bags for 48 h. Inoculation was carried out when a minimum of four ears had reached mid-anthesis (GS65). Eight whole plant replicates were prepared per treatment; aphids were applied at different timings to four treatments and one treatment was kept free of aphids as a control. Aphids were either added seven days or three days prior to *F. graminearum* inoculum, on the same day as the pathogen inoculation or four days afterwards. Plants were contained within large wooden-framed netted cages and aphids were added by placing a colonised leaf, containing a minimum of 30 adults, from the rearing cage adjacent to wheat ears which was then removed after 48 h.

Plants were monitored for aphid colonisation rate every seven days until considered to be thoroughly colonised (20 aphids per ear) and for visual signs of disease (17) every 3-4 days commencing at four days after *F. graminearum* inoculation and terminating at 28 days after inoculation.

DNA extraction and quantification of F. graminearum by real-time PCR

Individual wheat ears (n = 8) were harvested at 30 and 35 days after inoculation for the aphid transmission study and the aphid timing study, respectively. Whole ears were freeze dried and milled intact in a centrifugal mill (ZM 200 Retsch GmbH, Germany). Flour samples (0.5 g) were weighed into 15 ml tubes and DNA extracted as described previously (18) in CTAB buffer. Volumes of CTAB and potassium acetate were adjusted to 3.75 ml and 1.25 ml respectively to account for the small sample mass, and incubation of flour and CTAB at 65°C was reduced to two hours. DNA concentration was calculated by examination of absorbance at wavelengths 260, 280, 328 and 360 nm by a CARY® 50Probe UV-visible spectrophotometer (Varian, CA, USA) then computed with Simple Reads software, adjusted to 20 ng μl^{-1} and stored at -20°C.

Diagnostic PCR assay was carried out to confirm the presence of fungal DNA using ITS4&5 primers (5'-3': ITS4, TCCTCCGCTTATTGATATGC; ITS5, GGAAGTAAAAGTCGTAACAAGG) using a program as described previously (19). The resulting samples were run on a 1% gel and visualised under UV light (Gel Doc 2000, BioRad, USA). Then two technical repeats of a real time PCR assay were performed in a total volume of 13 µl comprising 2.5 µl template DNA, SYBR® Green 2x Master Mix (BioRad, USA) and 250 nM of *F. graminearum* specific primers (5'-3': Fg16NF: ACAGATGACAAGATTCAGGCACA; Fg16NR: TTCTTTGACATCTGTTCAACCCA) (20) with a template product size of 280 bp. The cycling protocol was as described previously (21) comprising a 1.5 minute initial denaturation step at 95°C, then 35 cycles of 30 seconds at 94°C, 45 seconds at 64°C, and 45 seconds at 72°C, and finally five minutes at 72°C for elongation. Target DNA from plant extracts was quantified using six DNA standards in ten-fold dilutions, originally extracted from a pure culture of *F. graminearum* isolate #212 (University of Nottingham). Linear regression was used to calculate the quantity of target DNA. Results for pathogen DNA (pg. ng^{-1}) denotes target DNA (pg) as a proportion of total DNA (ng).

Analysis of mycotoxin content

The remaining flour after DNA extraction was used in mycotoxin extractions for deoxynivalenol (DON). No flour remained for any of the replicates of ears treated with spore-coated aphids, thus mycotoxin quantification could not be performed for this treatment.

For calibration, flour samples (1 g) were spiked with a C^{13} DON internal standard (Biopure, Romer Labs, Runcorn, UK) and C^{13} zearalenone internal standard (Biopure, Romer Labs) to produce a final concentration of 20 ppb each. Extraction was performed in acetonitrile (84%) for one hour on a rotary shaker, then acetonitrile (100%) was added and shaken by hand to mix, filtered through filter paper (Whatman No 1) and the filtrate passed through a clean-up column (Mycosep® 226, Romer Labs). The solvent of 5 ml filtrate was evaporated off to dryness with nitrogen gas and redissolved in 500 µl methanol (100%), solvent evaporated off once again then redissolved in 100 µl methanol (10%) for use in LC-MS.

Aliquots of the samples (10 µl) were chromatographed using a 5 µm Luna C18(2) HPLC column 250 x 3 mm (Phenomenex, Macclefield, UK) fitted to an Agilent 1100 HPLC system (Stockport, UK). The compounds were eluted (flow rate 0.5 $ml.min^{-1}$) using a linear gradient from 10% methanol (Sigma-Aldrich) to 100% methanol over 15 min. This was held at 100% for 7 min, then decreased to 0% over 0.2 min and finally held at 0% for 4.8 min.

Mass spectrometry was performed on a Micromass Platform LCZ (Micromass, Manchester, UK), controlled by MassLynx software (version 3.2). The mass spectrometer was equipped with an ESI source operated in negative ion mode. The source temperature was 75°C; desolvation temperature, 450°C; gas flow rate, 646 $l.h^{-1}$ capillary, 3.5 kV; cone, 21 V. DON and C^{13} DON were detected at m/z 331 and 346 in selected ion mode, with a dwell time of 0.1 s.

Calibration was achieved using a series of standards which contained 1000 ppb of each standard and 20 ppb of each internal standard. The peak area for DON in samples was compared with that of standards after compensating for variation in the peak area of the internal standard. Mycotoxin extraction was unsuccessful for the aphid timing study, and insufficient flour remained to repeat the extraction process.

Assessment of aphid mortality and population growth

Aphids that were transferred to aphid-only controls or transmission treatment ears in the single ear experiment were monitored to measure mortality and population growth on healthy versus diseased ears. Aphids were fed and contained in netting on control and symptomatic ears for 48 h (n = 8) after which time netting was opened and the ear and netting inspected for living and dead aphids. The numbers of living and dead aphids were recorded prior to transfer of surviving insects to the new pathogen-free hosts. Mortality was calculated as the number of dead as a percentage of the starting population. Following on from transfer to the new host ears aphids were once again entrapped in netting, after 48 h of aphid feeding the numbers of living and dead aphids were recorded again. Aphid population growth following transfer to the new host was calculated as the total number of both dead and alive aphids found on the ears after a 48 h feeding period expressed as a percentage of the starting population.

Volatile collection

Four symptomatic ears per plant, 14 days after inoculation, were entrained in partially sealed glass vessels using headspace sampling methods described previously (22). Controls ears were mock-inoculated with sterile distilled water instead of spore suspension. Charcoal filtered air was pumped in through the base at 600 ml min^{-1} and drawn out through a Porapak™ filter in the top of the jar at 400 ml min^{-1} for 48 h. Volatile samples were eluted from filters with 0.5 ml dichloromethane (DCM). Four collections were carried simultaneously comprising two pathogen-free control plants and two plants point inoculated with *F. graminearum*.

Olfactometer bioassays

Behavioural assays were done using a Perspex® four-arm olfactometer (23) lit from above by diffuse, uniform lighting and maintained at 23°C. The bottom of the apparatus was lined with filter paper (Whatman No. 1) and air was drawn through the four arms towards the centre at 350 ml min^{-1}. Single winged *S. avenae* virginoparae (females reproducing by parthenogenesis; n = 10) were introduced into the central chamber and the time spent and number of entries into each arm were recorded using specialist software (OLFA, Udine, Italy) over a 16 minute period. The apparatus was rotated a quarter of a turn every 2 minutes to eliminate any directional bias. Headspace sample (10 µl) was applied to a filter paper strip and the solvent allowed to evaporate for 30 seconds. The filter paper was then placed at the end of the treated side arm. The three control arms were similarly treated with 10 µl of solvent alone on filter paper.

Chemical analysis and identification

Headspace samples were analysed by GC-MS using 4 μl per sample. A capillary GC column (50 m × 0.32 mm i.d. HP-1) fitted with a cold on-column injector was directly coupled to a mass spectrometer (VG Autospec, Fisons Instruments, Manchester, UK). Ionization was by electron impact at 70 eV, 250°C. The oven temperature was maintained at 30˚C for five minutes and then programmed at 5°C.min^{-1} to 250˚C. Tentative GC-MS identifications were confirmed by peak enhancement with authentic samples on both the polar and nonpolar GC columns (24).

Electroantennography

Electroantennogram (EAG) recordings were made from aphid antennae using Ag-AgCl glass microelectrodes filled with Ringer solution (7.55 g.l^{-1} sodium chloride, 0.64 g.l^{-1} potassium chloride, 0.22 g.l^{-1} calcium chloride, 1.73 g.l^{-1} magnesium chloride, 0.86 g.l^{-1} sodium bicarbonate, 0.61 g.l^{-1} sodium orthophosphate). The head of a *S. avenae* winged virginopara was separated from the body with a scalpel, and the tips of the antennae were removed to ensure good contact with the electrode. The head was placed into the tip of the indifferent electrode, and the tips of the antennae were positioned in the end of the recording electrode. The coupled GC-electrophysiology system, in which the effluent from the GC column is simultaneously directed to the antennal preparation and the GC detector, has been described previously (25). Separation of the volatiles was achieved on a 50 m × 0.32 mm i.d. HP-1 column fitted in an HP6890 GC equipped with a cold on-column injector and a flame ionization detector. The oven temperature was maintained at 40°C for two minutes and then programmed at 5°C min^{-1} to 100°C and then at 10°C.min^{-1} to 250°C. The carrier gas was hydrogen (flow rate 42 cm.s^{-1}). The outputs from the EAG amplifier and the FID were monitored simultaneously and analyzed with a software package (Syntech, the Netherlands).

Preparation of synthetic components

Synthetic versions of six electrophysiologically-active chemicals were acquired (suppliers listed in Table 2.2) and 1 mg.ml^{-1} solutions were prepared in hexane. Blends of synthetic chemicals were prepared using the same ratio and concentration as in natural samples of *Fusarium graminearum*-induced wheat volatiles (FgVOCs). A two-component blend, comprising 2-pentadecanone and 2-heptanone in hexane and a six-component blend, comprising all six chemicals listed in Table 2.2, also in hexane, were made.

Statistical analysis

Genstat version 15.1, (VSN International, UK) was used for all data analyses. Disease severity data were angularly transformed prior to analysis of disease progress over time by repeated measurement ANOVA. DNA and mycotoxin data were $\log_{10}$ transformed prior to ANOVA to normalise the residuals. Aphid mortality and population growth were analysed using one-way two sample t-tests.

Mean time spent in and number of entries into treated and control arms were compared using a paired t-test. Data from olfactometer bioassays of replicate FgVOC samples were subjected to the Bonferroni correction for multiple t-tests. Analysis of time spent in arms of the comparative olfactometer assays was by general ANOVA. Treatment means generated by individual and repeated measurement ANOVA were considered significantly different at $P \leq 0.05$.

Results

The role of aphids in FHB disease progression and accumulation of Fusarium graminearum DNA and DON in wheat ears

The first differences between treatments for disease severity were observed six days after inoculation (DAI) when ears treated with both aphids and *Fusarium graminearum* inoculum showed significantly more symptoms than the rest of the treatments ($P < 0.001$; Fig. 2.1). For the duration of the experiment, ears from this treatment developed the greatest disease severity, and by 28 DAI 83.1% mean disease severity was observed. Ears point inoculated with *F. graminearum* developed significantly greater disease in comparison to negative control ears at 18 DAI, spore-coated aphid treated ears and aphid transmission ears, with a final disease severity of 41.6% by 21 DAI. Aphid transmission ears and ears treated with spore-coated aphids developed a mean disease severity of 15.7% and 14.3% respectively, which was not significantly different from negative controls.

Ears treated with aphids and *F. graminearum* inoculum had two- and five-fold greater quantity of pathogen DNA and DON respectively than pathogen only controls (Fig. 2.2). A significantly greater quantity of pathogen DNA was found in pathogen plus aphid treated ears compared to pathogen only ears, and both treatments had significantly greater pathogen DNA quantities than negative controls. DON accumulated more in pathogen plus aphid treated ears than all other treatments. However, DON accumulation was not significantly different in pathogen only controls compared to negative controls. All other treatments, with the exception of spore-coated aphids for which DON was not quantified, contained pathogen DNA and DON quantities that were not significantly different to that of negative controls. The treatment means for quantity of pathogen DNA and DON across treatments ranged from 0.001 – 48 $pg.ng^{-1}$ and 180 – 36000 ppb respectively. Both pathogen DNA and DON were detected in negative controls and aphid-only controls at low levels, presumed to be due to cross–contamination aided by the circulation of air within the controlled environment chamber. Furthermore, regression analysis of log_{10} transformed DON concentration data on log_{10} transformed DNA concentration data showed that pathogen DNA and DON levels were significantly positively related ($P > 0.001$, $R^2 = 0.48$) showing an increase in DON in samples with increased pathogen DNA concentrations,

The effect of timing of aphid colonisation on FHB disease progression and F. graminearum DNA

Although there were no significant differences in visual disease development between treatments with different timings of aphid colonisation at 5%LSD, plants with 7 days duration of aphid colonisation produced the highest disease severity (T-7, 19.13%), followed by plants treated with aphids three days before inoculation (T-3, 14.43%) (Fig. 2.3). Plants treated either with aphids simultaneously (T0), four days after pathogen inoculum (T+4) or the aphid-free mean produced similar visual severities (13.51, 13.22% and 11.0%, respectively.

Timing of aphid colonisation in relation to FHB infection significantly influenced the quantity of pathogen DNA in flour samples (*P* = 0.003). Plants treated with inoculum in the absence of aphids reaped significantly lower levels of pathogen DNA than all treatments where aphids were present (Fig. 2.4). The amount of pathogen DNA increased with the duration of aphid colonisation, thus the greatest levels were found in plants treated with aphids seven days prior to the addition of pathogen inoculum which contained a seven-fold greater quantity than aphid-free controls. Significant differences in pathogen DNA quantity were also observed between aphid-free controls and plants treated with aphids three days prior to and simultaneously with inoculum, with mean quantities for the treatments ranging from 2.9 – 22.0 $pg.ng^{-1}$ of total DNA.

Aphid mortality and reproductive rate

After a 48h feeding period, aphids fed on ears heavily infected with *F. graminearum* had a mean mortality rate of 59.4%, which was significantly greater than the mortality rate of 36.5% for aphids fed on control ears (*P* = 0.011, Fig. 2.5A). Surviving aphids from *F. graminearum* infected ears transferred onto pathogen-free wheat ears incurred a depressed reproductive rate (*P* = 0.042), producing on average 1.43 offspring per aphid compared to 2.0 when the previous host ear was not infected (Fig. 2.5B).

Olfactometer bioassays with natural samples

Headspace samples from healthy wheat ears did not alter aphid behaviour, and there were no significant differences in the amount of time spent in olfactometer arms treated with healthy wheat volatiles versus pure solvent (data not shown). However, volatiles from FHB exposed plants (FgVOCs) elicited a repellent response from aphids, which consistently spent significantly less time in areas treated with headspace samples from *F. graminearum* infected ears than in areas treated with pure solvent (*P* = 0.03, Fig. 2.6).

Analysis of FgVOCs by GC-MS and GC-coupled electroantennography

Gas chromatograms of FgVOCs showed an increase in 2-pentadecanone compared to healthy ear samples and the presence of several other compounds not found in healthy ear samples. GC coupled electroantennography (GC-

EAG) of FgVOCs identified several compounds which were detectable by grain aphids, including 2-pentadecanone; six of which were used in further olfactometer assays and are listed in Table 2.2.

Olfactometer assays with synthetic chemicals

Of the six individual synthetic components of the FgVOCs that were used in olfactometer bioassays, 2-pentadecanone and 2-heptanone were strongly and weakly repellent respectively (P = 0.013, P = 0.091), (-)-α-gurjunene was weakly attractive (P = 0.058) and phenyl acetic acid, (-)-α-cedrene and 2-tridecanone had no statistically significant influence on aphid behaviour (Fig. 2.6).

A two-component blend of 2-pentadecanone and 2-heptanone was significantly repellent to aphids in olfactometer bioassays (P < 0.001, Fig. 2.6), as was a six-component blend of all the individually tested chemicals (P = 0.004). In choice test olfactometer bioassays, designed to compare aphid responses to the natural FgVOC sample and synthetic blends, there was a preference for the 2-component blend over the natural sample (P < 0.001, Fig. 2.7A). However, in bioassays using the six-component blend there was no significant difference in time spent in areas treated with either FgVOCs or the blend, but both of these treatments had significantly different means to the control arms (P = 0.004, Fig. 2.7B) indicating that both treatments were repellent.

Discussion

Wheat ears treated with a combination of aphids and *Fusarium graminearum* inoculum showed greatly accelerated disease progression, increased disease severity and mycotoxin accumulation compared to plants treated only with *F. graminearum* inoculum. However, aphid fitness was markedly reduced on diseased ears. Thus, in this pathosystem, co-localisation of organisms has significant effects on fitness and effects are unequal with the fitness of one organism rising substantially but that of the other declining sharply. It appears that the host colonisation strategy of the fungal pathogen, *Fusarium graminearum*, is detrimental to the aphids but that host colonisation by aphids may act to supress plant defences against the pathogen, with increased accumulation of pathogen DNA in the aphid-colonised hosts occurring in a dose-dependent manner. The sequence of arrival of attacking organisms on a plant may influence their performance due to differences in activation of induced defence pathways (15).

Sitobion avenae did not transmit the pathogen to new hosts, it only increased development and within-ear spread of the disease when already present, perhaps because aphid feeding damage makes it easier for the pathogen to colonise the plant. The interaction has large benefits for the pathogen overall as aphid feeding increased susceptibility of the host to the pathogen which reached advanced stages of disease earlier in the host life cycle. Our data showed a larger area under the disease progress curve when aphids were present indicating that the presence of aphids facilitated faster colonisation by *F. graminearum*. This could provide larger sources of inoculum early enough in the growing season to overlap with the flowering period of neighbouring plants or later emerging ears, and facilitate further spread of the pathogen. In this case the influence of insects on pathogenesis is synergistic and positive, meaning that aphids are highly relevant for disease epidemiology. This will impact disease management protocols but to our knowledge has not been previously reported except as anecdotal field observations.

These results also have implications for the management of mycotoxin accumulation as we observed a five-fold increase in DON accumulation in wheat ears when both aphids and *F. graminearum* occurred together compared to in hosts with only *F. graminearum*. There was a larger difference in DON concentration between *F. graminearum* only and *F. graminearum* plus aphids treatments than the difference in visual symptoms of disease between the two treatments. Presence of aphids resulted in infected plants accumulating increased levels of DON. Crucially, pathogen inoculation with added aphid stress resulted in an increased mycotoxin output per unit of pathogen DNA compared to pathogen inoculation alone. DON is considered to be important for virulence during plant pathogenesis (26). Conversely, host resistance has been correlated with reduced mycotoxin contamination (27) via mechanisms including resistance to initial infection and pathogen movement throughout host tissues and by the detoxification of mycotoxins within the ear (28, 29).

Aphids appear to suppress plant defences against *F. graminearum* because when aphid and pathogen stresses occur together, or aphids have already established themselves prior to pathogen infection, the host plant may be less able to inhibit pathogen spread or to detoxify DON that is produced in ears.

In corroboration with these findings, previous studies of FHB in maize found interaction with insects to result in increased mycotoxin accumulation. When transgenic Bt maize plants and non-transgenic controls were manually infested with European corn borer larvae (ECB), not only did the Bt maize have less ECB feeding compared to wild-type plants but they also had less *Fusarium* ear rot symptoms and up to a ten-fold lower concentration of fumonisin mycotoxins (30). Spray application of pyrethroid pesticides to reduce ECB infestation in Italian maize fields reduced fumonisin levels by 75% on average (31). It should be noted that in this case ECB larvae both damage the host and act as a vector to deliver *Fusarium* conidia to corn ears where infection is initiated (32). In contrast, in our study the aphids are not involved as a vector, and yet a rise in mycotoxin accumulation in the insect-affected was achieved through other mechanisms. In wheat field trials application of insecticide to reduce grain aphid populations at booting and/or heading was shown to reduce FHB incidence and severity by 20% and 31% respectively (33). In terms of direct evidence of aphid involvement increasing pathogen success on wheat, it has previously been shown that aphid feeding increases lesion frequency caused by *Microdochium nivale* on wheat leaves (34). Furthermore, wheat leaves fed on by the aphids *Rhopalosiphum padi* for a seven day period that was terminated seven days prior to infection of wheat ears with either *F. culmorum* or *F. graminearum* showed no significant difference in disease severity but significantly increased ergosterol and DON content in grain when compared to aphid-free infected plants (35). This work shows that aphid feeding does not necessarily need to be at the same site as pathogen inoculation for increased host susceptibility to the FHB disease.

It appears that the risk conferred to wheat is greater if existing aphid colonies are present on ears prior to infection than when both stressors occur simultaneously, and aphid feeding shortly before or after inoculum arrival increases the host susceptibility to the pathogen. This information is required to understand the impact of aphid involvement in FHB disease epidemiology and so as to inform management strategies of the importance of pest management at different epidemiological stages. The mechanism by which aphid feeding confers increased host susceptibility to this fungal pathogen as yet remains unknown. Honeydew deposits on plant organs following aphid feeding may promote successful colonisation of FHB-causing pathogens. It is also plausible that effectors within aphid saliva can act to down-regulate the host defences in such a way as to benefit the pathogen as well as the herbivore (36, 37). As it has been shown that aphid feeding on wheat leaves can lead to increased myocotoxin content in grain upon infection with *Fusarium* spp. (35) it appears that molecular mechanisms above and beyond the possible influence of honeydew may play a significant role in the mechanism by which hosts are rendered increasingly susceptible to FHB with coincidental aphid stress. The elucidation of this mechanism is necessary to fully understand the fundamental cause of the observed effects.

Olfactometer bioassays showed that aphids were significantly repelled by FgVOC headspace samples. In contrast, olfactometer bioassays using volatile samples from healthy infected ears showed no effect on aphid behaviour. Thus, pathogen-induced plant volatiles can affect the behaviour of herbivore insects so as to discourage infestation. This could have evolved as a means for aphids to avoid poor quality hosts. In this case, the aggressive pathogen *F. graminearum* turns aphid-hospitable wheat ears into strongly repellent sites through the production of deterrent semiochemicals. 2-pentadecanone was produced in *F. graminearum*-infected ear samples in greater amounts than healthy ear samples and was repellent to aphids by itself. However, the pathogen-induced volatiles were more potently repellent as a mixture than as individual compounds. The effect of 2-pentadecanone on aphid behaviour was not as strong as the effect observed with the full blend of electrophysiologically active FgVOCs. The six-component blend was of comparable repellence to natural samples, so it can be concluded that this is an adequate model for the main responsible compounds. 2-pentadecanone is a novel compound that is repellent to aphids and this effect should be explored for application within integrated pest management. The timing within the plant and pathogen life cycle at which healthy host volatile emissions begin to resemble FgVOC remains unknown. As the hosts were expressing visual symptoms at the time of VOC collection, we do not yet have evidence that disease-induced volatiles can be used for early detection of *F. graminearum* infection. While the in-field consequence of aphid repellence as a result of FHB symptom induction has not yet been explored, it is plausible that relocation of repelled aphids to hosts with fewer FHB symptoms could increase aphid stress on these plants and perpetuate the promotion of increased FHB severity and mycotoxin accumulation across whole field systems.

Fusarium pathogens have previously been shown to cause changes in volatile chemical profiles of their host, for example, young wheat plants (38), stored wheat grain (39) and maize plants (40). Inoculation of maize leaves with a composite inoculum comprising *Fusarium avenaceum, F. culmorum, F. graminearum*, *F. oxysporum* induced volatile emissions that were not present in controls. The concentration of these chemicals increased over time and particular components ((*Z*)-3-hexenal, (*Z*)-3-hexenyl acetate, β-caryophyllene and linalool) were attractive to cereal leaf beetles (40). Another study sampled volatiles prior to ear emergence from wheat plants inoculated with single species inoculum of *F. avenaceum*, *F. culmorum or F. graminearum* through the soil, damaged by cereal leaf beetle herbivory or damaged mechanically (38). Of the chemicals not found in controls, (*Z*)-3-hexenyl acetate was the most abundant in *F. graminearum* infected plants. The abundances of induced volatiles differed between *Fusarium* species used to inoculate the host. Moreover, volatiles induced by the fungi were distinct in both quantity and quality from those induced by either herbivory or mechanical damage.

The cause of the observed increase in aphid mortality and decreased reproductive rate on diseased plants is unknown. Changes to leaf free amino acid composition has been suggested as a mechanism behind both the increased fitness of aphids on birch leaves infected with *Marssonina betulae* (41) and the decreased fitness of aphids *Aphis fabae* when feeding on bean plants infected with the necrotroph *Botrytis cinerea* (14). As FHB symptoms develop on the host ears, green tissue becomes bleached due to fungal blockages in plant vasculature (42) from which aphids would otherwise feed, therefore removal of feeding sites could contribute to increased aphid mortality. However, the decreased rate of reproduction following aphid relocation to healthy hosts implies that aphid contact with toxins produced in the infected host may cause a lasting deleterious effect on the aphid. It is of interest for putative chemical pest control to identify the chemicals responsible for this effect although they may be the same mycotoxins which are deleterious to human health.

The repellent effect observed in olfactometer bioassays implies *Sitobion avenae* would be an incompatible vector for *F. graminearum*, and this is further demonstrated by the failure of aphids fed on symptomatic ears to produce disease in subsequent hosts. This work strongly suggests that *Fusarium graminearum* is not transmissible by grain aphids. Instead, as is summarised in Figure 2.8, colonisation of aphids prior to FHB symptom induction improves the spread and development of *F. graminearum.* However, as the disease progresses the host is transformed into a hostile and toxic environment for the aphid colonies. Aphid dispersal as a result of the creation of an inhospitable host environment can be viewed as a behavioural adaptation by the herbivore to escape a harmful environment. There may also be a further benefit to the pathogen if relocating aphids spread throughout a crop, enhancing susceptibility to the pathogen in less affected hosts. Overall, interactions between *F. graminearum* and grain aphids on wheat ears benefit the pathogen at the expense of the herbivore, and result in increased damage to the host plant by the disease.

Acknowledgements

This work was supported by a Lawes' Trust PhD studentship. Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council (BBSRC).

References

1. **Parry D. W., P. Jenkinson, and L. McLeod.** 1995. Fusarium ear blight (scab) in small-grain cereals - a review. Plant Pathol. **44:** 207-238.

2. **Glynn N. C., M. C. Hare, D. W. Parry, and S. G. Edwards.** 2005. Phylogenetic analysis of EF-1 alpha gene sequences from isolates of *Microdochium nivale* leads to elevation of varieties majus and nivale to species status. Mycol. Res. **109:** 872-880.

3. **Desjardins A. E.** 2006. Fusarium *mycotoxins: chemistry, genetics, and biology*. APS Press St. Paul, MN.

4. **Anon. 2006.** Commission Regulation (EC) No 1881/2006 setting maximum levels of certain contaminants in foodstuffs. Official Journal of the European Union L365, 5-24.

5. **Nganje W. E., D. A. Bangsund, F. L. Leistritz, W. W. Wilson, and N. M. Tiapo.** 2004. Regional economic impacts of Fusarium Head Blight in wheat and barley. Appl. Econ. Perspect. Pol. **26:** 332-347.

6. **Zadoks J. C., T. T. Chang, and C. F. Konzak.** 1974. Decimal code for growth stages of cereals. Weed Res. **14:** 415-421.

7. **Wratten S. D.** 1975. Nature of effects of aphids *Sitobion avenae* and *Metopolophium dirhodum* on growth of wheat. Ann. Appl. Biol. **79:** 27-34.

8. **De Zutter N., K. Audenaert, G. Haesaert, and G. Smagghe.** 2012. Preference of cereal aphids for different varieties of winter wheat. Arthropod Plant Interact. **6:** 345-350.

9. **Mayer C. J., A. Vilcinskas, and J. Gross.** 2008. Phytopathogen lures its insect vector by altering host plant odor. J. Chem. Ecol. **34:** 1045-1049.

10. **Stout M. J., Thaler J. S. and B. P. H. J. Thomma.** 2006. Plant-mediated interactions between pathogenic microorganisms and herbivorous arthropods. Annu. Rev. Entomol. **51:** 663-689.

11. **Goswami R. S. and H. C. Kistler.** 2004. Heading for disaster: *Fusarium graminearum* on cereal crops. Mol. Plant Pathol.**5:** 515-525.

12. **Al-Naemi F. and P. E. Hatcher.** 2013. Contrasting effects of necrotrophic and biotrophic plant pathogens on the aphid *Aphis fabae*. Entomol. Exp. Appl. **148:** 234-245.

13. **Zebitz C. P. W. and H. Kehlenheck.** 1991. Performance of *Aphis fabae* on chocolate spot disease-infected faba bean plants.Phytoparasitica. **19:** 113-119.

14. **T. Ohgushi.** 2005. Indirect interaction webs: herbivore-induced effects through trait change in plants. Ann. Rev. Ecol. Evol. Syst. **36:** 81-105.

15. **Bruce T. J. A., and J. A. Pickett.** 2007. Plant defence signalling induced by biotic attacks. Curr. Opin. Plant Biol. **10:** 387-392.

16. **Tuite J.** 1969. Plant pathological methods. Fungi and bacteria. Burgess Publishing Company, Minneapolis, MN.

17. **G. Bai.** 1996. Variation in *Fusarium graminearum* and cultivar resistance to wheat scab. Plant Dis. **80:** 975-979.

18. **Edwards S. G., S. R. Pirgozliev, M. C. Hare, and P. Jenkinson.** 2001. Quantification of trichothecene-producing *Fusarium* species in harvested grain by competitive PCR to determine efficacies of fungicides against fusarium head blight of winter wheat. Appl. Environ. Microbiol. **67:** 1575-1580.

19. **White T. J., T. Bruns, S. Lee, and J. Taylor.** 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, 315-322. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), Pcr Protocols: a Guide to Methods and Applications. Academic Press, San Diego, CA.

20. **Nicholson P., D. R. Simpson, G. Weston, H. N. Rezanoor, A. K. Lees, D. W. Parry, and D. Joyce.** 1998. Detection and quantification of

Fusarium culmorum and *Fusarium graminearum* in cereals using PCR assays. Physiol. Mol. Plant Pathol. **53:** 17-37.

21. **Brandfass C., and P. Karlovsky.** 2008. Upscaled CTAB-Based DNA Extraction and Real-Time PCR Assays for *Fusarium culmorum* and *F. graminearum* DNA in Plant Material with Reduced Sampling Error. Int. J. Mol. Sci. **9:** 2306-2321.

22. **Birkett M. A., T. J. A. Bruce, J. L. Martin, L. E.Smart, J. Oakley, and L. J. Wadhams.** 2004. Responses of female orange wheat blossom midge, *Sitodiplosis mosellana,* to wheat panicle volatiles. J Chem. Ecol. **30:** 1319-1328.

23. **Petersson J.** 1970. An aphid sex attractant. Part 1 Biological Studies. *Entomologica Scandinavia* **1:** 63-73.

24. **Pickett, J.** 1990. Gas chromatography-mass spectrometry in insect pheromone identification: three extreme case histories, 299-309. *In* A. R. McCaffery, and I. D. Wilson (ed.), Chromatography and isolation of insect hormones and pheromones. Plenum Press, New York, NY.

25. **Wadhams L. J.** 1990. The use of coupled gas chromatography electrophysiological techniques in the identification of insect pheromones, 289-298. *In* A. R. McCaffery, and I. D. Wilson (ed.), Chromatography and isolation of insect hormones and pheromones. Plenum Press, New York, NY.

26. **Maier F. J., T. Miedaner, B. Hadeler, A. Felk, S. Salomon, M. Lemmens, H. Kassner, and W. Schäfer.** 2006. Involvement of trichothecenes in fusarioses of wheat, barley and maize evaluated by gene disruption of trichodiene synthase (Tri5) gene in three field isolates of different chemotype and virulence. Mol. Plant Pathol. **7:** 449-461.

27. **Mesterhazy A.** 2002. Role of deoxynivalenol in aggressiveness of *Fusarium graminearum* and *F. culmorum* in Resistance to *Fusarium* Head Blight. Eur. J. Plant Pathol. **108:** 675-684.

28. **Bai G. H., A. E. Desjardins, and R. D. Plattner.** 2002. Deoxynivalenol-nonproducing *Fusarium graminearum* causes initial infection,

but does not cause disease spread in wheat spikelets. Mycopathologia **153:** 91-98.

29. **Boutigny A. L., F. Richard-Forget, and C. Barreau.** 2008. Natural mechanisms for cereal resistance to the accumulation of *Fusarium* trichothecenes. Eur. J. Plant Pathol. **121:** 411-423.

30. **Munkvold G. P., R. L. Hellmich, and L. G. Rice.** 1999. Comparison of Fumonisin concentration in kernals of transgenic Bt maize hybrids and nontransgenic hybrids. Plant Dis. **83:** 130-138.

31. **Saladini M. A., M. Blandino, A. Reyneri, and A. Alma.** 2008. Impact of insecticide treatments on *Ostrinia nubilalis* (Hubner) (Lepidoptera: Crambidae) and their influence on the mycotoxin contamination of maize kernels. Pest Manag. Sci. **64:** 1170-1178.

32. **Sobek E. A., and G. P. Munkvold.** European corn borer (Lepidoptera: Pyralidae) larvae as vectors of *Fusarium moniliforme*, causing kernel rot and symptomless infection of maize kernels. J. Econ. Entomol. **92:** 503-509.

33. **Bagga P. S.** 2008. Fusarium head blight (FHB) of wheat: Role of host resistance, wheat aphids, insecticide and strobilurin fungicide in disease control in Punjab, India. Cereal Res. Comm. **36:** 667-670.

34. **Diehl T., and H. Fehrmann.** 1989. Wheat fusarioses - influence of infection date, tissue-injury and aphids on leaf and ear attack. Z. Pflanzenk. Pflanzen. **96:** 393-407.

35. **Liu Y., and H. Buchenauer.** 2005. Interactions between *Barley Yellow Dwarf virus* and *Fusarium* spp. Affecting development of Fusarium head blight of wheat. Eur. J. Plant Pathol. **113:**283-295.

36. **Anathakrishnan R., D. K. Sinha, M. Murugan, K. Y. Zhu, M. S. Chen, Y. C. Zhu, and C. M. Smith.** 2014. Comparative gut transcriptome analysis reveals differences between virulent and avirulent Russian wheat aphids, *Diuraphis noxia*. Arthropod Plant Interact. **8:** 79-88.

37. **Bos J. I., D. Prince, M. Pitino, M. E. Maffei, J. Win, and S. A. Hogenhout.** 2010. A functional genomics approach identifies candidate effectors from the aphid species *Myzus persicae* (Green Peach Aphid). PLoS Genet. **6:** e1001216.

38. **Piesik D., G. Lemnczyk, A. Skoczek, R. Lamparski, J. Bocianowski, K. Kotwica, and K. J. Delaney.** 2011. Fusarium infection in maize: Volatile induction of infected and neighboring uninfected plants has the potential to attract a pest cereal leaf beetle, *Oulema melanopus*. J. Plant Physiol. **168:** 1534-1542.

39. **Presicce D. S., A. Forleo, A. M. Taurino, M. Zuppa, P. Siciliano, B. Laddomada, A. Logrieco, and A. Visconti.** 2006. Response evaluation of an E-nose towards contaminated wheat by *Fusarium poae* fungi. Sensor. Actuat. B-Chem. **118:** 433-438.

40. **Piesik D., A. Wenda-Piesik, R. Lamparski, P. Tabaka, T. Ligor, and B. Buszewski.** 2010. Effects of mechanical injury and insect feeding on volatiles emitted by wheat plants. Entomol. Fennica **21:** 117-128.

41. Johnson S. N., Douglas A. E., Woodward S., Hartley S. E. 2003. Microbioal impacts on plant-herbivore interactions: the indirect effects of a bitch pathogen on a birch aphid. Oecolgica **134:** 388-396.

42. **Guenther J. C., and F. Trail.** 2005. The development and differentiation of *Gibberella zeae* (anamorph : *Fusarium graminearum*) during colonization of wheat. Mycologia **97:** 229-237.

Figures

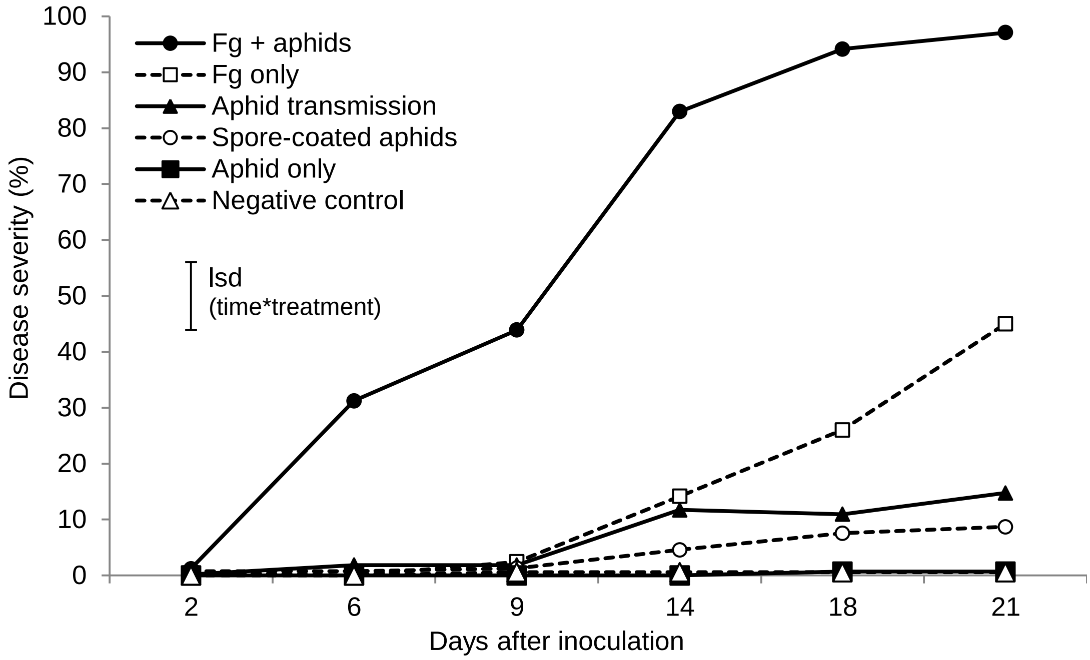

Fig. 2.1. Disease progress curve for aphid transmission experiment analysed by repeated measurements ANOVA, in which treatment, time and treatment*time were all significant (n = 8; $P < 0.001$, 5% lsd = 12.14, Greenhouse-Geisser $\varepsilon = 0.3932$). Treatments are labelled according to description in Table 2.1.

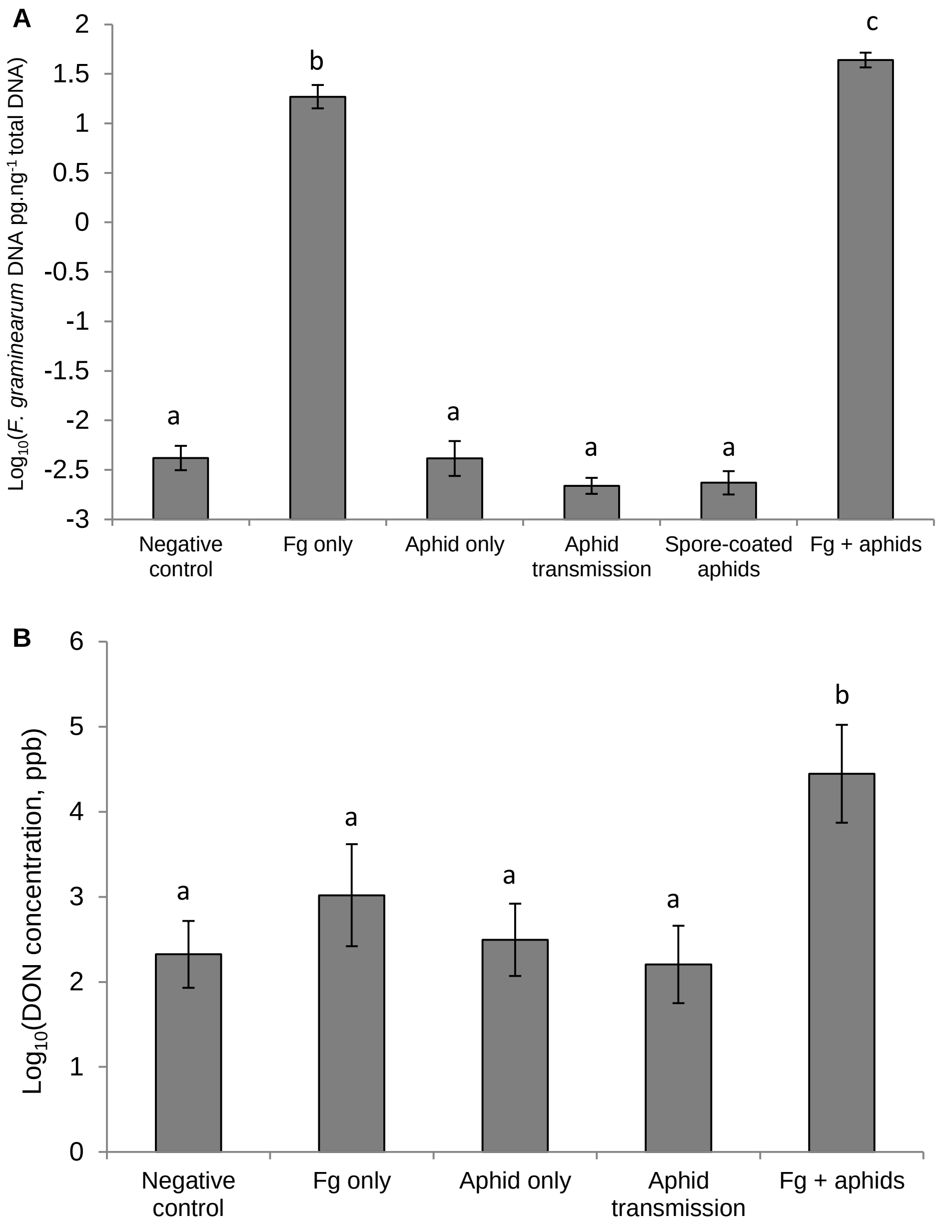

Fig. 2.2. Mean log transformed concentration (±SEM) of (a) *F. graminearum* DNA (pg per ng of total DNA) and (b) DON (ppb) in treated ears. (Bars marked with the same letter are not significantly different at 5% lsd. 5% lsd: (a) = 0.34, (b) = 0.91). Treatment was significant for both variables (n = 8; $P < 0.001$).

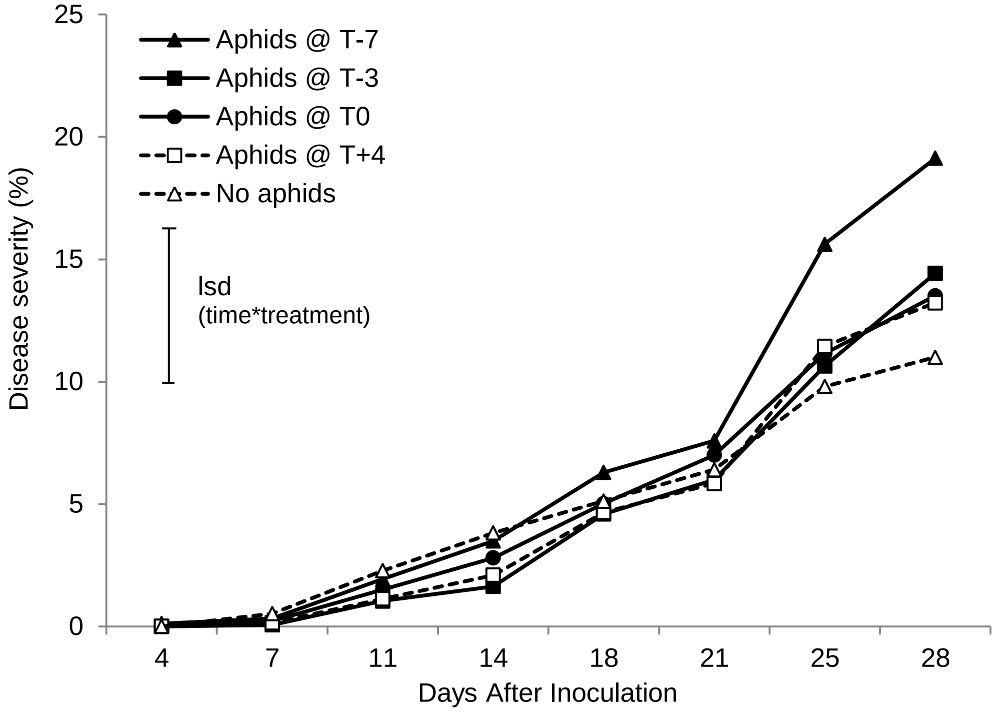

Fig 2.3. Disease progress curve for *F. graminearum*-inoculated ears with aphids applied at different timings, analysed by repeated measurements ANOVA (n = 8; $P = 0.53$; 5% lsd = 6.20, Greenhouse-Geisser $\mathcal{E} = 0.1845$).

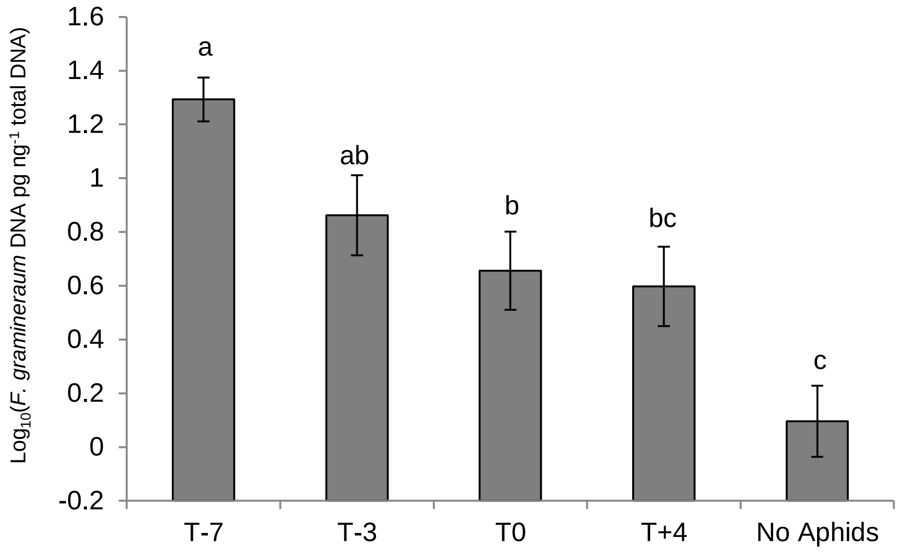

Fig. 2.4. Mean log transformed concentration of *F. graminearum* DNA (±SEM) in *F. graminearum*-inoculated plants treated with aphids applied at different timings. Treatment was significant (n = 8; *P* = 0.003; 5% lsd = 0.55) and bars marked with the same letters are not significantly different the 5% lsd.

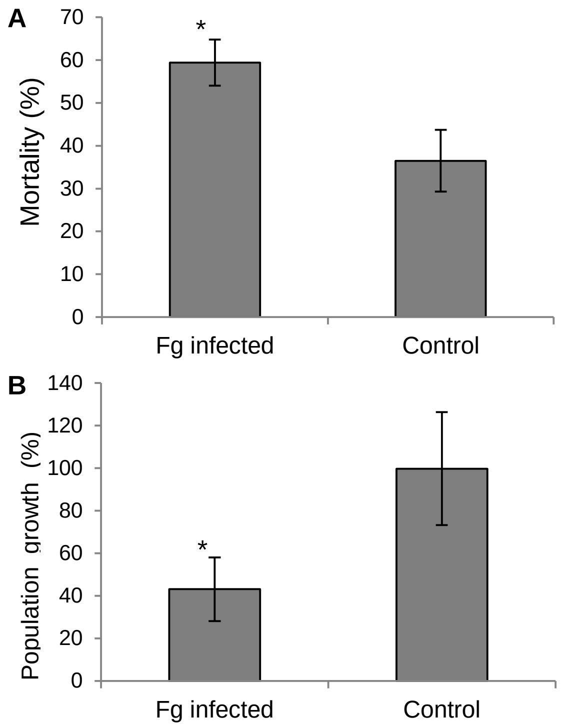

Fig. 2.5. (a) Aphid population mortality (± SEM) after 48h feeding on mock inoculated or *F. graminearum* inoculated ears (P = 0.011); (b) Aphid population growth (as a proportion of starting population) after surviving aphids from (a) were transferred onto pathogen-free wheat ears (P = 0.042).

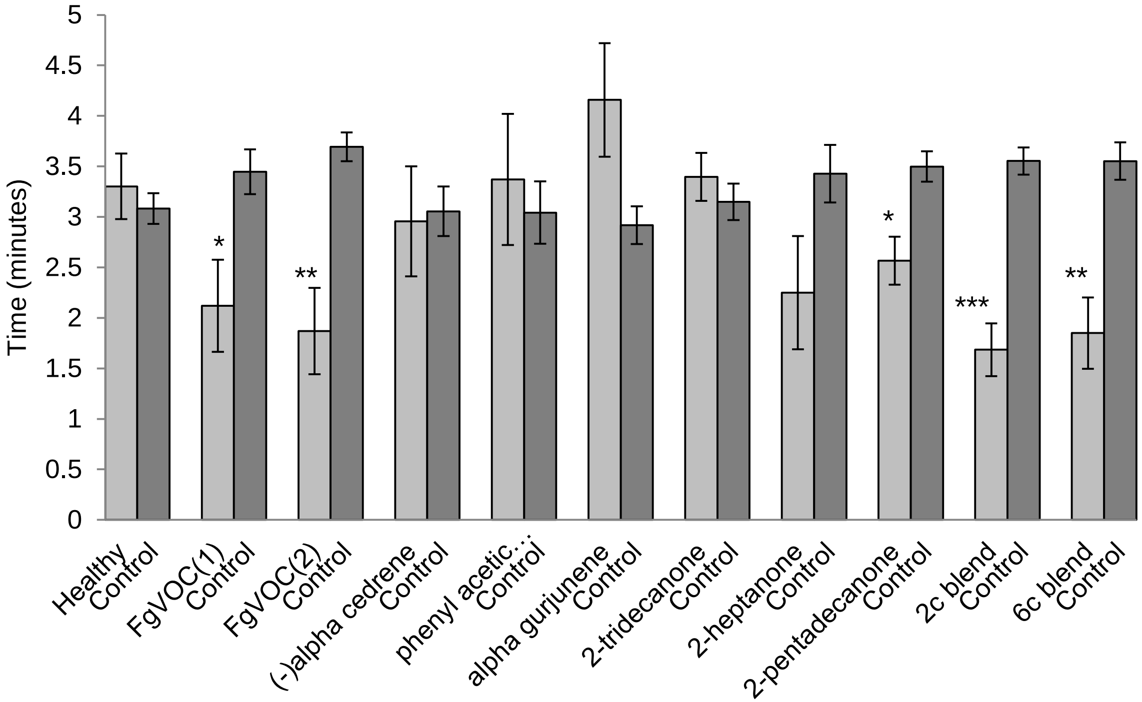

Fig. 2.6. Olfactometer bioassays showing mean time spent in olfactometer arms treated with healthy wheat VOCs, pathogen-induced plant VOCs, individual chemical components of the natural sample (FgVOCs), and blends of chemicals according to natural abundances compared to time spent in control arms (± SEM; *, ** and *** denote significant differences from controls at the level $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively).

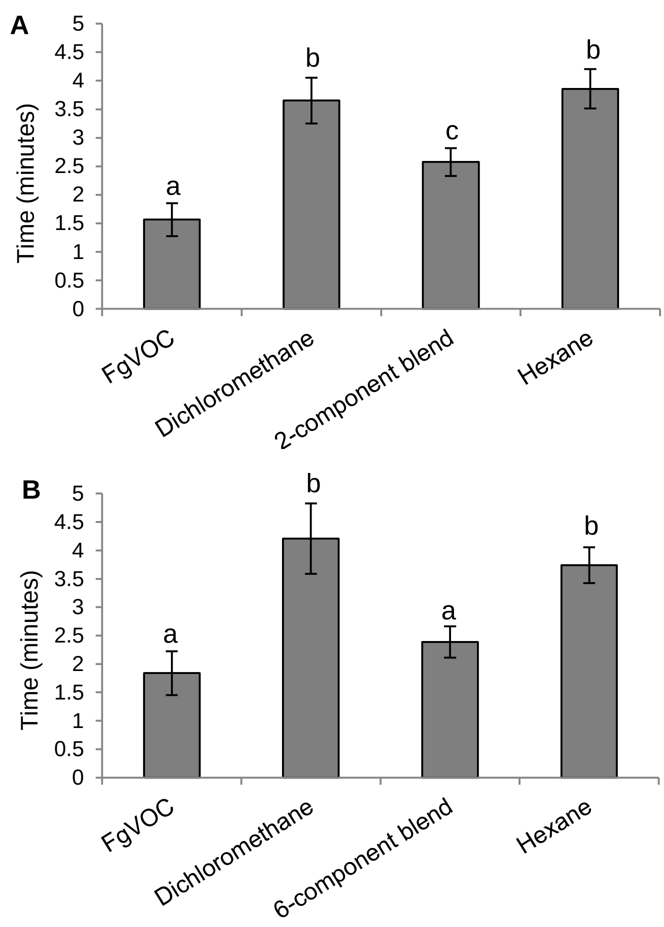

Fig. 2.7. Choice test olfactometer bioassay comparing natural sample (FgVOC) to (a) 2-component and (a) 6-component blends of synthetic chemicals. (Bars marked with same letter are not significantly different at 5% lsd. 5% lsd: (a) = 0.94, (b) = 1.35). Treatment was significant in both cases, (a) $P < 0.001$, (b) $P = 0.004$.

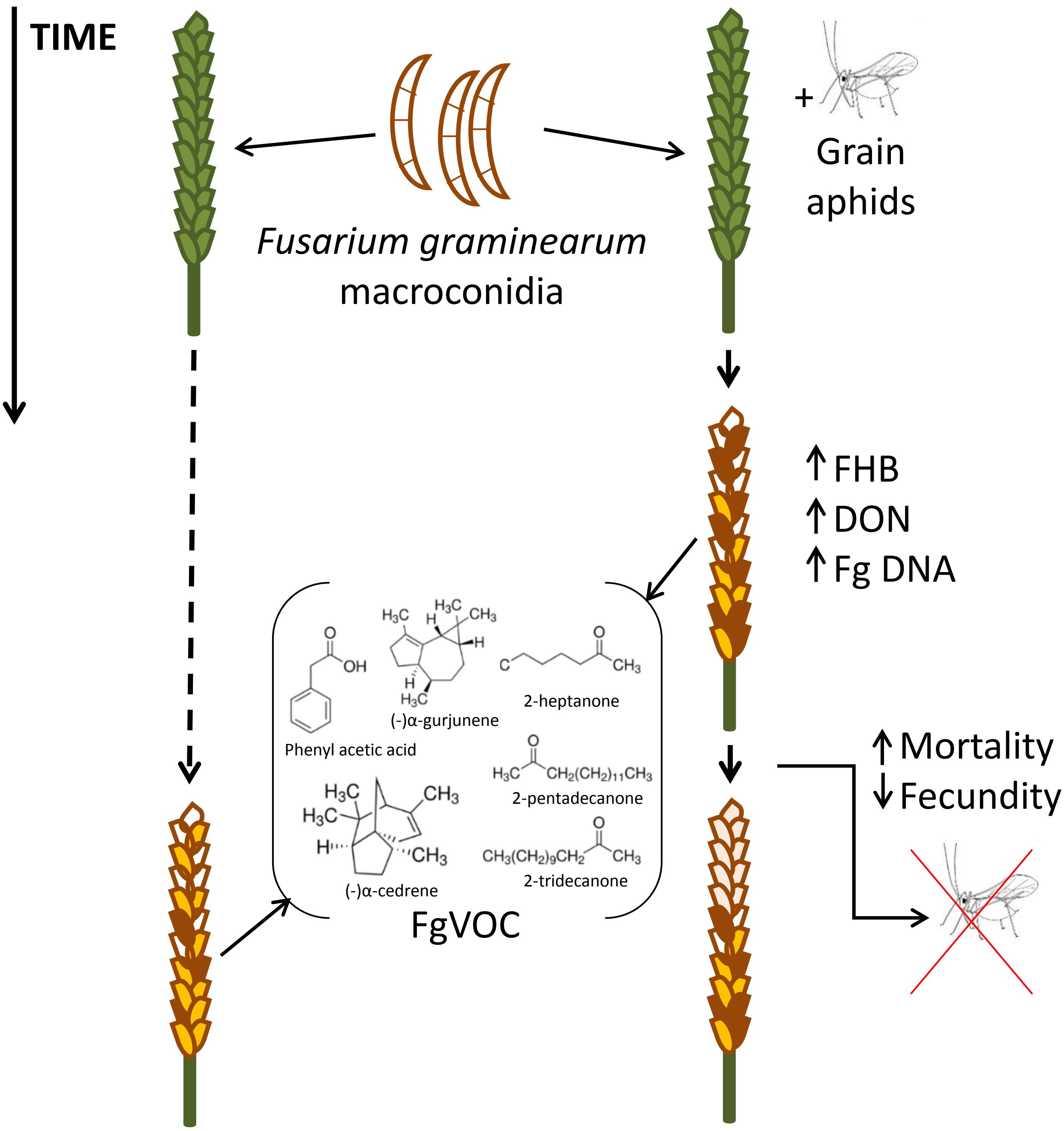

Fig. 2.8. Post-anthesis interactions that occur on wheat ears between *Fusarium graminearum* and *Sitobion avenae*.

Tables

Table 2.1. Treatment structure for single-ear experiment

Treatment name	Aphids added	Previous host of aphids	Inoculum added
Negative control	None	N/A	Point inoculation with 10µl sterile distilled water
Fg only (positive control)	None	N/A	Point inoculation with 10µl of 2.5 x 10^5 *F. graminearum* spores ml^{-1} pipetted between lemma and palea
Aphid only	10 adult wingless virginoparae	Healthy wheat ears	None
Aphid transmission	“	*F. graminearum* infected wheat ears	Only that occurring on aphids following feeding on infected previous host
Spore-coated aphids	“	Rearing cage wheat leaves	Aphids shaken in spore suspension as above that had been concentrated by heating at 40°C for 2h
Fg + aphids	“	Rearing cage wheat leaves	Point inoculation with 10µl of 2.5 x 10^5 *F. graminearum* spores ml^{-1} pipetted between lemma and palea

Table 2.2. Electrophysiologically active FgVOC components used in olfactometer bioassays

Chemical name	Abundance in FgVOCs (ng µl^{-1})	Supplier
2-heptanone	0.058	Aldrich
2-tridecanone	150.1	Aldrich
(-)-α-gurjunene	0.380	Aldrich
α-cedrene	0.65	Fluka
Phenyl acetic acid	0.027	Aldrich
2-pentadecanone	128.8	Alfa Aesar

Chapter 3: Aphid infestation increases *Fusarium langsethiae* and T-2 and HT-2 mycotoxins in wheat

The capacity of grain aphids to act as an insect vector for *Fusarium langsethiae* was studied with a series of transmission experiments, and is presented in this chapter. This work has been under review by *Applied and Environmental Microbiology*, and so has been presented in 'paper format'.

Author contribution

This paper was composed by J. Drakulic, and all practical work was conducted by J. Drakulic with the exception of the analysis of mycotoxin extracts by LCMS/MS, which was performed by O. Ajigboye. The work was supervised by R. V. Ray and T. J. Bruce.

RESEARCH ARTICLE

Aphid infestation increases *Fusarium langsethiae* and T-2 and HT-2 mycotoxins in wheat

Suggested running title: Aphids increase T-2 and HT-2 toxins in wheat

Jassy Drakulic[1], Olubukola Ajigboye[1], Toby Bruce[2], #Rumiana Ray[1]

1: University of Nottingham, Sutton Bonington Campus, College Road, Sutton Bonington, LE12 5RD, UK.

2: Rothamsted Research, West Common, Harpenden, Herts AL5 2JQ, UK.

Author for correspondence:
Rumiana Ray
Tel: +44 115 951 6049
E-mail: Rumiana.ray@nottingham.ac.uk

Abstract

Fusarium langsethiae is a fungal pathogen of cereal crops that is an increasing problem in Northern Europe, but much of its epidemiology is poorly understood. The species produces the mycotoxins T-2 and HT-2 which are highly toxic. It was hypothesised that grain aphids, *Sitobion avenae*, might transmit *F. langsethiae* inoculum between wheat plants, and a series of transmission experiments were performed to test this. Manual translocation of aphids from inoculated to uninfected hosts induced pathogen DNA accumulation in hosts. However, free movement of wingless aphids from infected to healthy plants did not. Addition of winged aphids reared on *F. langsethiae* inoculated wheat seedlings to wheat plants also did not achieve successful pathogen transfer. While our data suggested that aphid transmission of the pathogen was not very efficient, we observed an increase in disease when aphids were present. After seedling inoculation, an increase in pathogen DNA accumulation in seedling leaves was observed upon treatment with aphids. Furthermore, the presence of aphids on wheat plants with *F. langsethiae*-inoculated ears not only lead to a rise in the amount of *F. langsethiae* DNA in infected grain but also to an increase in concentration of T-2 and HT-2 toxins. Aphid infested diseased plants had more than 3-fold higher T-2 + HT-2 mycotoxin levels than diseased plants without aphids. This work highlights that aphids increase the susceptibility of wheat host plants to *F. langsethiae* and aphid infestation is a risk factor for accumulating increased levels of T-2 in wheat products.

Keywords: FHB, epidemiology, pathogen-insect interactions, mycotoxins

Introduction

Fusarium head blight (FHB) is a fungal disease of cereal crops caused by a pathogen complex comprising several species from the genus *Fusarium* (1) and two from the genus *Microdochium* (2). *Fusarium* species produce mycotoxins upon infecting host plants, which can be harmful if consumed by mammals with different health impacts depending on the specific toxins produced (3). As well as causing mycotoxin contamination, infection can also lead to reduced yield and grain quality. The disease affects crops in all major cereal growing regions worldwide, and different species prevail in different geographic areas (4). Globally, the most dominant and aggressive pathogens are *F. graminearum* and *F. culmorum* producing the mycotoxins deoxynivalenol (DON) and nivalenol (NIV) (5), however weaker pathogens such as *F. langsethiae* (6) or *F. poae* are able to coexist on infected hosts alongside the more aggressive species, and in doing so contribute to the mycotoxin contamination of the grain (7). *F. langsethiae* is a newly identified species, first described in 2004 (8) that has been observed in Norway (9), Sweden (10), Denmark (11), UK (12), Poland (13), Belgium (14), Italy (15), Germany (16), Russia (17) and Finland (18). There is also evidence that in northern Europe *F. langsethiae* is increasing in importance (19) with confirmed hosts including oats, wheat and barley (20).

Previously *F. langsethiae* was considered one and the same as *F. poae* due to its similar spore morphology, although phylogenetically, *F. langsethiae* is more closely related to *F. sporotrichioides* than *F. poae* (21). The mycotoxin profile reflects this, as the highly toxic T-2 and HT-2 type A trichothecenes are produced by both *F. langsethiae* and *F. sporotrichioides* (12; 22). Levels of T-2 and HT-2 are not yet under EU regulation, but it is likely that they will be soon. The discussed limits of these toxins reflect the lower level of toxins detected in wheat compared to oats and the large reduction in toxin content that occurs following the processing of oats (6; 12; 23; 24): 1000 µg. kg^{-1} for oats compared to 100 µg. kg^{-1} in wheat.

The epidemiology of *F. langsethiae* has been challenging to unravel since the species causes few outwards symptoms of FHB in cereals, having preferential pathogenicity towards oats then barley and wheat (24). However, the mycotoxins it produces in cereals mean that it is a major threat to grain production (25; 26). The ability of *F. langsethiae* to successfully cause diseases of the Fusarium complex was investigated under controlled environment by Imathiu *et al*. (27) who inoculated oat (cv. Gerald) and wheat (cv. Claire) seeds with fungal conidia and observed that *F. langsethiae* failed to cause visible seedling blight or subsequent head blight symptoms. However, only diseased tissues were used to re-isolate the pathogen and the success of *F. langsethiae* colonisation of non-symptomatic seedling tissues was not assessed. While this study provides evidence against *F. langsethiae* being pathogenic to seedlings, it does not exclude that seeds could be congenitally colonised with the species, that cryptic infection may be possible or that other biological processes, such as herbivory or wounding, may be needed to facilitate infection. Indeed, detached leaf assays demonstrated that *F. langsethiae* required a wound site to cause lesions on wheat leaves (24).

Under field conditions, weather patterns play a role in which *Fusarium* species contribute most to FHB disease and mycotoxin accumulation at different times. Edwards (28) reported that following a wet harvest in 2004, UK wheat was highly contaminated with NIV and DON, but T-2 and HT-2 were not present in high amounts. Conversely, T-2 and HT-2 were at their highest levels in 2005 which had been a dry summer. The observed pattern of mycotoxin accumulation may represent the ability of different *Fusarium* spp. to dominate the FHB pathogen complex when conditions reflect the differing optimal conditions of the species, for example the DON-producing *F. graminearum* requires a high water availability to grow and produce mycotoxins (29). Increased T-2 and HT-2 during drier weather may indicate that species capable of surviving on marginal water availability, such as *F. langsethiae*, may out-compete the more frequently dominant species during drought-stress. The optimum temperature for *F. langsethiae* growth and T-2 and HT-2 production is also lower than for *F. graminearum* growth and DON production (29; 22), and accordingly this species is of greatest concern in cooler, drier geographical regions (19).

Drier weather also favours increased levels of insect pests, such as aphids. English grain aphids, *Sitboion avenae*, have been described as ear-feeders (30) which are more strongly influenced by the volatile organic chemical (VOC) emissions of wheat ears over wheat seedlings (31) and have been recorded as being present during ear emergence in Belgian wheat fields (32). We hypothesised that grain aphids, because they occur on cereal crop ears alongside FHB pathogens, could influence disease epidemiology and have recently shown that FHB caused by *F. graminearum* was influenced by aphid activity when the insect and pathogen shared the same host plant (33). Our previous study showed that grain aphids were unable to transmit *F. graminearum* between wheat ears, but that coincidence of aphids and the pathogen lead to increased disease severity, fungal biomass and DON accumulation in hosts. While oat is a more suitable host plant for *F. langsethiae*, in order to compare the role of aphids in the epidemiology of these two diverse FHB pathogens, wheat was also used in the present study. *F. graminearum* is a species that produces large macroconidia which are several-fold larger than the microconidia produced by *F. langsethiae* (34). The smaller spore size and the prevalence of *F. langsethiae* in dry seasons are factors that could make *F. langsethiae* more compatible for insect transmission by aphids than *F. graminearum*.

The interactions of insect herbivores with host plants can be influenced by a number of sensory factors, with olfactory detection of host volatile odours being just one way that herbivores can recognise suitable host plants (35). The way in which aphids may respond to volatile chemical emissions from wheat hosts infected with *F. graminearum* has previously been shown to be aversion (33), and as such aphids would be expected to avoid hosts infected with this species. While this might provide a stimulus to induce emigration from infected hosts, this is unlikely to directly affect spread of the disease as aphids were shown to be incapable of transmitting the pathogen. By studying the response of aphids to volatiles from *F. langsethiae*-infected wheat plants, this would provide more information to the compatibility of the insect to fulfil the niche of vector to the pathogen. The present study aimed (1) to assess if aphids can transmit *F. langsethiae* from diseased to healthy wheat plants and seedlings; (2) to compare the speed of disease progression and mycotoxin levels in infected plants and seedlings with and without cohabiting aphids; and (3) to elucidate the nature of diseased host-insect volatile chemical interactions.

Materials & Methods

Wheat plants at ear emergence

Wheat seeds (cv. Gallant), treated with 10 g of prothioconazole and 50 g clothianidin 100 kg^{-1} seed (Redigo® Deter®, Bayer CropScience) were sown in compost (Levington's F2+S) and vernalised for 6 weeks at 4°C, then potted into individual pots (5 l) and grown in a glasshouse at maximum 18°C, minimum 15°C daytime temperature, maximum 12°C/ minimum 10°C night time temperature with a 16h photoperiod until ear emergence (GS 59) (36).

Wheat seedlings

Wheat seeds (cv. Gallant) were surface sterilised in sodium hypochlorite for 2 minutes followed by three 2 minute washes in distilled water and dried by blotting. Individual seeds were sown into compost (Levington's F2+S) into seedling trays comprising 3 x 4 modules of size 3cm x 3cm x 10cm (l x w x d). Seedlings were grown in a controlled environment chamber at 18°C daytime temperature, 12°C night time temperature under a 12h photoperiod for 14 days, until at the three-leaf growth stage (GS13) (36).

Preparation of fungal inoculum

Single-spore isolates of *Fusarium langsethiae* from the University of Nottingham collection (#154, 221 & 229) that had originally been isolated from wheat or oat samples were grown on PDA plates for 10 days and plugs of colonise agar (10 per flask) were used to inoculate carboxymethyl cellulose liquid media flasks (37). Sterile L-shaped spreaders were added to flasks to increase turbulence and flasks were sealed with cotton wool and foil, and shaken at 90 rpm at 23°C for 4 days. After this time, flask contents were filtered through two layers of sterile muslin and spores concentrations calculated under light microscope with a Neubauer haemocytometer (Marienfeld-Superior, Germany). Spore suspensions were adjusted to 1 x 10^6

spores ml^{-1} for ear inoculations and 1 x 10^7 spores ml^{-1} for seedling inoculations, with composite inoculum suspension made by combining spores of the three isolates together in equal measure.

Aphid rearing

Grain aphids, *Sitobion avenae*, were obtained from colonies maintained at Rothamsted Research (Harpenden, UK) and reared on wheat seedlings (cv. Gallant) in cages within a glasshouse, under the same temperature and light settings as for plant growth (18°C daytime temperature, 12°C night time; 12h:12h L:D photoperiod), at the University of Nottingham (Sutton Bonington, UK).

Transmission of Fusarium langsethiae via manual movement of apterous (wingless) aphids from infected to healthy host ears (Experiment 1)

Wheat ears were infected with a suspension of *Fusarium langsethiae* spores by spray inoculation until runoff. Ears were bagged in polythene bags for 7 days, and then grown for a further seven days while disease became established in the host. Approx. 20 apterous (wingless) adult aphids were placed on infected ears (n = 6) and trapped on the ear in netting sealed at the base of the ear. After a 72h feeding period, aphids were removed from the diseased plants, by carefully handling them by the rear limbs using sterile needle-nosed forceps. Ten individuals were transferred to each new healthy host ear (n = 6), and again trapped there inside netting sealed below the ear. After 72h on the host ears the aphids were removed. A disease-free aphid control treatment was prepared in the same way, but the initial aphid host ears were mock inoculated with water. Two further treatments were prepared: a pathogen-only treatment was prepared by point inoculation of ears at mid-anthesis (GS65) with spore suspension with 10µl added to each of ten spikelets per ear, and a negative control which was prepared as in the pathogen-only treatment but using distilled water instead of the spore suspension.

Following the application of treatments, ears were assessed visually for disease symptoms (dark lesions at the base of spikelets or bleached spikelets). The number of symptomatic spikelets was taken as a proportion of the total number of spikelets per ear to calculate disease severity (%). Assessments were conducted at nine time points, roughly twice weekly beginning at 4 days after inoculation (DAI) until 29 DAI. Ears were then left to develop until maturity when they were harvested, freeze-dried whole and milled without threshing to obtain flour samples. From the flour, DNA extractions were performed and samples analysed by two technical repeats of QPCR as described below.

Volatile collection and olfactometer bioassays

Under the same controlled environment chamber conditions as described above, four ears per wheat plant were spray inoculated with *F. langsethiae* spore suspension or mock inoculated with sterile distilled water. Following inoculation ears were bagged in polythene for 72h, then bags were removed and plants incubated for a further 14d to allow the infection to develop, until lesions were visible on the infected ears. The volatile organic chemical (VOC) emissions were then collected using headspace sampling methods described previously (33; 38) and VOC samples were used in 4-arm olfactometer bioassays using individual alate (winged) *Sitobion avenae* (n = 10) using one treated arm and three solvent arms. The assays were conducted over a period of 16 mins, rotating the chamber every 2 mins to eliminate lighting bias, at 23°C under uniform lighting, the chamber was lined with filter paper (Whatman No. 1) and air was drawn out through the centre at a rate of 350 ml min^{-1}. After bioassays which used either diseased or healthy host volatiles, comparative bioassays were performed that directly compared the preference of aphids to healthy versus diseased volatiles. The olfactometer bioassays were performed in the same manner as above (n = 10), except two oppositely positioned arms were loaded with each healthy and diseased host volatiles, and the remaining two were treated only with dichloromethane solvent.

Inoculation of wheat seedlings with* Fusarium langsethiae *and transmission between seedlings by apterous* Sitobion avenae *(Experiment 2)

Wheat seedlings were placed into individual plastic boxes of size 25cm x 25cm x 15cm (l x w x h) with netting panels in the lid and absorbent paper on the bottom. Seedlings (n = 10) were infested with aphids by placing a colonised wheat leaf from the rearing cage in proximity to seedling leaves to allowing aphids to move onto the new hosts. A further set of seedlings (n = 5) were kept aphid free during this period as controls. After a period of 48h, the detached leaf used to import aphids to the seedlings was removed. Of the seedlings infested with aphids, half (n = 5) were then cleared of aphids by hand. All seedlings were then removed from their containers and spray inoculated with *Fusarium langsethiae* spore suspension until runoff, using a plastic sheet to prevent runoff reaching the compost, and replaced in the containers for seven days. A second 14-day old seedling was added to the opposite side of each container to avoid direct contact between seedling leaves. The experiment was terminated 21 days after the addition of the second seedling, the target of the transmission experiment (target seedlings), by manual removal of any aphids and excision at the base of all the leaves of the seedlings. Green leaf material was stored at -18°C until DNA extraction.

Transmission of* Fusarium langsethiae *via natural movement of apterous aphids from infected to healthy host plants (Experiment 3)

Wheat plants at ear emergence (GS59) were inoculated on all ears by spray inoculation until runoff with *Fusarium langsethiae* spore suspension and bagged in polythene bags for 7d. A single infected plant was placed into each of 20 compartments of large timber framed and net covered cages of size 105 cm x 30cm x 120cm (l x w x h). Each cage was accessible on the narrow side by a Velcro-sealed door and fed with automatic watering drippers. Infected plants were placed furthest from the cage door, and into half of the cages (n=10) aphids were added to the plant ears, according to a randomised design, by placing a colonised wheat leaf from the rearing colony on to the infected ears, held in place between the top spikelets. Aphids were allowed to move to the new host over a period of 48h before the removal of the detached leaves. Seven days after the addition of aphids a second wheat plant, uninoculated and at ear emergence (GS59), was then added to all cages and positioned near the cage door at a distance of approx. 1m from the infected plant in each cage. Visual disease assessments were conducted from four days after the addition of the target plant to each cage (4 DAI) at twice weekly intervals until 35 DAI. Disease symptoms (lesions and bleaching) were recorded for each ear of each plant that had emerged when plants were added to the cages. Disease severity was calculated for individual ears and averaged over the whole plant. Plants were allowed to mature before harvesting, with each ear being harvested individually, freeze-dried and milled without threshing to gain flour samples that were used in DNA and mycotoxin extractions.

Primary transmission of* Fusarium langsethiae *via alate* Sitobion avenae *(Experiment 4)

To prepare the alate aphids, wheat seedlings were grown to create the feedstock for two aphid rearing cages. Seedlings were grown under standard conditions as described and one pot spray inoculated with sterile distilled water for disease-free control aphids and the other pot spray-inoculated with *Fusarium langsethiae* spore suspension until runoff. Seedlings were added to rearing cages along with aphids 24 h after inoculation. Following the addition of the pots of seedlings to their separate rearing cages and the colonisation of the seedlings with aphids for a period of 7d, colonised plants were re-sprayed with spore suspension or distilled water. Colonised plants were maintained for another 5 weeks to induce crowding and encourage the production of alate morphs. Leaf material from the rearing cages was sampled 4 weeks after the addition of aphids to the cages for use in DNA extraction as described for the seedling transmission experiment to confirm the infection status of each cage.

Single wheat plants at ear emergence (GS59, n = 20) were added to each of the 20 individual compartments of a large timber-framed cage (as for Experiment 3). 10 alate aphids were harvested from the rearing cages with a vacuum pooter (Watkins & Doncaster, UK) and released into each of the cage compartments. Aphids from the infected rearing colony were used in half of the cages (n = 10) and the other half treated with aphids from the control colony according to a randomised design. No visual assessment of plants was conducted to prevent the need to open the cages and the escape of winged aphids. When all tillers were mature the oldest 4 ears per plant were harvested, freeze dried and milled into flour for use in DNA and mycotoxin extractions.

DNA extraction and quantification of* Fusarium langsethiae *DNA by QPCR

Freeze-dried samples of whole wheat were milled without threshing in a centrifugal mill (ZM 200 Retsch GmbH, Germany). Flour (0.5g) was shaken with CTAB buffer (3.75 ml) in 15ml tubes and incubated at 65°C for 2 h. Tubes were then chilled on ice and potassium acetate was added (1.25 ml). The extraction then proceeded as previously described (39). For green leaf material, the oldest three seedling leaves were chopped finely with scissors, and 0.5g leaf material added to CTAB (3.75 ml) with a sterile ball bearing and shaken mechanically for two 20 second intervals using a FastPrep-24™ homogeniser (MPBio, USA). Extraction proceeded as described for flour samples from this point. The concentration of DNA was determined by spectrophotometry measuring the absorbance at wavelengths 260, 280, 328 and 360 nm using a CARY® 50Probe UV-visible spectrophotometer (Varian, CA, USA). Simple Reads software was used to calculate the DNA concentration from absorbance values and then sample concentrations were adjusted to 20 ng. μl^{-1} and stored at 4°C until use in PCR assays.

Sample DNA was analysed with diagnostic PCR using ITS 4 & 5 primers to confirm the presence of fungal DNA, as described previously (40). Samples were then analysed further to quantify the amount of *Fusarium langsehtiae* DNA present by QPCR using species-specific primers FlangF3 (5'-CAAAGTTCAGGGCGAAAACT-3') and LanspoR1 (5'-TACAAGAAGACGTGGCGATAT-3') and a cycling program previously described (41) which amplified a 310 bp product. Ten-fold dilutions of pure *F. langsethiae* DNA was included in the assay to generate a standard curve from $1 – 10^{-4}$ ng μl^{-1}.

Mycotoxin extraction from flour samples and analysis by LCMS/MS

Flour samples (12.5 g) were spiked with ^{13}C-labelled DON toxin internal standard (Biopure, Romer Labs, Runcorn, UK) to produce a final concentration of 20 µg. kg^{-1}, and blended with 50ml methanol (100%) for three minutes. Samples were filtered through filter paper (Whatman No. 1) and 2 ml of the filtrate made up to 50 ml with PBS buffer, which was then filtered through fibreglass. The filtrate (20 ml) was passed over a DZT cleanup column by gravity over a 30 minute period, and then the column was washed with sterile water prior to elution of samples by passing through 1 ml methanol (100%). Samples were dried under a stream of nitrogen gas then redissoved in 500 µl methanol (10%) in preparation for LCMS/MS.

The LCMS/MS analysis was performed on an Agilent 1100 series LC system (Agilent Technologies, Germany) coupled to a triple quadrupole Micromass Quattro Ultima V4.0 SP4 (Waters) and equipped with Luna® C18 Å100 LC (5 µm, 250 mm x 3 mm) column (Phenomenex, Torrance, CA, USA). The flow rate was set at 0.5 ml min^{-1} and the injection volume was 30 µl. Mobile phase A was 90% water with 10% methanol, mobile phase B was 100% methanol. A linear binary gradient was applied from 0 to 100% phase B within 15 min. The content of phase B was held for 7 min and then lowered to 0% within 20 s followed by equilibration of the column for 5 min.

Quantitative determination of all compounds was performed by operating the mass spectrometer in ESI positive and negative ionisation mode. Optimized instrument settings include capillary voltage 3.5 kV, source temperature 100^{o}C, desolvation temperature 450°C, desolvation gas flow rate of 672 L hr^{-1} cone voltage, a cone gas flow rate of 76 L. hr^{-1}, and multiplier 650 V. Argon was used as the collision gas at a pressure sufficient to increase the collision cell Pirani gauge to a reading of 8.74 e^{-4} mbar. Parent/daughter ions for T-2, HT-2 and ^{13}C-labelled DON were detected at 489/245, 447/354 and 279/230 m/z respectively with a dwell time of 0.1 s. Masslynx 4.0 software was used for data acquisition and processing. Quantification of the samples was carried out using a matrix of standards prepared in-house. The limit of detection for both T-2 and HT-2 was 10 µg kg^{-1}.

Toxicity of T-2 to* Sitobion avenae *through artificial feeding

To determine the toxicity of T-2 on *Sitobion avenae*, an artificial feeding assay was performed. Sucrose solution (0.5 g ml^{-1}) was spiked with T-2 toxin in varying concentrations ranging from 1 µg ml^{-1}, down in ten-fold dilutions to 1 ng.ml^{-1}. Toxin free sucrose solution was used as a control. Sachets of feeding solution were prepared in Parafilm suspended over Perspex rings (approx. 2 cm tall, and 2 cm inner radius). Ten adult aphids were added to the underside of each sachet, sealed at the base of the tube, and allowed to feed for a total of 7 days. Feeding apparatus was covered with a green acetate sheet and placed under direct lighting for a 12h photoperiod in a controlled environment chamber of daytime temperature 18°C and night time temperature of 12°C. The number of living and dead aphids was recorded after 24h, 48h and 7 days, and any newly born nymphs were removed. To calculate the mortality, the number of dead aphids as a proportion of the starting population was subjected to Abbot's correction for mortality, where the control mortality (M_c) is deducted from the treatment mortality (M_t) and from 100 to correct the numerator and the denominator of the percentage calculation. This can be summarised in the following formula:

Corrected mortality = $[(M_t – M_c)/(100 – M_c)]*100$.

The concentration at which 50% corrected mortality was observed, the EC_{50}, was calculated from the gradient of the trendline generated by plotting the means for corrected mortality against the $\log_{10}$ transformed concentrations of T-2.

Statistics

Disease progress was analysed with repeated measurements ANOVA, and calculated values for the area under the disease progress curve (AUDPC) were analysed using general ANOVA for experiments with multiple treatments, or t-test for experiments with two treatments, (and in experiment 1 where disease symptoms and pathogen DNA were only detectable in two treatments), along with disease severity at individual time points, pathogen DNA and [T-2 + HT-2] concentration data. To normalise the residuals, DNA and toxin concentrations were $\log_{10}$ transformed and disease severity data was angularly transformed. Aphid mortality data was analysed with general ANOVA separately for each time point, and due to the Abbot's correction analysis was only performed on data from T-2-containing diets. All statistical analyses were conducted using Genstat v. 17.1 (Harpenden, UK).

Results

Experiment 1: Transmission of Fusarium langsethiae *via manual movement of apterous (wingless) aphids from infected to healthy host ears*

Infection levels were low for all treatments as expected for this pathogen species. The final disease severity (29 DAI) was zero for the mock inoculated and aphid only treatments, so these were excluded and aphid transmission and point inoculated treatments were compared using two sample t-tests. The final disease severity observed was similar for the aphid transmission treatment (12.0%) and the point inoculated treatment (10.1%) which were not significantly different to one another (*P* = 0.781). Values calculated for the area under the disease progress curve (AUDPC) were also not significantly different between treatments (*P* = 0.288), and the overall mean AUDPC across both symptomatic treatments was 96.8.

Only samples from ears from point inoculated and aphid transmission treatments accumulated detectable levels of *F. langsethiae* DNA. The mean concentrations were 0.019 pg *F. langsethiae* DNA ng^{-1} of total extracted DNA for point inoculated samples and 0.014 pg ng^{-1} from the aphid transmission treatment, which were not significantly different to one another. The mean concentration in spray inoculated ears used as a feedstock for aphids prior to transfer to new host ears was 0.49 pg ng^{-1} (±0.11 pg ng^{-1}) for those inoculated with spores and lower than the limit of detection in ears mock inoculated with water. The limit of detection for the assay was 0.005 pg ng^{-1}.

Aphid behavioural responses to VOCs of hosts with or without* Fusarium langsethiae *infection

There was no significant difference in time spent by aphids in areas of the olfactometer treated with healthy wheat VOC samples compared to areas treated only with solvent. There was also no significant difference in time spent in areas treated with *F. langsethiae*-infected host VOC samples compared to solvent treated areas. In comparative bioassays, the time spent by aphids in areas treated with *F. langsethiae*-infected host volatiles, healthy host volatiles, or solvent was also not significantly different.

***Experiment 2: Inoculation of wheat seedlings with* Fusarium langsethiae *and transmission between seedlings by apterous (wingless)* Sitobion avenae**

Aphids moved quickly from the inoculated plant to the target seedlings and multiplied to over 100 aphids per seedling on the new seedling within 10 days for all replicates. Despite this, no pathogen DNA was detected in target seedlings thus transmission of *Fusarium langsethiae* between inoculated and susceptible wheat seedlings was not observed in any treatment, with or without aphids present.

Seedlings that were treated with aphids prior to spray inoculation with *Fusarium langsethiae* spores were observed to have elevated levels of pathogen DNA in leaf tissue (P = 0.043). Seedlings with aphid exposure throughout the experiment had a mean pathogen DNA concentration of 0.148 pg ng^{-1}, whilst those with aphid only for 48h prior to inoculation had 0.137, pg ng^{-1}, which were not significantly different to one another. Seedlings without aphids had a three-fold lower mean level of pathogen DNA, 0.044 pg ng^{-1} (Fig.3.1).

Experiment 3: Transmission of* Fusarium langsethiae *via natural movement of apterous (wingless) aphids from infected to healthy host plants

Wheat plants placed at a distance of 1 m from *Fusarium langsethiae* infected wheat plants with or without aphids present showed no significant difference in the concentration of pathogen DNA. There was also no difference in the number of tillers per plant that became infected between the two treatments, neither was there a significant difference in AUDPC or the final disease severity at 28 days after inoculation. A low level of both pathogen DNA and visual signs of disease was observed in the target plants, with a mean concentration of 0.01 pg ng^{-1} for target plants across both treatments, and 0.02 pg ng^{-1} for target plants when uninfected tillers were excluded. The final disease severity was 0.6% and 0.4% for plants with and without aphids present, respectively.

Wheat plants that were spray inoculated with *Fusarium langsethiae* spores did show differences in fungal biomass and mycotoxin contamination when aphids were present compared to plants without aphids. The presence of aphids significantly increased the concentrations of *F. langsethiae* DNA (P = 0.015) and T-2 + HT-2 (P < 0.001) (Fig. 3.2) in infected flour samples. For pathogen DNA concentrations, aphid-free inoculated wheat had a mean of 3.12 pg. ng^{-1} compared to 5.57 pg ng^{-1} in inoculated plants to which aphids were added. Mean T-2 and HT-2 toxin concentrations for aphid-free inoculated plants were 15.2 µg kg^{-1} compared to 52.2 when aphids were present. Levels of T-2 and HT-2 were increased 2.7 and 7.6 times respectively when aphids were present. Furthermore, a significant, strong and positive relationship was observed between $\log_{10}$ transformed concentrations of pathogen DNA and T-2 + HT-2 ($P < 0.001$; $R^2 = 0.73$) (Fig. 3.3).

***Experiment 4: Primary transmission of* Fusarium langsethiae *via alate (winged)* Sitobion avenae**

Leaf material from the *F. langsethiae* inoculated aphid rearing cage had 0.10 pg ng^{-1} of *F. langsethiae* DNA. In leaf material from the healthy rearing cage no *F. langsethiae* DNA was detected. No *F. langsethiae* DNA was detected in real-time PCR assays using DNA samples extracted from wheat ears treated with alate aphids reared on either *F. langsethiae* infected wheat seedlings or healthy wheat seedlings.

***Toxicity of T-2 on* Sitobion avenae**

After 7 days of aphid feeding on T-2 spiked sucrose solution, the concentration of T-2 in the aphid diet was a significant factor accounting for aphid mortality ($P = 0.007$). The concentration of T-2 that induced 50% mortality, the EC_{50}, after 7 days of feeding was 407 pg ml^{-1} (Table 3.1). At 24 h and 48 h of artificial feeding no significant change in mortality was observed between T-2 treatments.

Discussion

The current results show that aphid infestation results in significantly increased fungal biomass of *F. langsethiae* and Type A trichothecenes, T-2 and HT-2, in wheat. Aphid infested diseased plants had more than 3-fold higher T-2 + HT-2 mycotoxin levels than diseased plants without aphids as well as elevated levels of fungal biomass. The amount of T-2 + HT-2 in of one of ten aphid-infested inoculated wheat plants breached the recommended tolerable daily limit of 100 micrograms per kilogram. A similar finding has recently been reported for *F. graminearum* in association with grain aphids on wheat (33). The authors showed a rise in DON and pathogen DNA in ears treated with aphids and *F. graminearum*, and that increased time of aphid colonisation prior to inoculation lead to increased pathogen DNA accumulation. The present study goes one step further, examining the impact of aphids on fungal biomass accumulation in seedlings as well as ear infection. It was not previously known if *F. langsethiae* was capable of colonising wheat seedlings systemically as a result of seed infection, infection attempts have only been measured visually and no symptoms are seen (27). This study showed both that seedling colonisation measured by pathogen DNA can be achieved using spray inoculation with a high concentration of inoculum (10^7 spores ml^{-1}), and that pre-treatment of seedlings with aphids increases fungal biomass accumulation four-fold in seedlings following inoculation. Thus, the effect of elevated biomass of *F. langsethiae* with aphid feeding on hosts that become colonised is consistent across two different wheat developmental stages tested.

Volatile organic chemical (VOC) emissions from wheat ears infected with *F. langsethiae* produced no change in aphid behaviour during olfactometer bioassay experiments. This is in contrast to *F. graminearum* infected wheat plants, which produced repellent VOCs (33). *F. graminearum* rapidly produces visible symptoms on the host plant, including ear bleaching, which reduces the green area of the ear available for aphid feeding. As *F. langsethiae* produces fewer or no symptoms the ear remains green for longer, this too has implications for the compatibility of aphids to interact with hosts infected with *F. langsethiae,* and potentially act as a vector. Our current data show that aphids do not distinguish between hosts infected with *F. langsethiae* and healthy ones. It is likely, based on our results, that aphids are not repelled by *F. langsethiae* infected wheat volatiles because the plants remain good quality hosts unlike *F. graminearum* infected plants which have lower aphid survival (33).

Another difference between the infection process of *F. graminearum* and *F. langsethiae* is production of the Type A trichothecenes, T-2 and HT-2, during *F. langsethiae* infection, compared to Type B trichothecenes, such as DON, which are produced by *F. graminearum*. Accordingly, the role of DON in the production of host volatile metabolites versus that of T-2 and HT-2 could differ, as could the comparative toxicity to aphids. Data presented in this work show that aphids were sensitive to T-2 after prolonged exposure to high concentrations of this mycotoxin. After 48h, the concentration of T-2 in artificial diets did not influence aphid mortality. This is in contrast to the previously observed effect of elevated mortality of aphid populations after feeding on wheat plants infected with *F. graminearum* for the same short timeframe of 48h (33). Thus, while aphids were sensitive to T-2, the effect on aphid populations may be less affected from feeding on hosts infected with *F. langsethiae* than with *F. graminearum*.

Early indications from the manual transmission experiment showed that grain aphids are capable of picking up sufficient inoculum from *F. langsethiae* infected wheat ears to induce disease when directly placed on a new susceptible host ear. Low levels of visible symptoms were observed, as is typical for *F. langsethiae* infection in wheat (26). However, both the disease severity and the level of pathogen DNA within ears were raised in the aphid transmission treatment compared to controls treated with aphids from healthy previous hosts, and were comparable to positive point inoculated controls. DNA levels were low in the experiment, and suggest that point inoculation is a less robust method for inducing *F. langsethiae* disease in wheat ears than spray inoculation. The level of disease induced by aphid transmission with manual transfer between hosts was not significantly different to that induced by point inoculation, and indicates that there is potential for aphid bodies to act as suitable vehicles for *F. langsethiae* inoculum given an unhindered route to the susceptible host or secondary spread on the ear.

Transmission experiments simulating natural movement of aphids from colonised plant to target plant did not provide further evidence that transmission of *F. langsethiae* is possible by either apterous or alate aphids. Apterous aphids move slowly from plant to plant to relieve crowding on any one host, and do not possess wings to move directly to wheat ears without moving up the length of the host plant to reach them. Emigrating aphids that had previously colonised infected host tissues may have originated from the site of infection with sufficient levels of inoculum to infect a new host being carried on their bodies; by the end of the journey to the new host this capacity to transmit appears to have diminished. It remains possible that these results would be affected by decreasing the distance of the infected and healthy hosts from 1 m to a distance more typical of field conditions, or with greater wind currents that could assist the translocation of aphids from infected ears to susceptible ones.

Alate aphids present an opportunity for aphids to reach new host tissues without the need for crawling long distances and potentially losing carried inoculum in the process. However, from the experiment performed, in which alate aphids were reared from *F. langsethiae* infected seedlings, the aphids were unable to transmit the inoculum when brought into proximity with a new host. Contact between immigrating alate aphids and wheat ears was not guaranteed, so it remains possible that aphids chose not to alight on wheat ears, but might have colonised other parts of the plant first with growing aphid colonies then spreading to the ears of the target plants. Also, while seedlings were successfully inoculated by *F. langsethiae* spores, the nature of the fungal growth through seedling tissue and the level of spore production of the pathogen on the surfaces of the seedlings are not known. It has been discussed whether *F. langsethiae* can exist as a non-pathogenic endophyte (42). As such, failure for alate aphids to transmit inoculum may have been due to the use of foliar tissues as a source of the fungus rather than infected ears. Further work to examine the infection process of *F. langsethiae* in wheat seedlings and the nature of the relationship between the host and the fungus is required to clarify the appropriateness of the method used in this instance.

From these results, the major implications for *F. langsethiae* epidemiology are that wheat seedlings that are attacked by aphids, either late in summer providing there is a mild autumn or in early in spring if a mild winter, may be more vulnerable to opportunistic attack by *F. langsethiae* and that ears colonised with aphids may be at greater risk of accumulating dangerous levels of mycotoxins upon fungal infection. It has been mentioned previously that the mycotoxin output of *F. langsethiae* cannot be predicted by visual disease assessment (25) but this study and others (11; 12) provide support for a strong correlation to be expected between pathogen DNA levels at harvest and T-2 and HT-2 contamination. If aphid presence on wheat can increase the susceptibility of the host to accumulate increased levels of T-2 and HT-2 during *F. langsethiae* infection, it is possible that this same effect could persist across other cereal hosts. Of particular interest for investigation would be oats, which are a more compatible host for *F. langsethiae* (24; 41). Control of aphids in high risk geographical locations could be of benefit for the limitation of toxin levels in grain and grain products.

This study, along with the previously described study on *F. graminearum* (33), show that the effect of aphid feeding on increased host susceptibility to FHB is not limited to a single *Fusarium* species; other fungal pathogen infections may also be enhanced in aphid-infested wheat hosts. While the mechanism by which aphids can increase the susceptibility of the host towards *F. langsethiae* is not addressed in this study, this pathogen species has been observed to be unable to produce lesions in detached wheat leaves without a wound site (24), indicating that physical degradation of tissues upon aphid feeding could contribute to improved infection success by the pathogen. While aphids do not produce large mechanical wounds, components of aphid saliva are able to alter host biochemistry so as to cause discolouration in the tissues surrounding feeding sites and can influence plant defence responses (43; 44). Suppression of host defences, or upregulation of plant defence pathways in response to aphid attack that operate antagonistically with the pathways responsible for coping with attack by fungal pathogens could lead to a muted defence response to the fungal attack and increased disease. It has been shown that the salicylic acid pathway is important in wheat defence against *Fusarium* (45) and it is possible that aphid attack may supress these defences because aphid saliva contains proteins that supress plant defence (46).

In conclusion, despite indications that grain aphids could be compatible vectors of *F. langsethiae* between wheat plants, experiments that mimicked the natural movement of aphids between host plants in the field did not show aphid transmission. However, aphid herbivory on hosts that became infected from other sources was shown to increase the amount of fungal biomass accumulated in wheat seedling foliar tissues and in ears as well as the concentration of T-2 and HT-2 toxin in infected grain. In the light of this, given that aphids are not repelled by infected hosts, as has been shown to occur with the more aggressive FHB pathogen *F. graminearum*, our study indicates that persistent interaction between infected hosts and aphids may be possible and that this interaction could lead to an increase in the toxin contamination of wheat crops. These findings help to fill knowledge gaps surrounding the life cycle and epidemiology of this newly identified pathogen species, particularly with respect to seedling infection and the role that aphids may play in enhancing the infection success of this weak pathogen of wheat.

Funding Information

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Acknowledgments

This work was supported by a Lawes' Trust PhD studentship. Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council (BBSRC). The authors would like to thank Mark Meacham for technical assistance.

References

1. **Wegulo SN, Baenziger PS, Nopsa JH, Bockus WW, Hallen-Adams H.** 2015. Management of Fusarium head blight of wheat and barley. Crop Prot **73:**100-107.

2. **Glynn NC, Edwards SG.** 2010. Evaluation of PCR assays for quantifying seed-borne infection by *Fusarium* and *Microdochium* seedling blight pathogens. J Appl Microbiol **108:**81-87.

3. **Marin S, Ramos AJ, Cano-Sancho G, Sanchis V.** 2013. Mycotoxins: Occurrence, toxicology, and exposure assessment. Food Chem Toxicol **60:**218-237.

4. **Parry DW, Jenkinson P, McLeod L.** 1995. Fusarium ear blight (scab) in small-grain cereals - a review. Plant Pathol **44:**207-238.

5. **Goswami RS, Kistler HC.** 2004. Heading for disaster: *Fusarium graminearum* on cereal crops. Mol Plant Path **5:**515-525.

6. **Imathiu SM, Edwards SG, Ray RV, Back MA.** 2013. *Fusarium langsethiae* - a HT-2 and T-2 Toxins Producer that Needs More Attention. J Phytopath **161:**1-10.

7. **Logrieco A, Bottalico A, Mule G, Moretti A, Perrone G.** 2003. Epidemiology of toxigenic fungi and their associated mycotoxins for some Mediterranean crops. Eur J Plant Path **109:**645-667.

8. **Torp M, Nirenberg HI.** 2004. *Fusarium langsethiae* sp nov on cereals in Europe. Int J Food Microbiol **95:**247-256.

9. **Halstensen AS, Nordby K-C, Klemsdal SS, Elen O, Clasen P-E, Eduard W.** 2006. Toxigenic *Fusarium* spp. as determinants of trichothecene mycotoxins in settled grain dust. J Occup Environ Hyg **3:**651-659.

10. **Fredlund E, Gidlund A, Sulyok M, Borjesson T, Krska R, Olsen M, Lindblad M.** 2013. Deoxynivalenol and other selected Fusarium toxins

in Swedish oats - Occurrence and correlation to specific Fusarium species. Int J Food Microbiol **167:**276-283.

11. **Nielsen LK, Jensen JD, Nielsen GC, Jensen JE, Spliid NH, Thomsen IK, Justesen AF, Collinge DB, Jorgensen LN.** 2011. Fusarium Head Blight of Cereals in Denmark: Species Complex and Related Mycotoxins. Phytopathology **101:**960-969.

12. **Edwards SG, Imathiu SM, Ray RV, Back M, Hare MC.** 2012. Molecular studies to identify the Fusarium species responsible for HT-2 and T-2 mycotoxins in UK oats. Int J Food Microbiol **156:**168-175.

13. **Lenc L.** 2015. Fusarium head blight (FHB) and *Fusarium* populations in grain of winter wheat grown in different cultivation systems. J Plant Prot Res **55:**94-109.

14. **Dedeurwaerder G, Ghysselinckx J, Hellin P, Janssen F, Duvivier M, Legreve A.** 2014. Detection of *Fusarium langsethiae* on wheat in Belgium. Eur J of Plant Pathol **139:**453-455.

15. **Infantino A, Santori A, Aureli G, Belocchi A, De Felice S, Tizzani L, Lattanzio VMT, Haidukowski M, Pascale M.** 2015. Occurrence of *Fusarium langsethiae* Strains Isolated from Durum Wheat in Italy. J Phytopath **163:**612-619.

16. **Denschlag C, Rieder J, Vogel RF, Niessen L.** 2014. Real-time loop-mediated isothermal amplification (LAMP) assay for group specific detection of important trichothecene producing *Fusarium* species in wheat. Int J Food Microbiol **177:**117-127.

17. **Minaeva LP, Korotkevich YV, Sheveleva SA.** 2013. Rapid determination of food grain infection by fungi Fusarium and their species detection (part 1). Vopr Pitan **82:**61-66.

18. **Yli-Mattila T, Paavanen-Huhtala S, Jestoi M, Parikka P, Hietaniemi V, Gagkaeva T, Sarlin T, Haikara A, Laaksonen S, Rizzo A.** 2008. Real-time PCR detection and quantification of *Fusarium poae, F. graminearum*, *F. sporotrichioides* and *F.*

langsethiae in cereal grains in Finland and Russia. Arch Phytopathol PFL **41:**243-260.

19. **Kokkonen M, Ojala L, Parikka P, Jestoi M.** 2010. Mycotoxin production of selected Fusarium species at different culture conditions. Int J Food Microbiol **143:**17-25.

20. **Torp M, Adler A.** 2004. The European Sporotrichiella project: a polyphasic approach to the biology of a new *Fusarium* species. Int J Food Microbiol **95:**241-245.

21. **Yli-Mattila T, Mach RL, Alekhina IA, Bulat SA, Koskinen S, Kullnig-Gradinger CM, Kubicek CP, Klemsdal SS.** 2004. Phylogenetic relationship of *Fusarium langsethiae* to *Fusarium poae* and *Fusarium sporotrichioides* as inferred by IGS, ITS, beta-tubulin sequences and UP-PCR hybridization analysis. Int J Food Microbiol **95:**267-285.

22. **Nazari L, Pattori E, Terzi V, Morcia C, Rossi V.** 2014. Influence of temperature on infection, growth, and mycotoxin production by *Fusarium langsethiae* and *F. sporotrichioides* in durum wheat. Food Microbiol **39:**19-26.

23. **Anon**. 2013. Commission recommendation of 27th March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products. 2013/165/EU.

24. **Imathiu SM, Ray RV, Back M, Hare MC, Edwards SG.** 2009. *Fusarium langsethiae* pathogenicity and aggressiveness towards oats and wheat in wounded and unwounded in vitro detached leaf assays. Eur J Plant Path **124:**117-126.

25. **Opoku N, Back M, Edwards SG.** 2013. Development of *Fusarium langsethiae* in commercial cereal production. Eur J Plant Path **136:**159-170.

26. **Imathiu SM, Ray RV, Back MI, Hare MC, Edwards SG.** 2013. A Survey Investigating the Infection of Fusarium langsethiae and

Production of HT-2 and T-2 Mycotoxins in UK Oat Fields. J Phytopath **161:**553-561.

27. **Imathiu SM, Hare MC, Ray RV, Back M, Edwards SG.** 2010. Evaluation of pathogenicity and aggressiveness of *F. langsethiae* on oat and wheat seedlings relative to known seedling blight pathogens. Eur J Plant Path **126:**203-216.

28. **Edwards SG, Barrier-Guillot B, Clasen PE, Hietaniemi V, Pettersson H.** 2009. Emerging issues of HT-2 and T-2 toxins in European cereal production. World Mycotoxin J **2:**173-179.

29. **Hope R, Aldred D, Magan N.** 2005. Comparison of environmental profiles for growth and deoxynivalenol production by *Fusarium culmorum* and *F-graminearum* on wheat grain. Lett Appl Miocrobiol **40:**295-300.

30. **Wratten SD.** 1975. Nature of effects of aphids *Sitobion-avenae* and *Metopolophium-dirhodum* on growth of wheat. Ann Appl Biol **79:**27-34.

31. **De Zutter N, Audenaert K, Haesaert G, Smagghe G.** 2012. Preference of cereal aphids for different varieties of winter wheat. Arthropod-Plant Inte **6:**345-350.

32. **De Roissart A, Audenaert K, Smagghe G, Haesaert G.** 2010. Monitoring Wheat Fields in Belgium to Assess the Role of Aphids in the Spread of Fusarium. *In* Fusarium, 11th European Seminar, Abstracts.

33. **Drakulic J, Caulfield J, Woodcock C, Jones SPT, Linforth R, Bruce TJA, Ray RV.** 2015. Sharing a Host Plant (Wheat *Triticum aestivum*) Increases the Fitness of *Fusarium graminearum* and the Severity of Fusarium Head Blight but Reduces the Fitness of Grain Aphids (*Sitobion avenae*). Appl Environ Microb **81:**3492-3501.

34. **Leslie JF, Summerell BA.** 2006. The Fusarium laboratory manual / John F. Leslie and Brett A. Summerell ; photographs by Suzanne

Bullock. Blackwell.

35. **De Vos M, Jander G.** 2010. Volatile communication in plant-aphid interactions. Curr Opin Plant Biol **13**: 366-371.

36. **Zadoks JC, Chang TT, Konzak CF.** 1974. Decimal code for growth stages of cereals. Weed Res **14:**415-421.

37. **Tuite J.** 1969. Plant Pathological Methods. Fungi and Bacteria. Burgess Publishing Company, Minneapolis, MN, USA.

38. **Birkett MA, Bruce TJA, Martin JL, Smart LE, Oakley J, Wadhams LJ.** 2004. Responses of female orange wheat blossom midge, *Sitodiplosis mosellana*, to wheat panical volatiles. J Chem Ecol **30**: 1319-1328.

39. **Edwards SG, Pirgozliev SR, Hare MC, Jenkinson P.** 2001. Quantification of trichothecene-producing Fusarium species in harvested grain by competitive PCR to determine efficacies of fungicides against fusarium head blight of winter wheat. Appl Environ Microb **67:**1575-1580.

40. **White T. J., T. Bruns, S. Lee, and J. Taylor.** 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, 315-322. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), Pcr Protocols: a Guide to Methods and Applications. Academic Press, San Diego, CA.

41. **Wilson A, Simpson D, Chandler E, Jennings P, Nicholson P.** 2004. Development of PCR assays for the detection and differentiation of *Fusarium sporotrichioides* and *Fusarium langsethiae*. Fems Microbiol Lett **233:**69-76.

42. **Divon HH, Razzaghian J, Udnes-Aamot H, Klemsdal SS.** 2012. Fusarium langsethiae (Torp and Nirenberg), investigation of alternative infection routes in oats. Eur J Plant Pathol **132:**147-161.

43. **Petersson J, Tjallingii WF, Hardie J.** 2007. Aphids as Crop Pests, p 87-113. *In* VanEmden HF, Harrington R (ed), Host-plant Selection and Feeding. CABI Publishing, Wallingford, UK.

44. **Walling LL.** 2008. Avoiding effective defenses: Strategies employed by phloem-feeding insects. Plant Physiol **146:**859-866.

45. **Makandar R, Nalam VJ, Lee H, Trick HN, Dong Y, Shah J.** 2012. Salicylic Acid Regulates Basal Resistance to Fusarium Head Blight in Wheat. Mol Plant Microbe In **25:**431-439.

46. **Elzinga DA, De Vos M, Jander G.** 2014. Suppression of Plant Defenses by a *Myzus persicae* (Green Peach Aphid) Salivary Effector Protein. Mol Plant Microbe In **27:**747-56.

Figures

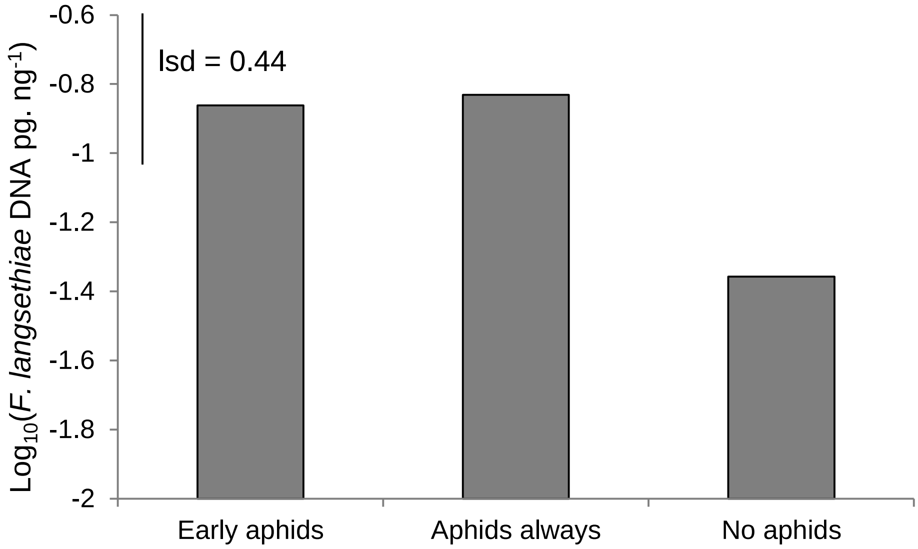

Fig. 3.1. *F. langsethiae* DNA concentration (pg. ng^{-1}) (log_{10}-transformed data) in inoculated wheat seedlings (Experiment 2) with aphid infestation prior to inoculation (early aphids), continued aphid infestation both before and after inoculation (aphids always) and without aphid infestation (no aphids). (P = 0.043).

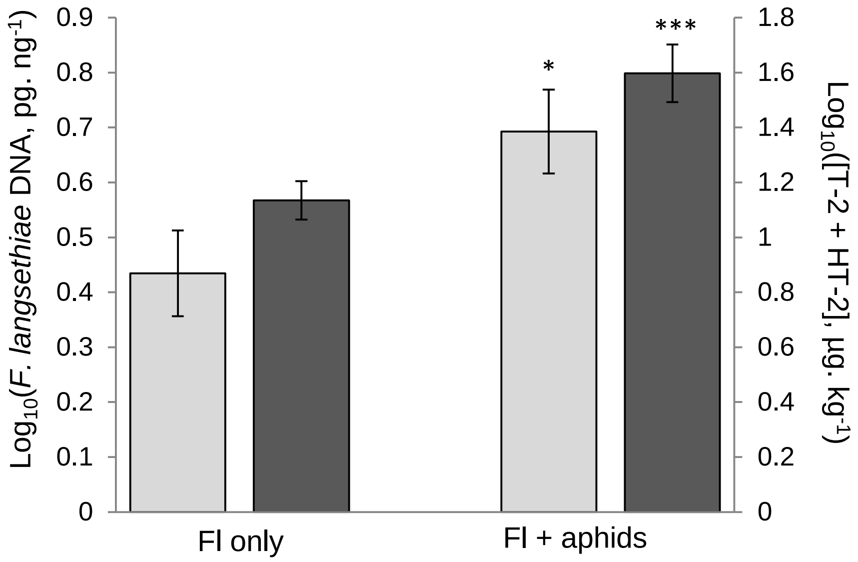

Fig. 3.2. Concentrations of (left axis) *F. langsethiae* DNA (pg ng^{-1}) (log_{10}-transformed) (P = 0.015) and (right axis) [T-2 + HT-2] toxins (µg kg^{-1}) (log_{10}-transformed) (P < 0.001), in inoculated feedstock plants in wingless aphid transmission experiment (Experiment 3) for plants with and without aphid infestation. Asterisks represent the level of significant difference (* 0.05 > P ≥ 0.01, ** 0.01 > P ≥ 0.001, *** P < 0.001).

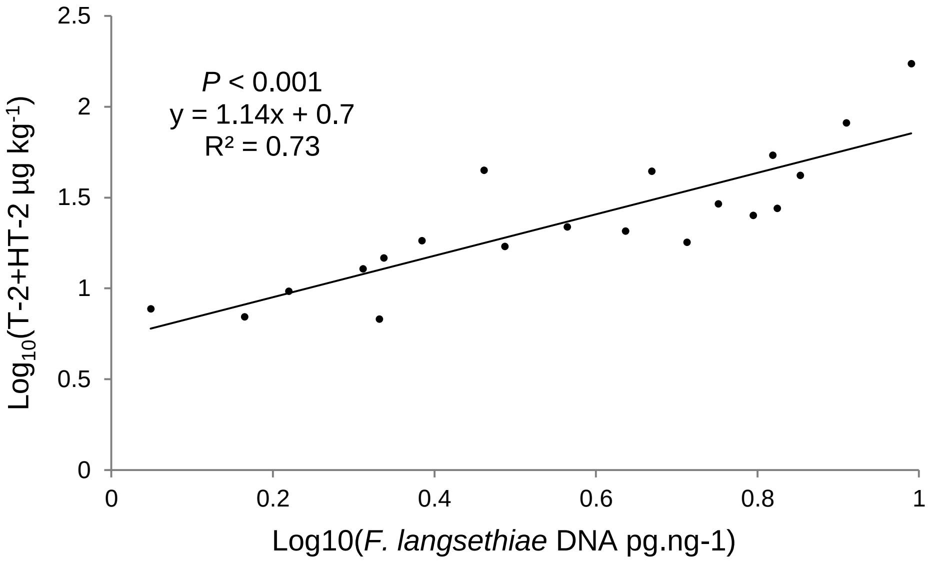

Fig. 3.3. Linear regression between $\log_{10}$-transformed concentrations of *F. langsethiae* DNA (pg ng^{-1}) and [T-2 + HT-2] toxins (µg kg^{-1}) in ears of inoculated wheat plants in wingless aphid transmission experiment.

Table 3.1.

Mortality of T-2 on *Sitobion avenae* during artificial feeding assay using T-2 toxin spiked sucrose solution. (Data marked with different letters are significant.)

Concentration of T-2 ($pg.ml^{-1}$)	**Abbott's corrected mortality (%) after 7 days**
1	5^{a}
10	11.4^{ab}
100	40^{bc}
1000	60^{c}
5% lsd	30.2
P	**0.007**
EC_{50} ($pg\ ml^{-1}$)	**407**

Chapter 4: Contrasting roles of deoxynivalenol and nivalenol in host-mediated interactions between *Fusarium graminearum* and *Sitobion avenae*

Experimental work examining the effect of the chemotype of *F. graminearum* isolates on the infection of wheat and the association of aphids with infected hosts is presented in this chapter. This work is a paper in preparation, and is presented in 'paper format'.

Author contribution

This paper was composed and experiments designed by J. Drakulic. Practical work was conducted jointly by M. Kahar and J. Drakulic, with the exception of the analysis of mycotoxin extracts by LCMS/MS, which was performed by O. Ajigboye. The work was supervised by R. V. Ray and T. Bruce.

RESEARCH ARTICLE

Contrasting roles of deoxynivalenol and nivalenol in host-mediated interactions between *Fusarium graminearum* and *Sitobion avenae*

Running title: F. graminearum chemotype affects wheat odour

Jassy Drakulic[1], Mohd Haziq Kahar[1], Olubukola Ajigboye[1], Toby Bruce[2], Rumiana V. Ray[1]

[1] University of Nottingham, Sutton Bonington Campus, College Rd, Sutton Bonington, LE12 5RD, UK

[2] Rothamsted Research, Harpenden, Herts AL5 2JQ, UK

Word count (excluding references, figure legends and tables): 5571

Abstract

Fusarium graminearum is the predominant causal species of Fusarium head blight in Europe and North America. Different chemotypes of the species exist, which produce different mycotoxins in infected grain, dependent on genetic differences. Nivalenol (NIV) and deoxynivalenol (DON) differ in toxicity to mammals and plants, and DON has been identified as a virulence factor that promotes pathogen spread. However, the effect of each mycotoxin on volatile emissions of plant hosts is not known. Host volatiles are interpreted by insect herbivores such as *Sitobion avenae,* the English grain aphid, during host selection. Previous work has shown that grain aphids are repelled by wheat infected with DON-producing *F. graminearum,* and this study seeks to determine the influence of pathogen mycotoxins to host volatile chemistry. Volatile collections from infected hosts and olfactometer bioassays with alate aphids were performed. Infections with isolates that produced DON and NIV were compared, as well as trichothecene-deficient transgenic mutants. This work confirmed the repellent nature of infected hosts with DON accumulation, although a failure of the knock-out mutant prevented the conclusion that DON is specifically responsible for this effect. Infection with NIV accumulation produced volatiles that were attractive to aphids. Attraction did not occur when infection was absent of NIV, and was therefore a direct consequence of NIV production. The responsible chemistry for attraction to NIV-producing infection could not be determined; however 2-pentadecanone was concluded to be a volatile signal that is elevated in *F. graminearum* pathogenesis independent of chemotype.

Keywords: Fusarium head blight, aphids, trichothecenes, volatiles

Introduction

Fusarium Head Blight (FHB) is a fungal disease caused by a complex of species from the genus *Fusarium*, and *F. graminearum* is the most prevalent species in North America (Goswami & Kistler, 2004) and Europe (Waalwijk *et al*., 2003; Xu *et al.,* 2005) at present. As a result of FHB disease, cereal host crops become contaminated with mycotoxins, grain quality is impaired and yield is lost (Parry *et al*., 1995). Trichothecene mycotoxins produced by *Fusarium* species are a group of toxic chemicals formed from tetracyclic sesquiterpenoids with an epoxy-ring at the C12,13 position and can be classed into Type A or B depending on the absence or presence, respectively, of a ketone functional group at the C8 position and a hydroxyl group at C7 (Alexander *et al*., 2011). *F. graminearum* produces Type B trichothecene mycotoxins, including deoxynivalenol (DON) and nivalenol (NIV) and acetylated derivatives of these 3-ADON, 15-ADON and 4-ANIV. A legal limit for DON has been set in Europe for different categories of grain products, with a maximum of 500 $\mu g.kg^{-1}$ DON in finished cereal products intended for human consumption (Anon, 2006).

The prevalence of different *F. graminearum* chemotypes varies according to geographical region, with NIV chemotypes prevailing in Asia and DON chemotypes prevailing in Europe and North America (Carter *et al*., 2002; van der Lee *et al*., 2014). The geographical distribution of the different chemotypes has also been recently described to be shifting, although the factors driving the change are not known. Among the DON producing strains these can be categorised into those producing 3-ADON and 15-ADON. In North America 15-ADON isolates are traditional, although emerging populations of the 3-ADON type or of the 15-ADON type with genetic similarities to the 3-ADON type have been observed (Ward *et al*., 2008; Foroud, *et al*., 2012). Shifting patterns of mycotoxin production by this species show that ongoing study into the impact of the different chemotypes on hosts and their environment is needed.

The different mycotoxins produced have differing levels of phytotoxicity (Eudes *et al*., 2000) and toxicity to mammalian consumers (Marin *et al*., 2013).

The mode of action for all is to inhibit protein synthesis by interacting with small 60S ribosomal subunits and peptidyltransferase enzymes. In mammals, the health implications of consuming DON and NIV include feed refusal and weight loss, plus DON has been reported to have immunosuppressive effects and NIV to cause oral lesions (D'Mello *et al*., 1999). In plant hosts, the phytotoxicity has been measured by using different model systems. Wheat coleoptile growth was severely inhibited by DON, whereas the effect of NIV was no different to negative controls (Eudes *et al*., 2000). In *Chlamydomonas reinhardtii* toxicity of NIV was detectable, but less still than that of DON (Suzuki & Iwahashi, 2014). Neither of these studies truly demonstrates the phytotoxic effects to cereal ears at the site of head blight disease, but indicates that the phytotoxicity of NIV is less than of DON and is host dependent.

The trichothecene biosynthetic pathway is governed by *Tri* genes, the first in the pathway being *Tri5* which encodes trichodiene synthase which catalyses the conversion of farnesyl pyrophosphate into trichodiene (Hohn *et al*., 1989). The genetic difference of *F. graminearum* chemotypes has been elucidated (Lee *et al*., 2002). NIV producers differ from DON producers in their presence of a functional *Tri13* and *Tri7* genes, which carry out hydroxylation and acetylation of the C4 position of the trichothecene ring (Kimura *et al*., 2003; Alexander *et al*., 2011). In the absence of these functioning genes, the trichothecene biosynthetic pathway is diverted at this point in DON-producing strains. To determine the role of mycotoxins as virulence factors, Maier *et al*. (2006) transformed DON- and NIV-producing isolates to knock out the *Tri5* gene, and these mutants were shown to be unable to produce any mycotoxins. Growth of *tri5* knock out mutants was impeded at the rachis node, indicating that DON assists spread of the fungus into other spikelets of the ear (Jansen *et al*., 2005). DON has been shown to be an important virulence factor in the colonisation of wheat by *F. graminearum*, whereas NIV is a more important virulence factor in the infection of maize by this species (Maier *et al*., 2006). Accordingly, different chemotypes can have different pathogenicity depending on the host infected, although NIV-producers in some cases have been shown to be less pathogenic to wheat than DON-producers (Carter *et al*., 2002). DON and NIV are water soluble, and DON has been shown to be able to travel in phloem sieve tubes and xylem vessels in wheat ears infected with *F. culmorum* (Kang & Buchenauer, 1999), occurring in tissues beyond those colonised by fungal hyphae. DON has specifically been linked to bleaching of ears upwards from the site of infection (Horevaj *et al*., 2012), which is less commonly observed with NIV-producing *F. graminearum* infection (Gosman *et al*., 2009).

Interactions can occur between arthropods and host plants infected with FHB (Drakulic *et al*., 2016), but information is lacking on the impact of the mycotoxin output of the infecting fungi on visiting herbivores. A previous study of wheat ears infected with *F. graminearum* showed that the volatile chemical emissions were repellent to English grain aphids, *Sitobion avenae* Fab. (Hemiptera: Aphididae), in olfactometer bioassays (Drakulic *et al*., 2015), and it was hypothesised that this effect is driven by the mycotoxin output of the pathogen. Thus, a series of volatile collection experiments were performed to test this hypothesis. Isolates described in Maier *et al*. (2006) of *F. graminearum* of known chemotype, along with *tri5* transformants of the same isolates, were used to infect wheat ears and volatile chemicals were collected at five time points from 6 hours to 7 days after inoculation. The aims of this work were to compare volatile chemical emissions of DON and NIV producers to their trichothecene deficient mutant counterparts and to one another, and to examine the behavioural response of aphids, *Sitobion avenae*, to those volatiles. Visual disease assessment, real-time PCR and mycotoxin extraction were used to validate the treatments.

Materials & Methods

Plant material

Wheat seeds (*T. aestivum* cv. Gallant) treated with 10 g of prothioconazole and 50 g clothianidin per 100 kg seed (Redigo Deter, Bayer) were planted into compost (Levington's F2+S) in module trays (l x w x d: 2cm x 2cm x 5cm) and vernalised for 6 weeks at 4°C. Seedlings were then potted out into individual 5 L pots into compost (John Innes Type 2) and grown in a glasshouse under a 12 h photoperiod at 15 - 18°C day and 12 - 15°C night temperatures. At ear emergence (GS59) (Zadoks *et al*., 1974) plants were moved to a controlled environment chamber with 16 h/8 h day/night photoperiod, at 23°C day and 15°C night temperatures, and acclimated for one week prior to the application of treatments.

***F. graminearum* isolates**

Isolates of *F. graminearum* used in these experiments were donated by Universitat Hamburg by the Schaefer group. They consisted of one DON-producing strain (FG8.1/ #290), a transformed version of the same isolate with a disrupted *Tri5* gene (#291), one NIV-producing strain (FG06/ #293) and its *Tri5* knockout transformant (#294). Transformed strains were disrupted in the *Tri5* gene by vector insertion of a truncated *Tri5* fragment as previously described (Maier *et al*, 2005; Maier *et al*., 2006). Treatment names abbreviated to DON-wt, DON-ko and NIV-wt and NIV-ko for each of the DON- and NIV-producing wild types and their corresponding *tri5* knock-out transformants. NTC denotes the non-treated controls.

Production of fungal inoculum and inoculation of wheat ears

Isolates were grown in PDA for 10 days then subcultured on synthetic nutrient deficient agar (SNA) for a further 10 days. SNA plates were washed with 1 ml sterile distilled water (SDW) and agitated using a sterile L-shaped spreader. Spore suspensions were removed by pipette and the concentration calculated using a Neubauer improved haemocytometer (Marienfeld-Superior, Germany). The concentration of the suspensions was adjusted to 2.5 x 10^5 spores ml^{-1} with SDW and kept for two days at 4°C until inoculation.

Wheat ears at anthesis were point inoculated on 10 spikelets per ear, by pipetting 10 µl spore suspension between the lemma and palea of individual spikelets. Control plants were inoculated in the same way using SDW. Three repeats of the inoculation experiment were performed, and 4 ears per plant were inoculated in the first and third experiments and 2 ears per plant in the second as limited numbers of same age tillers were produced. Two whole plant replicates were produced in the first and second repeats of the experiment, and four replicates were prepared in the third. After inoculation, ears were bagged in perforated plastic bags for 6 h to allow the inoculum to infiltrate the host tissues prior to volatile chemical collection.

Volatile chemical collection and olfactometer bioassays

In the first two repeats of the experiment, 6 hours after inoculation (HAI) volatile chemical collection apparatus was connected to wheat ears. All inoculated ears of each plant were contained within odour-free cooking bags which were tied around the stems. Air was pumped into the bags from the base at a rate of 600 ml min^{-1} and air was removed from the top corner, drawn out over a Porapak (TM) polymer matrix filter at a rate of 400 ml min^{-1}. Collections were commenced at 6, 24, 48, 96 HAI and 7 days after inoculation (DAI) and were carried out for approximately 24 h, with the exception of the 6 HAI collection which was only performed for 18 h. Between collections Porapak (TM) filters were removed, and volatile emissions were eluted into glass vials in 500 µl dichloromethane (DCM), and samples stored at -20°C until use in olfactometer bioassays. Filters were cleaned between samples with 6 ml DCM.

Disease assessment

On the eighth day after inoculation, after the final volatile collection, visual disease severity was assessed. The number of spikelets that displayed either dark lesions or bleaching was recorded as being symptomatic, and the disease severity was calculated as the percentage of symptomatic spikelets out of the total spikelets per ear. A second disease assessment was performed 14 DAI. The proportion of spikelets that were bleached out of the total number of spikelets per ear was calculated to give the severity of the bleaching symptoms at both 7 and 14 DAI.

DNA extraction and QPCR

Ears were harvested 21 DAI and freeze-dried without threshing. Samples were then ground to flour using a commercial coffee grinder (Krups). DNA was extracted from flour samples (0.5 g) using CTAB buffer (3.75 ml), following a method that has been described previously (Drakulic *et al*., 2015). DNA was resuspended in TE buffer (200 µl) by warming tubes in a hot block (65°C) for 2 h and vortexing. The concentration of DNA in each sample was determined by spectrophotometry at wavelengths 260, 280 and 328 nm (Cary50 UV Spectrophotometer, USA) recorded in software (Simple Reads). DNA stocks were then diluted to 20 ng μl^{-1} and the concentration of diluted stocks confirmed by spectrophotometry. Due to large quantities of target DNA being quantified after the first round of quantitative PCR, stocks were further diluted to 4 ng μl^{-1} for use in *F. graminearum* and *Tri5* specific assays.

DNA stocks were used in quantitative real-time PCR (QPCR) assays using four different primer sets, shown in Table 4.1. Primers for *F. graminearum*, NIV-producing isolates, 15-ADON producing isolates, 3-ADON producing isolates, and for the *Tri5* gene were used. All assays used a total volume of 13 µl comprising 2.5 µl template DNA, SYBR Green 2X MaterMix and 250 nM of each forward and reverse primers. The temperature cycling programs for each assay are listed in Table 4.1.

A standard curve of DNA of known concentration was prepared for each assay, using DNA from pure fungal cultures of either known DON-producing (#216) or NIV-producing isolates (#281) from the University of Nottingham collection for each of the assays that used 15- or 3ADON- and NIV-producer specific primers. Assays using primers not specific to chemotype were run twice, using each of the previous standards in each run. Quantifications were found to be similar with both standards, and results presented are a mean of the quantifications of both assays. Ten-fold dilutions of DNA standards were assayed, ranging from 1 to 10^{-6} ng μl^{-1}. This linear regression was used to estimate the starting quantity of target DNA in samples. The limit of quantification for the *F. graminearum* assay was 1 x 10^{-5} ng μl^{-1}, and for all others the limit of quantification was 1 x 10^{-4} ng μl^{-1}.

Mycotoxin extraction and LCMS/MS

To extract NIV, flour samples (0.5 g) were added to 15 ml tubes along with 4 ml methanol (70%) and shaken for four intervals of 20 s (MPBio FastPrep). Tubes were centrifuged at 2000 xg for 5 mins then 1 ml supernatant added to a glass vial and spiked with ^{13}C-labelled NIV as an internal standard to produce a final concentration of 20 µg kg^{-1}. Following this, samples were dried under Nitrogen gas and redissolved in methanol (10%) and stored at -20°C until analysis. On the day of analysis, samples were warmed to room temperature, liquid content transferred to a microcentrifuge tube and centrifuged for 5 minutes at 13,000 xg. The supernatant was then transferred to a new glass vial and then analysed by LCMS/MS.

To extract DON, flour samples (0.5 g) were added to 15 ml tubes with 4ml methanol (70%), spiked with ^{13}C-labelled DON at a concentration of 20 μg kg^{-1}, and shaken for four intervals of 20 s (MPBio FastPrep). Tubes were centrifuged at 2000 xg for 5 mins and 2 ml supernatant diluted into PBS (23 ml). The diluted samples were filtered through glass microfiber paper (VWS). The filtrate was then added to a 20 ml syringe attached to a DZT MS-PREP column (R-Biopharm) and allowed to pass through by gravity over a half hour period. The DZT column was washed by passing 20 ml deionised water through the column using the syringe plunger to maintain a flow rate of 5 ml min^{-1}, then dried by passing air over the column five times. Using a 2 ml syringe and plunger, 1 ml methanol (100%) was passed over the DZT column at a rate of one drop per second. During the passage of methanol over the column, the plunger was withdrawn to reverse the flow and draw liquid back up from the column three times to ensure maximum elution of the toxins contained on the column into the methanol. Methanol was evaporated off from the samples under a stream of nitrogen gas until dryness and redissolved in 500 μl methanol (10%) to prepare samples for analysis by LCMS/MS.

The LCMS/MS analysis was performed on an Agilent 1100 series LC system (Agilent Technologies, Germany) coupled to a triple quadrupole Micromass Quattro Ultima V4.0 SP4 (Waters) and equipped with Luna® C18 Å100 LC (5 μm, 250 mm x 3 mm) column (Phenomenex, Torrance, CA, USA). The flow rate was set at 0.5 ml min^{-1} and the injection volume was 30 μl. Mobile phase A was 90% water with 10% methanol, mobile phase B was 100% methanol. For DON analysis a linear binary gradient was applied from 0 to 100% phase B within 15 mins. The content of phase B was held for 7 mins and then lowered to 0% within 20 s followed by equilibration of the column for 5 mins. For NIV analysis a linear binary gradient was applied from 0 to 50% phase B over 7.5 mins, and from 50 to 100% over a further 30 s. The content of phase B was held for 1 min then lowered to 0% over 1 min and followed by equilibration of the column for 5 mins.

Quantitative determination of all compounds was performed by operating the mass spectrometer in ESI positive and negative ionisation mode. Optimized instrument settings include capillary voltage 3.5 kV, source temperature 100°C, desolvation temperature 450°C, desolvation gas flow rate of 672 L hr^{-1} cone voltage, a cone gas flow rate of 72 L hr^{-1}, and multiplier 650 V. Parent/daughter ions for DON, ^{13}C-labelled DON, NIV and ^{13}C-labelled NIV were detected at 265/217, 279/230, 281/191 and 295/198.7 m/z respectively with a dwell time of 0.1 s. Masslynx 4.0 software was used for data acquisition and processing. Quantification of the samples was carried out using a matrix of standards prepared in-house. The limit of detection was determined for DON and NIV was 10 μg kg^{-1}.

Analysis of VOCs by GC-MS

Headspace samples (4 μl) were analysed by GC-MS using a capillary GC column (50 m × 0.32 mm i.d. HP-1) fitted with a cold on-column injector which was coupled directly to a mass spectrometer (VG Autospec, Fisons Instruments, Manchester, UK). Ionization was by electron impact at 70 eV, 250°C. The oven temperature was maintained at 30˚C for 5 mins and then programmed at 5°C min^{-1} to 250˚C. Tentative GC-MS identifications were confirmed by peak enhancement with authentic samples on both the polar and nonpolar GC columns (Pickett, 1990). The peak area of confirmed chemicals was obtained from chromatograms and amounts were quantified using a known amount (100 ng) of authentic standards.

Statistics

Analyses of the full treatment structure were performed by general ANOVA, with the exceptions of cases where treatments contained zero values which were excluded from analyses. Experiment was not found to be a significant factor so all data was combined and analysed together. Direct comparisons between toxin-producing isolates and their trichothecene-deficient counterparts was analysed by ad hoc comparison of means with a critical threshold of 5% (P = 0.05). Abundance of VOCs over time was analysed by repeated measurements ANOVA. Disease severity and bleaching severity data was angularly transformed, and DNA, mycotoxin and volatile chemical abundance data was $\log_{10}$ transformed to normalise the residuals. Data from olfactometer assays was analysed by t-test, with a critical threshold of P = 0.05 for significance. All analyses were performed in Genstat v. 15.2 (Harpenden, UK).

Results

Visual disease assessment

Treatment was a significant factor accounting for disease severity at 7 DAI (P = 0.003), 14 DAI (P = 0.012) and bleaching severity at 7 DAI (P = 0.002) and 14 DAI (P < 0.001) (Fig. 4.1). Disease severity and bleaching severity were generally higher in the DON-wt, DON-ko and NIV-wt treatments and were lowest in the NIV-ko treatment, whilst controls showed no symptoms or bleaching whatsoever. At 7 DAI (Fig. 4.1A), the disease severities were similar for DON-wt (53.6%), NIV-wt (53.4%) and DON-ko (49.5%) but significantly fewer symptoms were observed in the NIV-ko treatment (42.1%) (Fig 4.1A). Bleaching at 7 DAI (Fig. 4.1B) was similar in DON-wt (39.9%) and DON-ko (34.7%) but significantly less bleaching occurred in NIV-wt (16.9%) and no signs of bleaching were observed in NIV-ko plants. At 14DAI the disease severity (Fig. 4.1C) had increased, with similar levels in DON-wt (64.2%), DON-ko (61.5%) and NIV-wt (61.7%) with significantly less seen in NIV-ko (53.7%). Bleaching at 14 DAI (Fig. 4.1D) also showed similar levels for DON-wt (55.6%), DON-ko (54.4%), but significantly less was observed in NIV-wt (40.9%). At this time point, NIV-ko showed some bleaching (7.3%) which was significantly less than the NIV-wt.

Characteristation of isolates by DNA extraction and QPCR

Treatment was a significant factor (P = 0.008) in accounting for target DNA levels in QPCR assays using *Fusarium graminearum* specific primers. Similar concentrations of target DNA were measured in flour samples from ears treated with DON-wt, DON-ko and NIV-wt isolates, but target DNA concentrations were significantly lower in those treated with the NIV-ko strain when compared with the 5% lsd value (Fig. 4.2A) and the quantity of target DNA was below the limit of detection in controls, as was also the case in assays using 15ADON- and NIV-producer and *Tri5* specific primers. *Tri5* DNA varied significantly with treatment (P = 0.005), there were greater *Tri5* DNA concentrations in DON-wt, DON-ko and NIV-wt treated samples compared to

NIV-ko treated samples (Fig. 4.2B). The concentration of NIV-producer DNA was lower than the limit of detection in all treatments except NIV-wt and NIV-ko samples. The difference in concentration of NIV-producer DNA between these two treatments only differed at the 10% level (P = 0.096) with the greater concentration occurring in the NIV-wt treatment (Fig. 4.2C). The quantity of 15ADON-producer DNA differed significantly between treatments (P < 0.001). DON-wt and DON-ko treatments accumulated significantly more target DNA compared to NIV wt and NIV-ko (Fig. 4.2D). There were no significant differences in the concentration of 3ADON DNA between with a (backtransformed) grand mean of 3ADON DNA across all treatments of 0.001 pg ng^{-1}.

Characterisation of isolates by mycotoxin extraction and LCMS/MS

DON concentration was significantly affected by treatment (P < 0.001) with the greatest concentrations in DON-wt and DON-ko treatments, and significantly less in NIV-wt and NIV-ko (Fig. 4.3), and amounts that were lower than the limit of detection in mock-inoculated controls. The concentration of NIV was below the limit of detection in all treatments except for NIV-wt samples, which accumulated a mean concentration of 115.2 mg kg^{-1} (backtransformed).

Behavioural responses of Sitobion avenae to host volatiles

Alate aphids spent significantly less time in areas of the olfactometer treated with volatiles sampled from hosts inoculated with DON-wt (P < 0.001) and DON-ko (P = 0.003) isolates compared to control areas, showing a repellent response. In contrast, aphids were attracted to volatiles from hosts inoculated with the NIV-wt isolate as they spent significantly more time in areas treated with infected host volatiles compared to control areas, in two repeats of the experiment (P = 0.011; P = 0.010). Volatiles from both mock-inoculated hosts and hosts inoculated with the NIV-ko isolate had no significant influence on aphid behaviour and there was no difference in mean time spent in treated areas compared to solvent-only controls for either treatment (Fig. 4.4).

Chemical composition of host volatiles

A shortlist of commonly occurring chemicals were provisionally identified from chemical libraries and co-injected with a host volatile sample to confirm the identification by peak enhancement GC/MS. By this process 2-pentadecanone, 2-heptanone, 2-undecanone, nonanal and (-)α-cedrene were confirmed, and abundance data for this shortlist was collected. The mean chemical abundances across all time points differed significantly between treatments for 2-pentadecanone ($P < 0.001$), 2-undecanone ($P < 0.001$), nonanal ($P < 0.001$) and (-)α-cedrene ($P = 0.006$), but ocimene and 2-heptanone did not (Fig. 4.5). 2-pentadecanone was most abundant in the NIV-wt treatment (1.07 ng μl^{-1}) and least in the DON-wt treatment (0.22 ng μl^{-1}) with mock-inoculated controls containing an intermediate concentration (0.51 ng μl^{-1}). 2-undecanone was also greatest in the NIV-wt treatment (0.21 ng μl^{-1}), which along with the NIV-ko treatment (0.14 ng μl^{-1}) contained a significantly greater abundance than either the DON-wt (0.009 ng μl^{-1}) or DON-ko treatments (0.030 ng μl^{-1}). Nonanal abundances were low across all treatments, but the greatest abundance was in mock-inoculated samples (0.077 ng μl^{-1}), followed by DON-ko (0.034 ng μl^{-1}) and NIV-ko (0.038 ng μl^{-1}) treatments. Both NIV-wt (0.018 ng μl^{-1}) and DON-wt (0.009 ng μl^{-1}) treatments produced significantly lower nonanal abundances than controls. NIV-ko (0.036 ng μl^{-1}), NIV-wt (0.038 ng μl^{-1}) and mock-inoculated controls (0.025 ng μl^{-1}) had significantly greater abundances of (-)α-cedrene than the DON-wt (0.005 ng μl^{-1}) treatment, with DON-ko samples containing an intermediate concentration (0.013 ng μl^{-1}).

There were significant differences between treatments in the evolution of 2-pentadecanone over time in host volatiles sampled ($P < 0.001$) (Fig. 4.6). Over time, the abundance of 2-pentadecanone in all treatments peaked at 24 HAI then decreased, but this drop in abundance was greatest in mock inoculated controls and the DON-wt treatment. Three of the four inoculated treatments, DON-ko (0.255 ng μl^{-1}), NIV-wt (0.305 ng μl^{-1}) and NIV-ko (0.198 ng μl^{-1}), produced greater amounts of 2-pentadecanone after a week of infecting the host compared to mock-inoculated controls (0.017 ng μl^{-1}). The abundance of 2-pentadecanone at 7 DAI in the DON-wt treatment (0.031 ng μl^{-1}) was not different from controls. The abundances of all other chemicals identified by peak enhancement GC/MS were not significantly affected by treatment, but time was a significant factor in all cases ($P < 0.001$).

Discussion

Each of the *Fusarium* isolates in the study was characterised through analysis of DNA and mycotoxins. NIV-wt and NIV-ko mutants contained genetic sequences typical of NIV-producing isolates, and NIV-wt samples contained high levels of NIV but NIV-ko samples did not, thus confirming that this mutant had the expected difference in mycotoxin production. The DON-wt and DON-ko isolates were shown to possess genetic sequences of 15ADON-producers, however, both isolates produced DON during host infection demonstrating reversion of the knock-out trait in the DON-ko isolate or contamination of the DON-ko isolate during subculture. Thus, the DON-ko isolate did not have the expected difference in mycotoxin production and the study was not able to differentiate between effects of *F. graminearum* with and without DON-production. In contrast, differences between the NIV-wt and NIV-ko treatments could be attributed to the presence/absence of NIV during infection. Confirmation of isolate character was corroborated with visual disease assessment of infected plants. The highest disease severity was observed in toxin-producing treatments, whilst bleaching was greatest in DON-producing treatments. Both DON and NIV were factors that promoted development of visible disease in host plants.

The most prominent finding of this work is the novel discovery that aphids were attracted to volatiles from hosts inoculated with the NIV-producer. This is in contrast to host volatiles from the mycotoxin deficient mutant of the same isolate to which aphids were neither attracted nor repelled. Therefore attraction can be linked to NIV production by the isolate. Furthermore, the attractiveness of the NIV-producer's volatiles is also in contrast to the repellent nature of volatiles emitted by hosts infected with DON-producing isolates. This aphid behaviour is consistent with previous work where volatiles from hosts infected with a composite inoculum of three DON-producing *F. graminearum* isolates were also shown to be repellent to aphids (Drakulic *et al.*, 2015). The different effects of each mycotoxin on the host semiochemical output are detected by the aphids, and differential behavioural adaptations towards them have evolved.

It has previously been shown that aphid mortality increases on hosts infected with DON-producing *F. graminearum* (Drakulic *et al*., 2015) and as such, that the behavioural response to DON-producer infected host volatiles is one of avoidance this behaviour is advantageous for aphid survival by evading an inhospitable environment. Whether aphid mortality would be affected on hosts infected with NIV-producing isolates is not known, and so we cannot conclude whether the aphids are being manipulated by the pathogen to be recruited to a poor host environment or whether the host environment is in fact not disadvantageous to the aphids and therefore avoidance would not be beneficial. Some *Fusarium* species are considered to be entomopathogenic (Gagkaeva *et al*., 2014), and the reactions of meal beetle larvae towards volatiles from wheat grain infected with different species followed a trend of avoidance of volatiles from entomopathogenic species and attraction or neutral response to non-entomopathogenic species, with the exception of *F. culmorum* (Guo *et al*., 2014). Despite negatively impacting on meal beetle larvae populations, the insects were attracted to the volatiles from *F. culmorum* infected grain, highlighting that despite their attraction the aphids tested here may still be negatively impacted on hosts infected with NIV-wt *F. graminearum*. The mechanism behind the generation of different host volatiles when infected by DON- and NIV-producing isolates of *F. graminearum* is also not known, although differences in phytotoxicity have been measured for DON and NIV, with DON being considered more phytotoxic (Eudes *et al*., 2000) and leads to enhanced bleaching of ears. Determining the mechanisms by which phytotoxicity differs would provide insight into the potential causes for the changes to host semiochemical production.

The aphid-repellent host volatiles from DON-producing *F. graminearum* infection were determined previously to be a combination of six chemicals including 2-pentadecanone, 2-heptanone, 2-tridecanone, (-)α-cedrene, α-gurjunene, and phenyl acetic acid (Drakulic *et al*., 2015). However, the chemical abundances of 2-heptanone and (-)α-cedrene did not differ significantly between treatments or over time for single treatments in the present study. Neither did these chemicals, or any others measured, differ in abundance between host volatiles from NIV-wt and NIV-ko infections. While these chemicals appear to be important as part of a blend of chemicals that elicit avoidance in aphids, their abundances alone are not linked to aphid behaviour, or the mycotoxins present in the infected host. The effect of chemical composition in hosts infected with and without accompanying NIV accumulation remains unknown. Aphids have been shown to be capable of detecting chemicals at concentrations lower than the limit of detection by GC/MS (Bruce *et al*., 2008). Further electroantennography work should be performed to identify components of the volatile samples that aphids are able to detect and accompanying behavioural assays are required to determine the attractive chemistry. Chemical abundances may be too simple a measure of identifying the subtle differences in chemical blends that combine to produce volatile signatures that are interpreted as being attractive to aphids. The abundances of 5 out of the 6 aphid-detectable chemicals from DON-producing *F. graminearum* infected hosts identified by Drakulic *et al.* (2015) were less than 1 ng μl^{-1}. Given that chemicals of very low abundance contribute to changes in behavioural responses by aphids, it can be appreciated why identification of chemicals from abundance data alone failed to produce suitable candidates for further investigation. The previous work also showed that several chemicals combined produced a repellent response, but components of the blend did not elicit a repellent response in aphids, and one, α-gurjunene was slightly attractive (Drakulic *et al*., 2015). A change in the proportion of α-gurjunene could be measured to reveal if this attractive component is increased in NIV-wt infected host volatiles.

While the present study found *F. graminearum* infection by DON-producing isolates to not consistently produce elevated 2-pentadecanone levels, as was observed previously (Drakulic *et al*., 2015), three of the four pathogen treatments did produce elevated levels of 2-pentadecanone at 7 DAI compared to mock-inoculated controls. We speculate therefore that elevated 2-pentadecanone is unlikely to be affected by mycotoxin output by pathogens, but is more likely to be elevated as a consequence of toxin-independent pathogenesis of the host. The development of symptoms in infected hosts was rapid during headspace sampling, and the continued entrainment of inoculated ears maintained high atmospheric moisture levels post-anthesis, which favour pathogen development (Cowger *et al*., 2009). Therefore the evolution of host volatiles may not be representative of the time scale within which chemical evolution would occur following infection in the field. It is possible that due to the accelerated infection process in this experiment, the DON-wt treatment, which also incurred the highest level of disease severity and bleaching at 7 DAI, had ceased to emit the volatiles expected during pathogenesis. In extension of the earlier discussion regarding the nature of aphid olfaction, blends of chemicals may be of a greater importance than the crude abundance of any one component. 2-pentadecanone levels were lower at 7DAI in the DON-wt compared to the NIV-ko treatment, despite the DON-wt VOCs eliciting a repellent response to aphids and the NIV-ko VOCs having a neutral effect. Despite the lower representation of 2-pentadecanone, which is itself repellent to aphids, additional chemicals present in the DON-wt are likely be responsible for maintaining the repellent effect on aphid behaviour that are not present in the NIV-ko VOCs.

In summary, this work provides evidence of differential behaviour of aphids towards wheat plants with FHB caused by pathogens with differing mycotoxin production. This shows that mycotoxins can impact upon insect-interactions with infected hosts, achieved through subtle changes to host volatile chemistry which remain undefined. If this effect still occurs in the context of a field environment and volatile background, the expected outcomes of aphid infestation around the time of FHB infection could have differing consequences in geographical regions with different prevailing chemotypes of *F. graminearum* populations.

Acknowledgements

This work was supported by a Lawes' Trust PhD studentship. Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council (BBSRC).

References

Anon. 2006. Commission Regulation (EC) No 1881/2006 setting maximum levels of certain contaminants in foodstuffs. *Official Journal of the European Union* L365, 5-24.

Alexander NJ, Mccormick SP, Waalwijk C, Van Der Lee T, Proctor RH, 2011. The genetic basis for 3-ADON and 15-ADON trichothecene chemotypes in Fusarium. *Fungal Genetics and Biology* **48**, 485-95.

Bruce TJ, Matthes MC, Chamberlain K, Woodcock C, Mohib A, Webster B, Smart LE, Birkett MA, Pickett JA, 2008. cis-Jasmone induces Arabidopsis genes that affect the chemical ecology of multitrophic interactions with aphids and their parasitoids. *Proceedings of the National Academy of Sciences* **105**, 4553-8.

Carter JP, Rezanoor HN, Holden D, Desjardins AE, Plattner RD, Nicholson P, 2002. Variation in pathogenicity associated with the genetic diversity of *Fusarium graminearum*. *European Journal of Plant Pathology* **108**, 573-83.

D'Mello JPF, Placinta CM, Macdonald AMC, 1999. *Fusarium* mycotoxins: a review of global implications for animal health, welfare and productivity. *Animal Feed Science and Technology* **80**, 183-205.

Drakulic J, Bruce TJA, Ray RV, 2016. Direct and host-mediated interactions between *Fusarium* pathogens and herbivorous arthropods in cereals. *Plant Pathology* **DOI:** 10.1111/ppa.12546.

Drakulic J, Caulfield J, Woodcock C, Jones SPT, Linforth R, Bruce TJA, Ray RV, 2015. Sharing a host plant (Wheat *Triticum aestivum*) increases the fitness of *Fusarium graminearum* and the severity of Fusarium head blight but reduces the fitness of grain aphids (*Sitobion avenae*). *Applied and Environmental Microbiology* **81**, 3492-501.

Edwards SG, Pirgozliev SR, Hare MC, Jenkinson P, 2001. Quantification of trichothecene-producing *Fusarium* species in harvested grain by competitive

PCR to determine efficacies of fungicides against Fusarium head blight of winter wheat. *Applied and Environmental Microbiology* **67**, 1575-80.

Eudes F, Comeau A, Rioux S, Collin J, 2000. Phytotoxicity of eight mycotoxins associated with *Fusarium* in wheat head blight. *Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie* **22**, 286-92.

Foroud NA, Mccormick SP, Macmillan T, Badea A, Kendra DF, Ellis BE, Eudes F, 2012. Greenhouse studies reveal increased aggressiveness of emergent Canadian *Fusarium graminearum* chemotypes in wheat. *Plant Disease* **96**, 1271-9.

Gagkaeva TY, Shamshev IV, Gavrilova OP, Selitskaya OG, 2014. Biological relationships between *Fusarium* fungi and insects (review). *Sel'skokhozyaistvennaya Biologiya* **3**, 13-23.

Gosman N, Srinivasachary, Steed A, Chandler E, Thomsett M, Nicholson P, 2010. Evaluation of type I Fusarium head blight resistance of wheat using non-deoxynivalenol-producing fungi. *Plant Pathology* **59**:147-157.

Goswami RS, Kistler HC, 2004. Heading for disaster: *Fusarium graminearum* on cereal crops. *Molecular Plant Pathology* **5**, 515-25.

Guo ZQ, Doll K, Dastjerdi R, Karlovsky P, Dehne HW, Altincicek B, 2014. Effect of fungal colonization of wheat grains with *Fusarium* spp. on food choice, weight gain and mortality of meal beetle larvae (*Tenebrio molitor*). *Plos One* [http://journals.plos.org/] 10.1371/journal.pone.0100112.

Hohn TM, Plattner RD, 1989. Expression of the trichodiene synthase gene of *Fusarium-sporotrichioides* in *Escherichia-coli* results in sesquiterpene production. *Archives of Biochemistry and Biophysics* **275**, 92-7.

Horevaj P, Brown-Guedira G, Milus EA. 2012. Resistance in winter wheat lines to deoxynivalenol applied into florets at flowering stage and tolerance to phytotoxic effects on yield. *Plant Pathology* **61**:925-933.

Jansen C, Von Wettstein D, Schafer W, Kogel KH, Felk A, Maier FJ, 2005. Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. *Proceedings of the National Academy of Sciences* **102**, 16892-7.

Kimura M, Tokai T, O'Donnell K, Ward TJ, Fujimura M, Hamamoto H, Shibata T, Yamaguchi I, 2003. The trichothecene biosynthesis gene cluster of *Fusarium graminearum* F15 contains a limited number of essential pathway genes and expressed non-essential genes. *Febs Letters* **539**, 105-10.

Lee T, Han YK, Kim KH, Yun SH, Lee YW, 2002. *Tri13* and *Tri7* determine deoxynivalenol- and nivalenol-producing chemotypes of *Gibberella zeae*. *Applied and Environmental Microbiology* **68**, 2148-54.

Maier FJ, Miedaner T, Hadeler B, Felk A, Salomon S, Lemmens M, Kassner H, Schaefer W, 2006. Involvement of trichothecenes in fusarioses of wheat, barley and maize evaluated by gene disruption of the trichodiene synthase (*Tri5*) gene in three field isolates of different chemotype and virulence. *Molecular Plant Pathology* **7**, 449-61.

Maier FJ, Maiz S, Losch AP, Lacour T, Schafer W, 2005. Development of a highly efficient gene targeting system for *Fusarium graminearum* using the disruption of a polyketide synthase gene as a visible marker. *Fems Yeast Research* **5**, 653-62.

Marin S, Ramos AJ, Cano-Sancho G, Sanchis V, 2013. Mycotoxins: occurrence, toxicology, and exposure assessment. *Food and Chemical Toxicology* **60**, 218-37.

Nicholson P, Simpson DR, Weston G, Rezanoor HN, Lees AK, Parry DW, Joyce D, 1998. Detection and quantification of *Fusarium culmorum* and *Fusarium graminearum* in cereals using PCR assays. *Physiological and Molecular Plant Pathology* **53**, 17-37.

Nielsen LK, Jensen JD, Rodriguez A, Jorgensen LN, Justesen AF, 2012. *TRI12* based quantitative real-time PCR assays reveal the distribution of trichothecene genotypes of *F. graminearum* and *F. culmorum* isolates in Danish small grain cereals. *International Journal of Food Microbiology* **157**, 384-92.

Parry DW, Jenkinson P, Mcleod L, 1995. Fusarium ear blight (scab) in small-grain cereals - a review. *Plant Pathology* **44**, 207-38.

Pickett JA, 1990. Gas chromatography-mass spectrometry in insect pheromone identification three extreme case histories. *In* Mccaffery, A. R. and I. D. Wilson (Ed.). Chromatography and Isolation of Insect Hormones and Pheromones; Plenum Press: New York, USA.

Suzuki T, Iwahashi Y, 2014. Phytotoxicity Evaluation of Type B trichothecenes using a *Chlamydomonas reinhardtii* model system. *Toxins* **6**, 453-63.

Van der Lee T, Zhang H, van Diepeningen A, Waalwijk C, 2015. Biogeography of *Fusarium graminearum* species complex and chemotypes: a review. *Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment* **32**,453-460.

Waalwijk C, Kastelein P, De Vries I, Kerenyi Z, van der Lee T, Hesselink T, Kohl J, Kema G, 2003. Major changes in *Fusarium* spp. in wheat in the Netherlands. *European Journal of Plant Pathology* **109**, 743-54.

Ward TJ, Clear RM, Rooney AP, O'Donnell K, Gaba D, Patrick S, Starkey DE, Gilbert J, Geiser DM, Nowicki TW, 2008. An adaptive evolutionary shift in Fusarium head blight pathogen populations is driving the rapid spread of more toxigenic *Fusarium graminearum* in North America. *Fungal Genetics and Biology* **45**, 473-84.

Xu XM, Parry DW, Nicholson P, Thomsett MA, Simpson D, Edwards SG, Cooke BM, Doohan FM, Brennan JM, Moretti A, Tocco G, Mule G, Hornok L, Giczey G, Tatnell J, 2005. Predominance and association of pathogenic fungi causing Fusarium ear blight in wheat in four European countries. *European Journal of Plant Pathology* **112**, 143-54.

Zadoks JC, Chang TT, Konzak CF, 1974. Decimal code for growth stages of cereals. *Weed Research* **14**, 415-21.

Figures

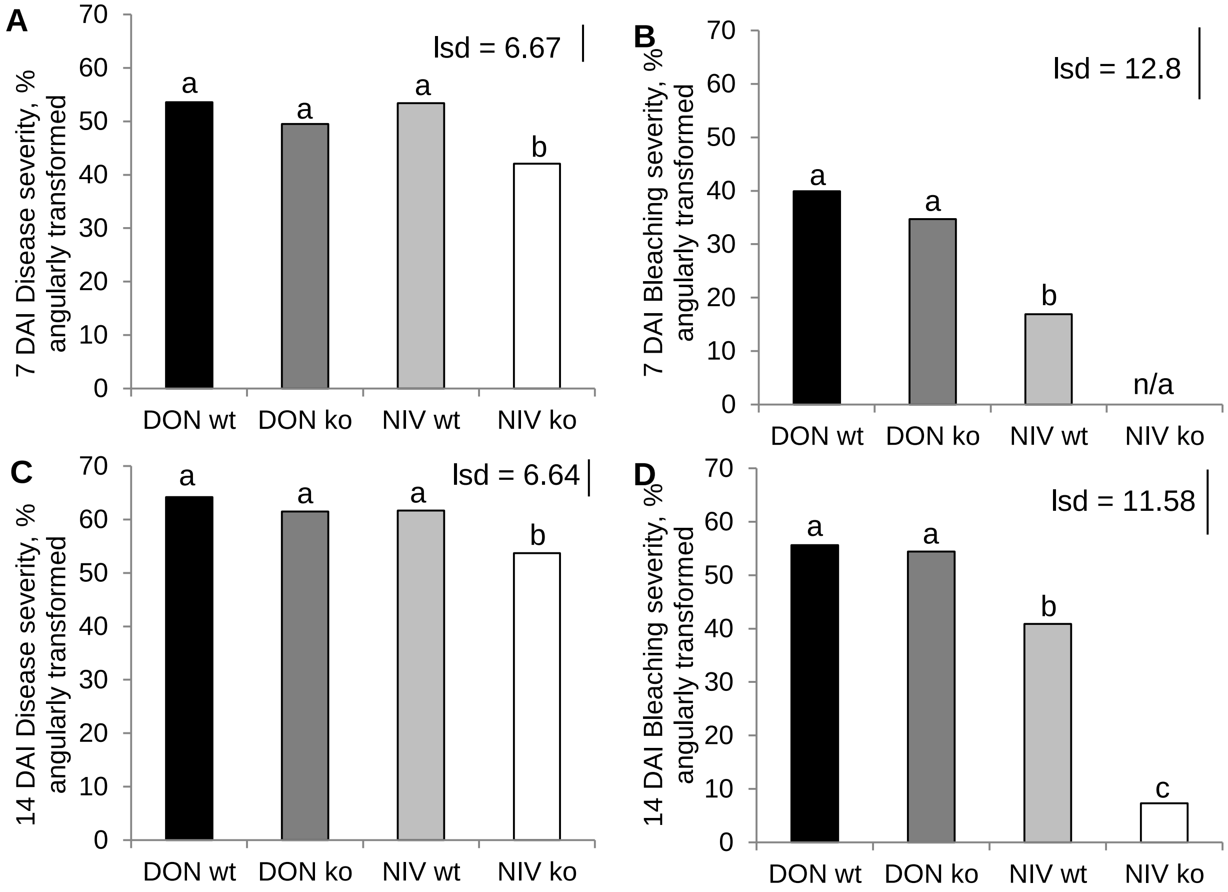

Fig. 4.1. Severity at 7 DAI of (A) disease (P = 0.003) and (B) bleaching (P = 0.012); and severity at 14 DAI of (C) disease (P = 0.002) and (D) bleaching (P < 0.001). All data were angularly transformed. DON-wt: DON-producer; DON-ko: tri5 knock out transformant of DON-wt; NIV-wt: NIV-producer; NIV-ko: tri5 knock out transformant of NIV-wt. Data marked with different letters are significantly different (lsd; P = 0.05), data marked n/a were not included in analysis.

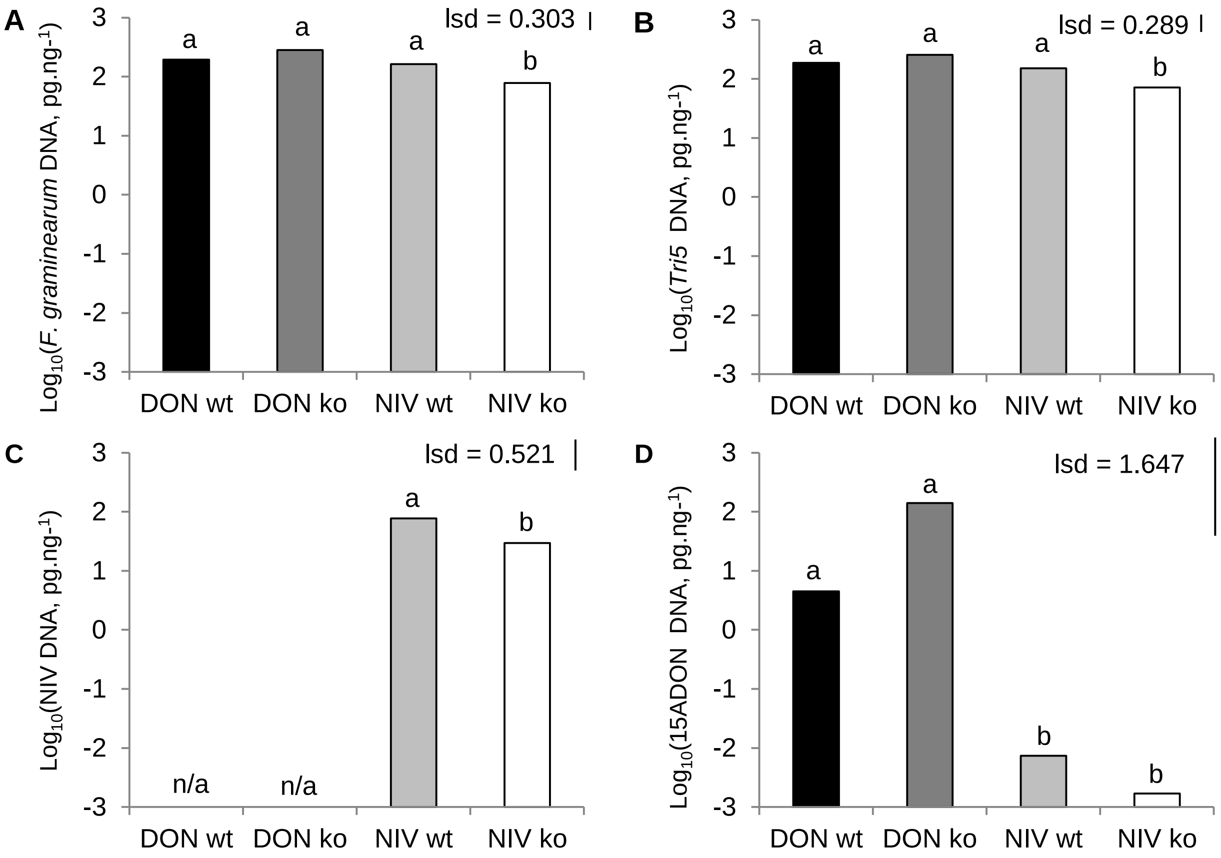

Figure 4.2. Results of QPCR with primers specific for (A) *F. graminearum* (P = 0.008), (B) *Tri5* (P = 0.005) (C) NIV chemotype (P = 0.096), and (D) 15ADON chemotype (P < 0.001). Data log10 transformed. DON-wt: DON-producer; DON-ko: tri5 knock out transformant of DON-wt; NIV-wt: NIV-producer; NIV-ko: tri5 knock out transformant of NIV-wt. Data marked with different letters are significantly different (lsd; P = 0.05), data marked n/a were not included in analysis.

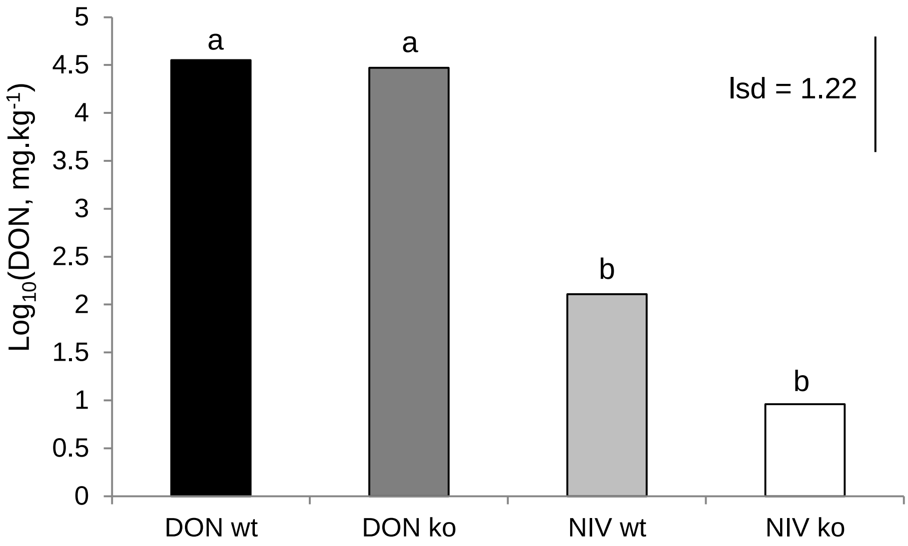

Fig. 4.3. Concentration of DON (log transformed) in ears treated with *F. graminearum* of different chemotypes. DON-wt: DON-producer; DON-ko: *tri5* knock out transformant of DON-wt; NIV-wt: NIV-producer; NIV-ko: *tri5* knock out transformant of NIV-wt. Data marked with different letters are significantly different (lsd; P = 0.05).

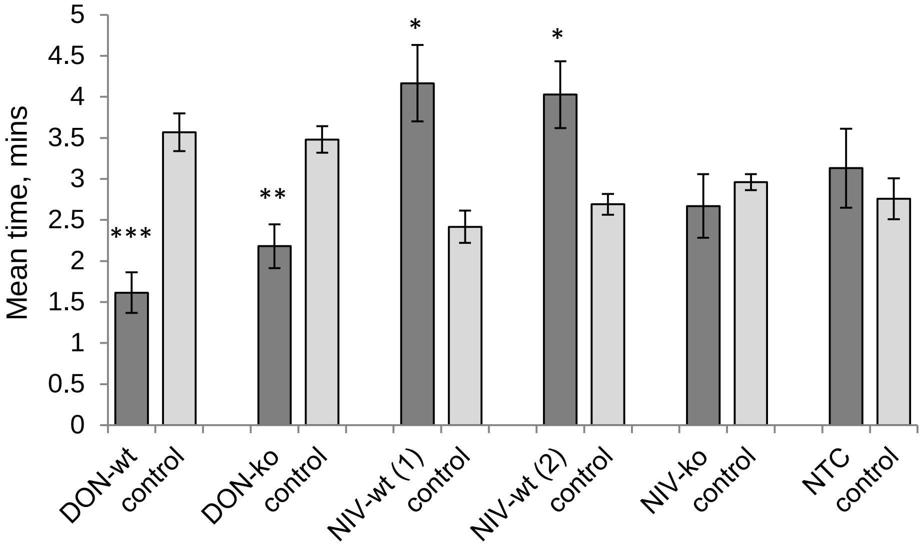

Fig. 4.4. Behavioural responses of *Sitobion avenae* to volatile chemicals sampled from *Fusarium*-infected hosts. *** $P < 0.001$, ** $0.001 \leq P < 0.01$, * $0.01 \leq P < 0.05$.

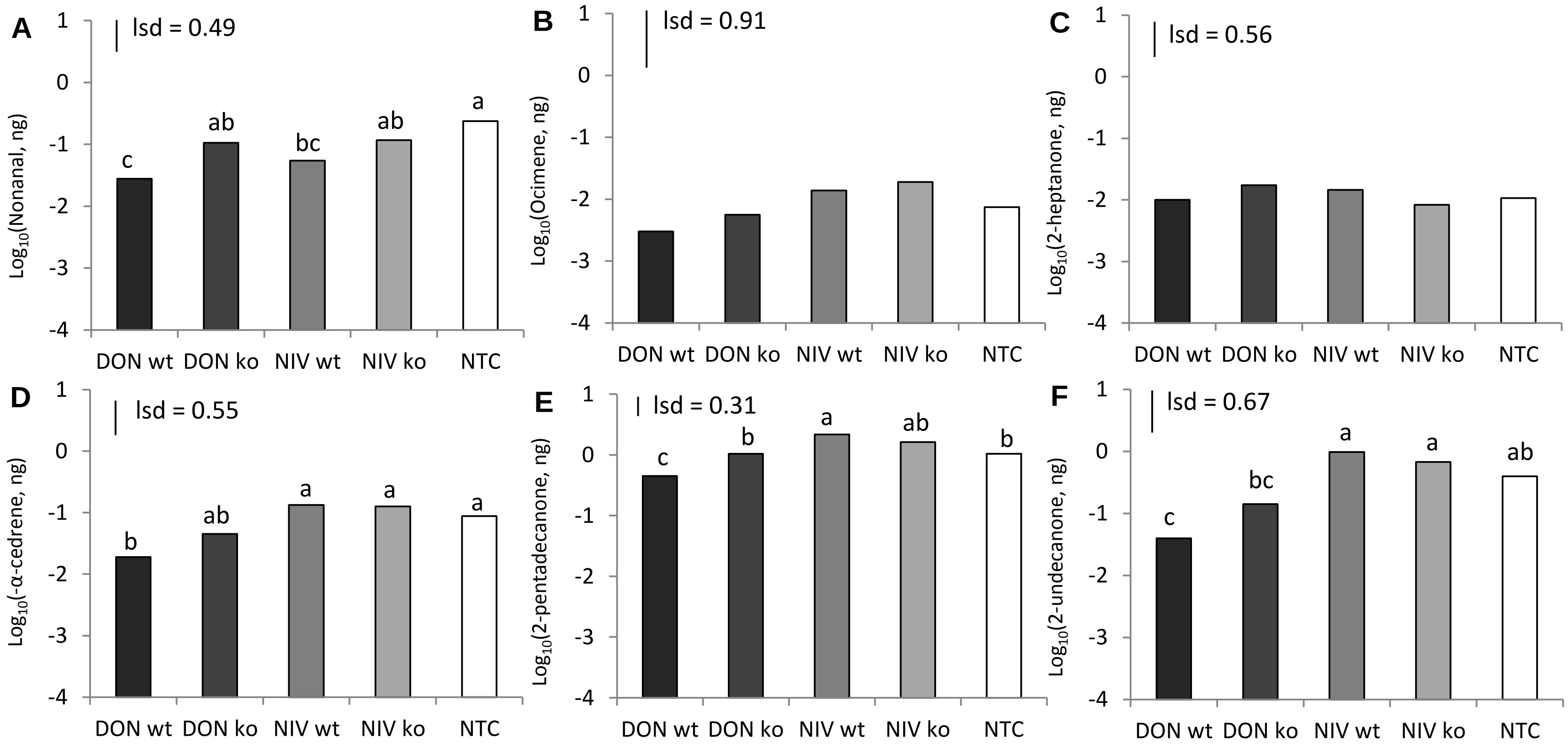

Fig. 4.5. Mean abundances of volatile chemicals, $\log_{10}$ transformed, across all time points. (A) Nonanal (P = 0.004), (B) ocimene (ns) (C) 2-heptanone (ns), (D) α-cedrene (P = 0.015), (E) 2-pentadecanone (P < 0.001), (F) 2-undecanone (P < 0.001). Data marked with different letters are significantly different.

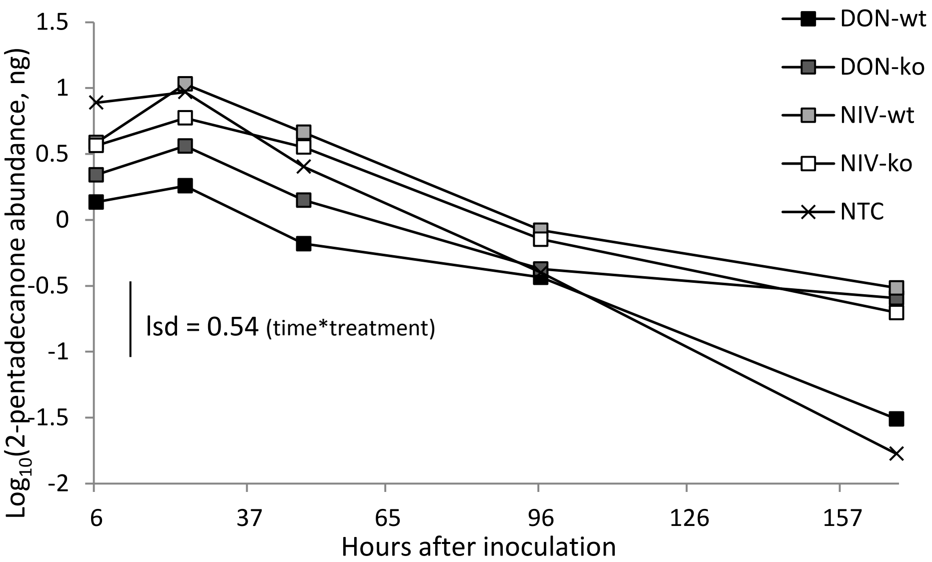

Fig. 4.6. Change in abundance of 2-pentadecanone over time. Treatment was significant (P = 0.019) as was the interaction between treatment and time (P < 0.001). DON-wt: DON-producer; DON-ko: tri5 knock out transformant of DON-wt; NIV-wt: NIV-producer; NIV-ko: tri5 knock out transformant of NIV-wt; NTC: non-treated control.

Tables

Table 4.1: Primers and programs used in QPCR confirmation of treatment characters.

Assay name	*Target*	*Primer sequences (5'-3')*	*Thermocycling programme*	*Product size (bp)*	*Reference*
F. graminearum	ITS	Fg16NF: ACAGATGACAAGATT CAGGCACA Fg16NR: TTCTTTGACATCTGTTCAACCCA	95°C for 90s; 35 cycles of 94°C for 30s, 64°C for 45s, 72°C for 45s; 72°C for 5 mins	280	Nicolson *et al*., 1998
NIV-producers	Tri12	NIV/f: GCCCATATTCGCGACAATGT NIV/r: GGCGAACTGATGAGTAACAAAACC	50°C for 2 mins; 95°C 10 mins; 40 cycles of 95°C for 15s, 60°C for 1 min	77	Nielsen *et al*., 2012
15ADON-producers	Tri12	15ADONfwd: GTTTCGATATTCATTGGAAAGCTAC 15ADONrev: CAAATAAGTATCGTCTGAAATTGGAAA	"	60	Nielsen *et al*., 2012
3ADON-producers	Tri12	3ADONf: AACATGATCGGTGAGGTATCGA 3ADONr: CCATGGCGCTGGGAGTT	"	57	Nielsen *et al*., 2012
Trichothecene producers	Tri5	HATri/F: CAGATGGAGAACTGGATGGT HATri/R: GCACAAGTGCCACGTGAC	94°C for 75s: 35 cycles of 94°C for 15 s, 62°C for 15 s, and 72°C for 45 s; 72°C for 4 min 15 s	260	Edwards *et al*., 2001

Chapter 5: The effect of aphid herbivory on host resistance to Fusarium Head Blight disease in wheat

In this chapter experimental work is presented which explores the relationship between aphid herbivory and disease progression of *F. graminearum,* with a comparison between cultivars of wheat which are each susceptible and resistant to FHB. The work is presented as a paper in preparation, in 'paper format'.

Author contribution

This paper was composed and experiments designed by J. Drakulic. Practical work was conducted jointly by B. Ó Loinsigh and J. Drakulic. The work was supervised by R. V. Ray and T. Bruce.

RESEARCH ARTICLE

The effect of aphid herbivory on host resistance to Fusarium Head Blight disease in wheat

Running title: Aphids on wheat ears weaken resistance to FHB

Jassy Drakulic[1], Brian Ó Loinsigh[1], Toby Bruce[2], Rumiana V. Ray[1]

[1] University of Nottingham, Sutton Bonington Campus, College Rd, Sutton Bonington, LE12 5RD, UK

[2] Rothamsted Research, Harpenden, Herts AL5 2JQ, UK

Abstract

Fusarium head blight (FHB) of wheat can be more severe on plants with current or previous aphid infestation than on hosts without exposure to aphid herbivory. Glasshouse studies were performed to explore the mechanisms responsible for this heightened host susceptibility to disease following aphid herbivory. Aphids were applied either to the flag leaf or the ear of wheat plants and disease progress on these was compared to aphid-free controls. Inoculation with *F. graminearum* followed one week of aphid infestation. Furthermore, responses of the cultivars 'Gallant' and 'Sumai 3', which are respectively susceptible and resistant to FHB, were compared. 'Gallant' treatments had both higher disease severity and fungal biomass than those of 'Sumai 3'. Ear infestation increased fungal biomass 2.7-fold in Gallant and 3.8-fold in Sumai 3 plants compared to aphid-free controls. Leaf infestation led to increased fungal biomass over controls to a lesser extent, by a factor of 1.7 for Gallant and 2.2 for Sumai 3. Ear infestation led to the highest disease severity and fastest disease progression; leaf infestation only caused a small rise in symptom development in both varieties. This shows that aphid infestation of wheat ears poses the greatest threat in exacerbating FHB severity, and that even the resistant cultivar, Sumai 3, can be compromised by ear infestation. The findings also indicate a need for aphid monitoring of wheat ears around heading to prevent FHB disease and for resistance breeding against FHB to consider the robustness of traits against insect attack.

Introduction

Severity and speed of symptom development of Fusarium head blight (FHB) of wheat has been shown previously to be enhanced with coincidental aphid infestation (Drakulic *et al*., 2015). However, the mechanism unperpinning this effect remains obscure. Honeydew produced by aphids feeding on the host is deposited on the surface of host tissues, containing sugars and amino acids that could be of benefit to colonising fungi (Fokkema *et al*., 1983). Sooty moulds have been shown to be able to grow on honeydew deposits (Dhami *et al*., 2013), but the capacity for honeydew to improve the growth and colonisation of *Fusarium* spp. has not been previously studied. Another hypothesis for the mechanism behind the rise in FHB severity when aphids also infest the shared host is that the aphids are able to alter the host plant defence response to the fungus. It is known that multiple attackers can alter plant defence responses (Bruce and Pickett, 2007) and aphid suppression of defence could benefit the fungus (Naessens *et al*., 2015). Host defence against aphid herbivory is not mediated wholly or by either salicylic acid (SA) or jasmonic acid (JA) defence pathways, and the contributions of each is a matter of debate (Ferry *et al*., 2011; Cao *et al*., 2014). Likewise, host defences against FHB have been attributed to SA- (Makandar *et al*., 2012), ethylene- dependent pathways (Chen *et al*., 2008) and the resistance of the cultivar 'Sumai 3' has been suggested to be mediated by both JA- and ethylene-dependent pathways (Li & Yen, 2008). While SA and JA are considered to act as antagonists, it remains unclear whether any such antagonism would be responsible for heightened disease impact in hosts with aphid herbivory. Protein effectors are secreted by aphids which can act as transcription factors (Bos *et al*., 2010). A recent study has shown that an aphid-secreted protein is responsible for impairing host innate defence responses, which may have an indirect effect of increasing host susceptibility to pathogen attack (Naessens *et al*., 2015). Heightened disease severity following aphid herbivory could be due to a combination of both systemic changes to host defences and local effects of honeydew deposits, damage to host tissues or release of nutrients.

The previous work which revealed the increased level of FHB symptom induction and pathogen biomass accumulation in plants with both pathogen infection and aphid infestation only studied the effect in a single variety of wheat: ‘Gallant’ (Drakulic *et al*., 2015). ‘Gallant’ is listed as being susceptible to FHB in the HGCA recommended list (rating 5), but other varieties exist which are known to be resistant to FHB including ‘Sumai 3’ and lines bred using this germplasm. Cultivar ‘Sumai 3’ resists the spread of infection by FHB pathogens to spikelets within the infected ear (Type II resistance) (Schroeder & Christensen, 1963) and as such the disease remains localised to points of primary infection. Glasshouse trials using point inoculation have shown that following inoculation of a single spikelet, ‘Sumai 3’ consistently limits the infection to that spikelet only (Bai & Shaner, 1996), and in field trials incurred a total disease severity of around 10 - 20%, compared to 35 - 45% in the susceptible cultivar ‘Emblem’ (Yoshida & Nakajima, 2010). Whether aphid herbivory can overcome the genetic resistance to spread of ‘Sumai 3’ has not been previously investigated, and so a comparison between the effect of aphids on the susceptible cultivar ‘Gallant’ and the resistant cultivar ‘Sumai 3’ was conducted in this study.

Resistance by the prevention of spread of the pathogen to other parts of the host has been referred to as Type II, with Type I referring to the prevention of initial colonisation of the host. Quantitative trait loci for ‘Sumai 3’’s Type II resistance have been identified on the short arm of chromosome 3B (Buerstmayr *et al*., 2003; Anderson *et al*., 2001; Bai *et al*., 2000; Waldron *et al*., 1999) and also on chromosome 5A (Buerstmayr *et al.*, 2003) and chromosome 6B (Anderson *et al*., 2001). The molecular mechanisms behind the increased resistance to spread of FHB in ‘Sumai 3’ are the subject of ongoing study, but so far results have been obtained to show that PR proteins from ‘Sumai 3’ conferred Type II resistance to transformed wheat seedlings in glasshouse studies, but not in field trials (Anand *et al*., 2003). Microscopy experiments using immunogold labelling revealed that upon *Fusarium graminearum* infection, ‘Sumai 3’ launches elevated cell wall reinforcement and chemical defences, shown by increased production of the hydrolases beta-1,3-glucanase and chitinase upon infection compared to the susceptible wheat variety Xiaoyan 22, and that lignin, thionins and hydroxyproline-rich glycoproteins increased in density on the cell walls (Kang *et al*., 2008). As a result of upregulation of defence responses, DON accumulation is reduced in 'Sumai 3', with 2.05 $mg.kg^{-1}$ compared to 30-105 $mg.kg^{-1}$ in susceptible cultivars (Mesterhazy, 2005), and a lower density of hyphae can be observed in infected spikelets (Kang *et al*., 2008). In this work it was hypothesised that if systemic changes from aphid herbivory lead to suppression of host defences, the genetic resistance of 'Sumai 3', which involves the upregulation of defensive compounds, could be affected by aphid herbivory.

The current study investigated whether aphid facilitation of disease spread was a local or systemic phenomenon and looked at the effect in a FHB resistant and susceptible wheat cultivar. To separate the influence of local and systemic herbivory on FHB disease, and to isolate the effects of honeydew and exclude the effect of aphid-enhanced dispersal about the host ear, aphids were added to host plants either locally at the site of infection (on ears and trapped there in netting) or distally to infected tissues (on the flag leaf confined in leaf clips) prior to *F. graminearum* inoculation and compared to aphid-free controls. Disease progression was observed alongside estimation of fungal biomass at maturity via quantification of *F. graminearum* DNA using QPCR. Experiments were conducted with 'Gallant' and 'Sumai 3' to explore these effects in two different host plant genotypes, and specifically whether the resistance to spread of 'Sumai 3' was robust against aphid herbivory.

Materials & Methods

Plant material

Seeds of winter wheat (*Triticum aestivum*) *cv.* 'Gallant' and spring wheat *cv.* 'Sumai 3' were used. 'Gallant' seeds were treated with Redigo Deter (10 g prothioconazole and 50 g clothianidin per 100 kg seed; Bayer) and sown in compost (Levington's F2+S) individually into module trays (2 cm x 2 cm x 5 cm, l x w x d, per module) and allowed to germinate in a glasshouse at minimum 15, maximum 18°C daytime temperature and minimum 12, maximum 15°C night time temperature for two weeks under a 16 h photoperiod. Seedlings were transferred to a polytunnel and kept at 4°C for 6-8 weeks for vernalisation. 'Sumai 3' seeds were sown at the same time as 'Gallant' seedlings were potted on, with one seed or seedling being planted directly into compost (John Innes Type 2) in individual 5 L pots, and fed by automatic irrigation in the glasshouse conditions described above until ear emergence.

F. graminearum *inoculum*

Isolates of *F. graminearum* were obtained from single spores cultures from the University of Nottingham isolate collection, (#212, 214 and 216). Isolates were grown on PDA for 7-10 days then on synthetic nutrient deficient agar (SNA) for a further 14 days to initiate spore production. SNA cultures were then washed with 1 ml sterile distilled water (SDW), agitated with a sterile L-shaped spreader and harvested by pipette. The concentration of harvested spores was calculated using a haemocytometer (Neubauer) and adjusted to 250,000 spores. ml^{-1} and each isolate combined in equal proportions to make a composite inoculum.

Aphid rearing

English grain aphids, *Sitobion avenae*, originally from colonies maintained at Rothamsted Research (Harpenden, UK) were used to initiate colonies within rearing cages at the University of Nottingham. Rearing cages were maintained in the glasshouse conditions described above, and aphids were reared on several winter wheat *cv.* 'Gallant' seedlings grown from unvernalised surface sterilised seeds grown in 5l pots and caged within a metal framed net cage that attached directly to the pot (Insectopia (TM), Watkins & Doncaster, Bolton, UK).

Treatment structure

The experiment was performed as a factorial design in randomised blocks. Two different varieties of wheat were used: 'Gallant' (susceptible to FHB) and 'Sumai 3' (Type II resistant to FHB i.e. resists spread throughout ear). The two varieties were either inoculated or mock inoculated. Inoculation was performed on two tillers per plant by point inoculation at the 10^{th} spikelet, as counted from the bottom up, with 25 µl of either spore suspension or SDW for mock inoculated controls. Following inoculation or mock inoculation, ears were bagged in perforated plastic bags for 48h. Inoculation was applied at mid-anthesis (GS65) (Zadoks *et al*., 1974). Three aphid treatments were used: (1) Treatment 'N', aphid-free controls; (2) Treatment 'L', aphids applied to the flag leaf for 7d prior to inoculation (systemic treatment), (3) Treatment 'E', aphids applied to ears for a period of 7d before inoculum was applied (local treatment). Adult aphids (n = 10) were added to leaves using a brush and trapped on the adaxial side of the leaf using leaf clips (inner radius = 2 cm). Aphids applied to ears (n = 10) were of similar age and trapped on the ears using netting. Seven days after aphid application, leaf clips and nets were removed and all plants were sprayed using 500 g/kg (50% w/w) pirimicarb (Aphox, Syngenta). The full treatment structure is detailed in full in Table 5.1. Visual disease assessment was performed on 5 intervals: 5 days after inoculation (DAI), 10, 16, 21 and 28 DAI. The number of visible symptoms (either dark lesions or bleached spikelets) that were observed on ears was recorded as the number of affected spikelets, which was divided by the total number of spikelets per ear to calculate the disease severity (%) and from this data the area under the disease progress curve (AUDPC) was also calculated.

Estimation of fungal biomass by QPCR

Ears were harvested at maturity, freeze dried whole and milled without threshing using a centrifugal mill (ZM 200; Retsch GmbH, Germany) with a sieve size of 1 mm. DNA was extracted from flour samples (0.5 g) which were firstly added to CTAB buffer (3.75 ml), heated in a water bath for 2 h, chilled on ice, then added to potassium acetate (1.25 ml). Tubes were mixed by inversion, centrifuged at 2000 xg for 15 mins and 1.2 ml supernatant removed and added to 600 µl chloroform (100%). The rest of the method proceeded as previously described (Edwards *et al*., 2001), and the final DNA pellets were resuspended using 200 µl TE buffer in a hot block at 65°C for 2 h. Quantification of DNA concentration was performed by measuring the absorbance of light at wavelength 260, 280 and 328 nm using a Cary 50 Probe UV-visible spectrophotometer (Varian, CA, USA). DNA stocks were diluted to 20 ng. µl^{-1} and used in QPCR assays with *F. graminearum* specific primers

(Fg16NF 5' - ACA GAT GAC AAG ATT CAG GCA CA -3'; Fg16NR 5'- TTC TTT GAC ATC TGT TCA ACC CA – 3') using a thermocycling program of: 90 s at 95°C; 35 cycles of 30 s at 94°C, 45 s at 64°C, 45 s at 72°C; and 5 min at 72°C which produced a 280 bp product (Nicholson *et al*., 1998). Quantification of pathogen DNA concentration as a proportion of DNA in the flour extracts was performed by quantitative real-time PCR (QPCR) using a total volume of 13 µl comprising 2.5 µl sample DNA, SYBR green 2X mastermix (Bio-Rad, USA) and 250 nM *F. graminearum* primers. DNA extracted from a pure culture of isolate #216 from the University of Nottingham isolate collection was used to prepare a standard curve of ten-fold dilutions ranging from 1 to 1 x 10^{-6} ng. μl^{-1}, and linear regression was used to calculate the starting quantity of target DNA in each sample from the standard curve. The limit of detection of the assay was 1 x 10^{-5} ng $\mu l^{-1.}$

Statistical analyses

All analyses were performed in Genstat v.15.2 (VSN International, United Kingdom). Repeated measurements analysis of variance (ANOVA) was used to analyse the visual severity data for the disease progress curves. AUDPC, final disease severity and fungal biomass as estimated by the concentration of pathogen DNA were analysed by ANOVA. Treatment means were compared using ad hoc testing with the lsd value at the 5% significance level. Analyses were carried out on the full treatment structure except in cases where uninoculated controls showed no visual signs of disease, when these treatments were excluded from the analysis.

Results

Visual disease severity

Overall mean disease severity and AUDPC were 49.8% and 579, respectively, for inoculated plants. Mock-inoculated plants showed no signs of diseas, so were excluded from analyses and inoculated treatments were directly compared. Aphid treatment and variety were both significant factors accounting for variation in disease progress (P = 0.025; P < 0.001) (Fig. 5.1) and AUDPC (P = 0.045; P < 0.001) (Fig. 5.2) although the interactions between the factors was not significant in either case. Final disease severity was significantly affected by variety ($P < 0.001$) but aphid treatment was only significant at the 10% level ($P = 0.074$), and the interaction between these factors was also not significant. 'Gallant' showed a greater mean final disease severity (71.3%) and AUDPC (839) than 'Sumai 3' (28.3%, 309). There was a consistent trend across varieties with the greatest AUDPC in the treatments with aphids on the ears (GIE: 1058; SIE: 400), followed by aphids on the leaves (GIL: 766; SIL: 279) then plants with no aphids (GIN: 693; SIN: 247). There were significant increases in the AUDPC between GIE compared to GIN, and in GIN compared to both SIN and SIL treatments when compared with the 5% lsd value. The final disease severity was also greatest for plants with aphids on the ears (GIE: 81.9%; SIE: 31.2%), but were followed by plants with no aphids (GIN: 67%; SIN: 28.8%) then plants with aphids on leaves (GIL: 65%; SIL: 24%).

F. graminearum *DNA quantification*

Inoculation ($P < 0.001$), variety ($P < 0.001$) and aphid treatment ($P = 0.028$) were all significant factors influencing the amount of *F. graminearum* DNA in samples, but there were no significant interactions. Of the inoculated treatments, mean pathogen DNA content in 'Gallant' treatments was greater than all the 'Sumai 3' treatments, which had overall means of 1.17 and 0.22 pg. ng^{-1} for 'Gallant' and 'Sumai 3' respectively.

All inoculated treatments amassed significantly more *F. graminearum* DNA in flour samples than their corresponding mock inoculated controls (Fig. 3.3) although there was some background contamination of the controls with *F. graminearum*, presumably due to transfer of small amounts of inoculum from inoculated plants in close proximity. A trend was observed which was consistent across both varieties tested. From the inoculated treatments of each variety, the greatest amount of *F. graminearum* DNA occurred in tillers with aphids added to the ears (GIE: 1.90; SIE: 0.41 pg. ng^{-1}), followed by tillers with aphids added to the flag leaves (GIL: 1.19; SIL: 0.24 pg. ng^{-1}) and then tillers with no aphids added had the lowest amount of pathogen DNA (GIN: 0.70; SIN: 0.11 pg. ng^{-1}). For uninoculated plants there was a significant increase in *F. graminearum* DNA when aphids were on the ear for 'Gallant' but not for 'Sumai 3'. Linear regression between DNA and final disease severity was significant ($P < 0.001$) and accounted for 34.1% of the variance.

Discussion

Increased FHB severity, pathogen DNA concentration and the mycotoxin DON in wheat plants, *cv*. 'Gallant', upon aphid herbivory of plants that were inoculated with *F. graminearum* has been reported previously (Drakulic *et al*., 2015). When the timing of the interaction between aphids and *F. graminearum* was examined, aphid infestation for 7 days prior to inoculation of the host led to the largest rise in pathogen DNA. The present study further supports the conclusions of the previous work, as aphid infestation led to the greatest disease progression and pathogen DNA concentration. The present study differs in the level of inoculum applied; in this case point inoculation of a single spikelet was performed in contrast to either spray inoculation of several tillers or point inoculation of 10 spikelets per spike as done previously. Inoculation of a single spikelet facilitates the study of pathogen spread throughout the spike, a trait which the Type II resistant variety, 'Sumai 3', is known to be resilient to (Bai & Shaner, 1996) and as such the capacity for aphid herbivory to impact on fungal spread could be observed. Another contrast occurred in the restriction of aphid feeding sites used in this study. In the previous work two methods were used: aphids were trapped on ears inside netting in one case, and in the second case aphids infested all parts of the host freely. In both cases, there could be no separation of the effect of aphid herbivory on systemic changes to host biochemistry and local effects such as direct damage due to competition for resources in phloem sap with growing tissues of the spike and enhancement of fungal colonisation due to honeydew deposits. Both of these differences could explain the lower concentration of pathogen DNA accumulated in inoculated hosts in the present study in comparison to the previous findings of Drakulic *et al*. (2015).

Previous infestation with aphids Inoculation raised pathogen DNA levels and disease progression in all treatments. Aphids on ears led to a greater increase in

AUDPC and *F. graminearum* DNA than in plants treated with aphids on leaves across both varieties tested. This indicates that direct damage to the host, honeydew deposits and local changes in host biochemistry promote infection during aphid herbivory. The treatments which included aphid herbivory on the flag leaf also lead to an increase in fungal biomass and disease severity compared to plants without any aphid treatment, which provides evidence that systemic changes in host defences are induced upon aphid feeding which can impact negatively on the host's resistance to FHB disease. The Type II resistance of 'Sumai 3' has been shown to be vulnerable to heightened disease pressure, as when several primary inoculations occur the resistance to spread becomes redundant (Anand, 2003; Bai & Shaner, 2004) That the trend of heightened susceptibility to disease upon aphid infestation was observed in both varieties shows that this effect is capable of reducing the efficacy of the innate Type II resistance of 'Sumai 3', and that this form of resistance is not robust when faced with mutual attack by aphids and FHB pathogens.

In mock inoculated plants, a low level of pathogen DNA was detected in all treatments. However, the amount of this background contamination that occurred in the GUE treatment, uninoculated 'Gallant' plants with aphids added to the ears, was significantly greater than in other uninoculated 'Gallant' treatments. This result corroborates the effect observed in inoculated plants: 'Gallant' plants which have incurred aphid herbivory on the ears are more likely to accumulate higher levels of pathogen colonisation in ear tissues. The adhesive nature of aphid honeydew is perhaps a factor that improved the uptake of inoculum from neighbouring inoculated ears in these control plants, and which would increase the likelihood of *F. graminearum* spore adhesion to wheat ears in the field during rainsplash or wind dispersal. Thus the impact of heightened FHB severity with concurrent aphid feeding on wheat could be due to both an increased chance of intercepting spores as well as increasing host susceptibility to disease due to internal molecular changes.

The mycotoxin deoxynivalenol (DON) is considered to be an important virulence factor facilitating the movement of the fungus past through the rachis and into neighbouring florets (Jansen *et al*., 2005) and is able to spread beyond the range of fungal growth and cause premature bleaching of tissues (Kang & Buchenauer, 1999). In susceptible wheat cultivars, strong correlations have been shown to occur between DON and both the disease severity and the fungal biomass in infected grain (Wegulo, 2012). Quantification of DON and other mycotoxins in the host tissue would reveal if the impact of local and/or distal aphid herbivory on hosts suppresses defences in such a way as to enhance mycotoxin accumulation, and would shed light onto the impact of systemic changes to host biochemistry and defences on mycotoxin accumulation.

Overall, this work suggests that for resistance breeding to be exploited successfully, the effect of insects on disease should be considered. To minimise fluctuations in FHB severity that occur, for instance with both differing weather patterns and insect pressures, resistance to aphids should also be incorporated, or an integrated approach combining pest control at ear emergence along with FHB resistance breeding should be used.

References

Anand A, Zhou T, Trick HN, Gill BS, Bockus WW, Muthukrishnan S. 2003. Greenhouse and field testing of transgenic wheat plants stably expressing genes for thaumatin-like protein, chitinase and glucanase against Fusarium graminearum. *Journal of Experimental Botany* **54:**1101-1111.

Anderson JA, Stack RW, Liu S, Waldron BL, Fjeld AD, Coyne C, Moreno-Sevilla B, Fetch JM, Song QJ, Cregan PB, Frohberg RC. 2001. DNA markers for Fusarium head blight resistance QTLs its two wheat populations. *Theoretical and Applied Genetics* **102:**1164-1168.

Bai GH, Shaner G. 1996. Variation in Fusarium graminearum and cultivar resistance to wheat scab. *Plant Disease* **80:**975-979.

Bai GH, Shaner G. 2004. Management and resistance in wheat and barley to Fusarium head blight. *Annual Review of Phytopathology* **42:**135-161.

Bai GH, Shaner G, Ohm H. 2000. Inheritance of resistance to Fusarium graminearum in wheat. *Theoretical and Applied Genetics* **100:**1-8.

Bos JIB, Prince D, Pitino M, Maffei ME, Win J, Hogenhout SA. 2010. A functional genomics approach identifies candidate effectors from the aphid species *Myzus persicae* (Green Peach Aphid). *PLoS Genetics* **6**.

Bruce TJ, Pickett JA. 2007. Plant defence signalling induced by biotic attacks. *Current Opinion in Plant Biology* **10:**387-392.

Buerstmayr H, Steiner B, Hartl L, Griesser M, Angerer N, Lengauer D, Miedaner T, Schneider B, Lemmens M. 2003. Molecular mapping of QTLs for Fusarium head blight resistance in spring wheat. II. Resistance to fungal penetration and spread. *Theoretical and Applied Genetics* **107:**503-508.

Dhami MK, Weir BS, Taylor MW, Beggs JR. 2013. Diverse honeydew-consuming fungal communities associated with scale insects. *PLoS One* **8**: e70316. doi:10.1371/journal.pone.0070306.

Drakulic J, Caulfield J, Woodcock C, Jones SPT, Linforth R, Bruce TJA, Ray RV. 2015. Sharing a Host Plant (Wheat Triticum aestivum) Increases the fitness of *Fusarium graminearum* and the severity of Fusarium head blight but reduces the fitness of grain aphids (*Sitobion avenae*). *Applied and Environmental Microbiology* **81:**3492-3501.

Edwards SG, Pirgozliev SR, Hare MC, Jenkinson P, 2001. Quantification of trichothecene-producing *Fusarium* species in hervested grain by competitive PCR to determine efficacies of fungicides against Fusarium head blight of winter wheat. *Applied and Environmental Microbiology* **67**, 1575-80.

Ferry N, Stavroulakis S, Guan W, Davison GM, Bell HA, Weaver RJ, Down RE, Gatehouse JA, Gatehouse AMR. 2011. Molecular interactions between wheat and cereal aphid (Sitobion avenae): Analysis of changes to the wheat proteome. *Proteomics* **11:**1985-2002.

Fokkema NJ, Riphagen I, Poot RJ, Dejong C. 1983. Aphid honeydew, a potential stimulant of *Cochliobolus-sativus* and *Septoria-nodorum* and the competitive role of saprophytic mycoflora. *Transactions of the British Mycological Society* **81:**355-363.

Jansen C, von Wettstein D, Schafer W, Kogel KH, Felk A, Maier FJ. 2005. Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted Fusarium graminearum. *PNAS* **102:**16892-16897.

Kang Z, Buchenauer H, Huang L, Han Q, Zhang H. 2008. Cytological and immunocytochemical studies on responses of wheat spikes of the resistant Chinese cv. Sumai 3 and the susceptible cv. Xiaoyan 22 to infection by *Fusarium graminearum*. *European Journal of Plant Pathology* **120:**383-396.

Li G, Yen Y. 2008. Jasmonate and ethylene signalling pathway may mediate Fusarium head blight resistance in wheat. *Crop Science* **48,** 1888-1896.

Mesterhazy A, Bartok T, Kaszonyi G, Varga M, Toth B, Varga J. 2005. Common resistance to different *Fusarium* spp. causing Fusarium head blight in wheat. *European Journal of Plant Pathology* **112:**267-281.

Naessens E, Dubreuil G, Giordanengo P, Baron OL, Minet-Kebdani N, Keller H, Coustau C. 2015. A secreted MIF cytokine enables aphid feeding and represses plant immune responses. *Current Biology* **25:**1898-1903.

Nicholson P, Simpson DR, Weston G, Rezanoor HN, Lees AK, Parry DW, Joyce D. 1998. Detection and quantification of *Fusarium culmorum* and *Fusarium graminearum* in cereals using PCR assays. *Physiological and Molecular Plant Pathology* **53:**17-37.

Schroeder HW, Christensen JJ. 1963. Factors affecting wheat scab to *Gibberalla zeae. Phytopathology* **42,** 720-727.

Waldron BL, Moreno-Sevilla B, Anderson JA, Stack RW, Frohberg RC. 1999. RFLP mapping of QTL for Fusarium head blight resistance in wheat. *Crop Science* **39:**805-811.

Yoshida M, Nakajima T. 2010. Deoxynivalenol and nivalenol accumulation in wheat infected with *Fusarium graminearum* during grain development. *Phytopathology* **100:**763-773.

Zadoks JC, Chang TT, Konzak CF. 1974. Decimal code for growth stages of cereals. *Weed Research* **14:**415-421.

Figures

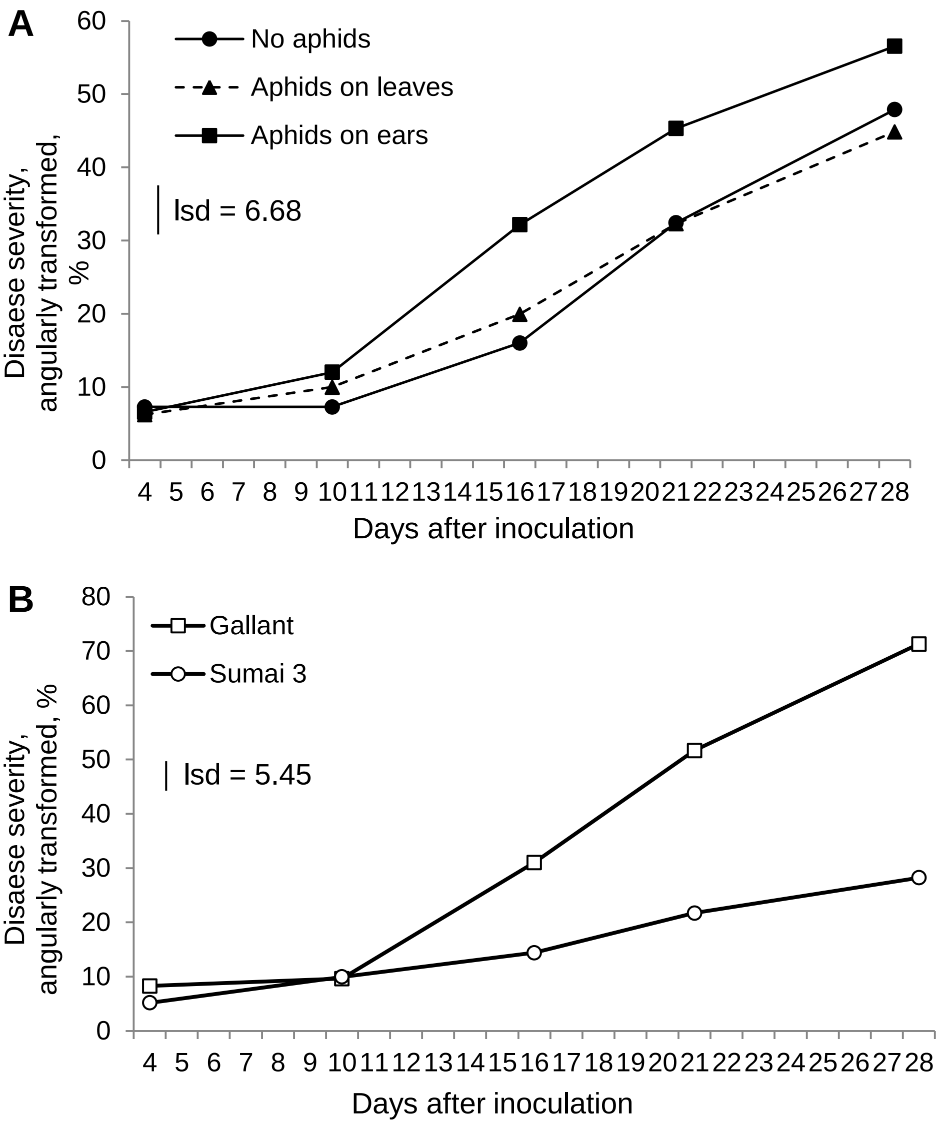

Fig. 5.1: Disease progress curve for inoculated tillers, using angularly transformed disease severity, grouped by (A) aphid treatment ($P = 0.025$) and (B) variety ($P < 0.001$).

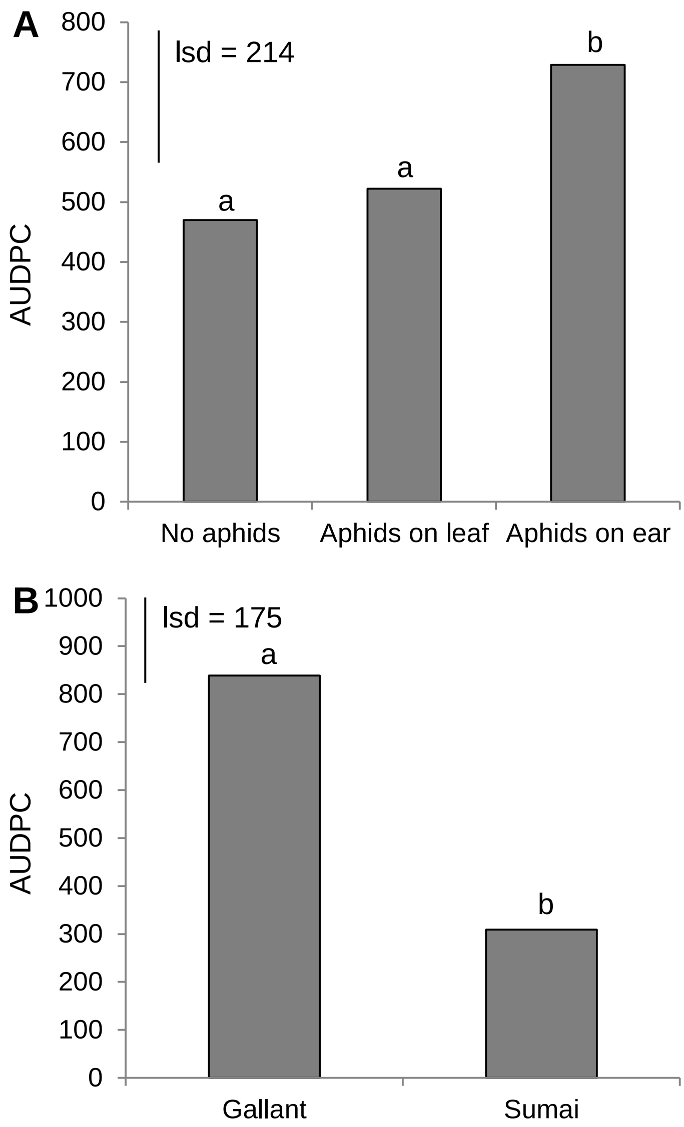

Fig. 5.2: Area under disease progress curve (AUDPC) for inoculated tillers, grouped by (A) aphid treatment (lsd = 214; P = 0.045) and (B) variety (lsd = 175; P < 0.001). Data marked with different letters are significantly different (lsd; P = 0.05)

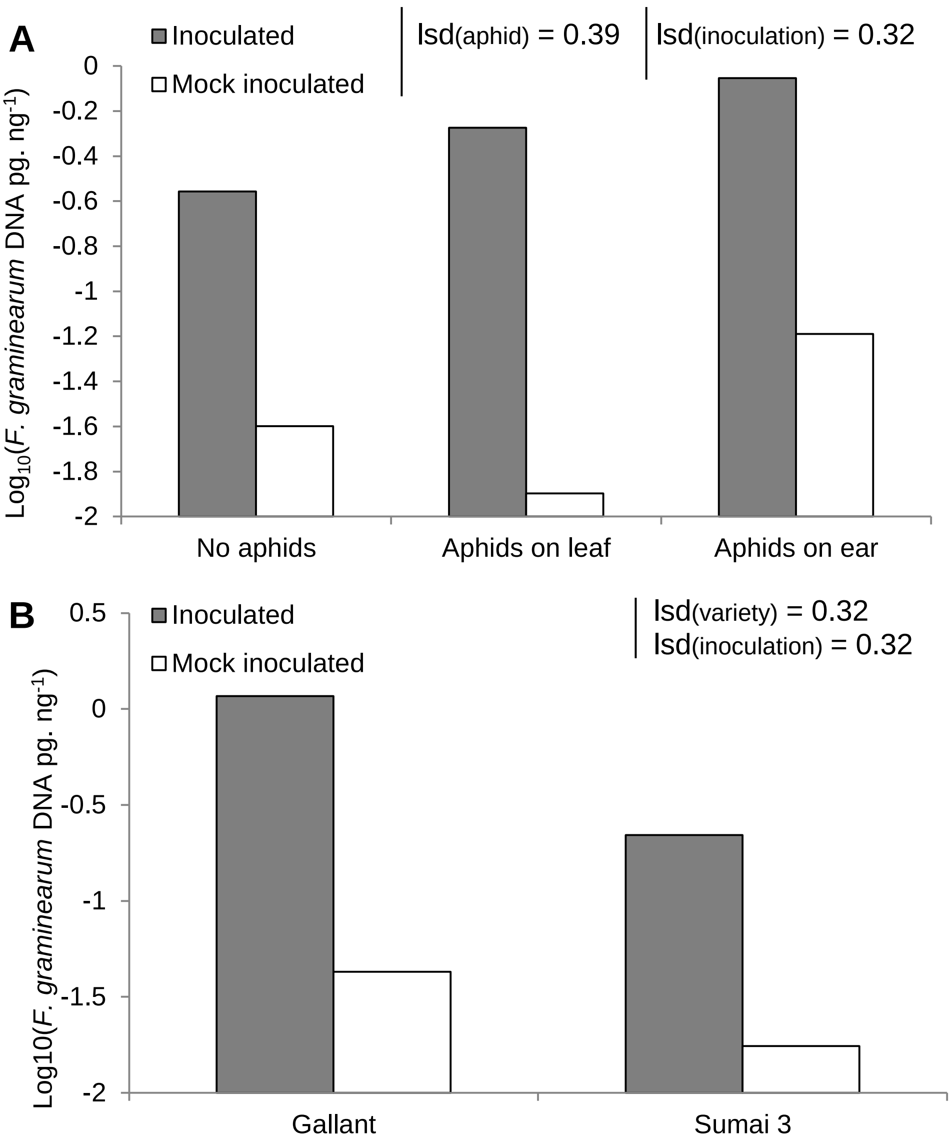

Fig. 5.3: *F. graminearum* DNA ($\log_{10}$ transformed) from treated ears grouped by (A) aphid treatment $P = 0.028$) and (B) variety ($P < 0.001$).

Tables

Table 5.1. Experiment design listing treatments in full along with treatment codes used. Letters in brackets correspond to the explanation of letters used in the treatment codes.

Treatment code	***Inoculation***	***Variety***	***Aphid treatment***
GIN	Inoculated (I)	Gallant (G)	No aphids (N)
GUN	Uninoculated (U)	"	"
SIN	Inoculated	Sumai 3 (S)	"
SUN	Uninoculated	"	"
GIL	Inoculated	Gallant	Aphids on Leaf (L)
GUL	Uninoculated	"	"
SIL	Inoculated	Sumai 3	"
SUL	Uninoculated	"	"
GIE	Inoculated	Gallant	Aphids on Ear (E)
GUE	Uninoculated	"	"
SIE	Inoculated	Sumai 3	"
SUE	Uninoculated	"	"

Chapter 6: General Discussion

The predominant theme that emerges from the results of this body of research is that the interaction between grain aphids and FHB pathogens can have a serious impact on the host plant in terms of heightened disease severity, and increased accumulation of fungal biomass and mycotoxins when aphids are present. Infection of wheat hosts with pre-existing aphid infestations, on any part of the plant, with either of two contrasting FHB pathogens reduced the host resistance to pathogen attack. This effect was found to vary according to the causal FHB head blight pathogen species: With *F. langsethiae,* symptoms and physical degradation of the grains are not increased, but the pathogen achieves increased levels of colonisation throughout the host tissues. As a result, an increase in pathogen biomass and T-2 and HT-2 toxins occurs (Chapter 3). With *F. graminearum*, the aggressive nature of the disease is enhanced, and premature bleaching of ears is accelerated in addition to the increased DON accumulation (Chapter 2). Consequently, in both cases the safety of the grain is reduced and economic losses may occur, due to heightened levels of mycotoxin contamination.

Weakening of pathogen resistance with aphid herbivory appears to affect the host plant more severely with increased duration of infestation. Thus, herbivory that commences after infection of the host with FHB pathogens has a *much more modest effect on fungal colonisation. While the order of events in* the tripartite interaction may be important, the importance of the site of aphid herbivory looks to be less so. Herbivory of tissues distant to the site of fungal infection led to a slight rise in fungal biomass accumulation and disease severity compared to infected hosts that were free of aphids (Chapter 5). Therefore a systemic effect of herbivory appears to be responsible, at least in part, for the impairment of host resistance to fungal infection. As previously discussed in this work, the molecular means by which aphids may be capable of altering host plant defence responses are under investigation by proteomics, transcriptomics and functional genomics (Thompson & Goggin, 2006; Reddy *et al*., 2013; Naessens *et al*., 2015). Manipulation of the host has evolved to facilitate successful prolonged feeding on hosts by aphids (Walling, 2008). Determination of the molecular mechanism by which aphids carry out suppression of host defences could reveal novel routes to breed crops resistant to aphid infestation or which can tolerate moderate levels of insect infestation without succumbing to heightened disease as a result.

Systemic suppression of host defences due to aphid herbivory was observed to overcome the Type II resistance (resistance to spread of infection) of 'Sumai 3', although the effect of aphids local to the site of infection caused a steeper rise in both fungal biomass and disease severity caused by *F. graminearum*. The same trend was observed in the susceptible variety, 'Gallant'. The spread of the pathogen was improved by aphid infestation in both varieties, and it is also important to note that in this experiment (Chapter 5) the aphids were only used as a pre-treatment of the hosts and were not still present on the ears upon fungal inoculation, so the increased spread cannot be attributed to aphid dispersal of inoculum to initiate several primary infections on the ear. The stimulation of fungal growth due to honeydew deposits remains possible (Dhami *et al*., 2013), perhaps encouraging external growth of the fungus enabling it to reach multiple penetration sites on the same ear. As a result of herbivory directly at the site of infection, tissues will have undergone competition for nutrients and water, as well as being the direct recipients of effectors from aphid saliva (Naessens *et al*., 2015). Thus, changes to the host biochemistry and phytohormones cannot be ignored as possible underlying factors that are likely to facilitate the enhanced disease spread observed in this study.

The contrast in disease severity observed between *F. langsethiae* and *F. graminearum* is in parallel with their different effects on the host volatile emissions, and consequently on host-aphid interactions. For crops infected with *F. graminearum*, the development of symptoms early on in the disease process, and the use of DON production to assist the spread of the pathogen through the ear (Maier *et al*., 2006), leads to a dramatic shift in the host condition from green to bleached, and fungal mycelia can quickly become visible on the external surfaces. Pre-existing aphid infestations are therefore subjected to a shift in their host environment that includes the loss of green feeding sites, the presence of fungi that may be capable of entomopathogenesis (Gagkaeva *et al*., 2014) and the presence of DON in sap (Kang & Buchenauer, 1999). Ecological fitness of populations of aphids fed on hosts infected with *F. graminearum* was negatively affected because elevated mortality and decreased fecundity were observed (Chapter 2). Grain aphids have co-evolved on cereal crops alongside FHB pathogens, and the observation that aphids are repelled by volatiles from infected hosts could be interpreted as an adaptation to avoid poor quality hosts. Infection of wheat ears with *F. langsethiae*, however, causes very few visible changes to the host and it is questionable that this fungus is truly pathogenic in this phase, and not merely a toxigenic endophyte. The volatile emissions of infected hosts are not repellent to grain aphids (Chapter 3), which may represent a lower selection pressure to adopt avoidance behaviours as hosts are less damaging to aphid populations than those infected with *F. graminearum*.

The persistence of aphids on ears that become infected with *F. langsethiae* is likely to be longer than on hosts infected with *F. graminearum*, and the pathogen may attain increased success of infection and dispersal as a result. It could be argued that the loss of aphid-repellent volatile chemistry and maintenance of prolonged aphid-induced host weakening could confer a fitness benefit to the relatively weak pathogen. Measurement of how well aphid infestation correlates with *F. langsethiae* prevalence in the field would be informative as to the importance of this interaction in the life cycle of the fungus. While this study did not seek to measure the impact on symptom development in wheat mutually attacked by *F. langsethiae* and grain aphids, this would be required to separate the impact of aphid herbivory on colonisation and virulence of *F. langsethiae* in wheat.

This work has also produced differing evidence for the possibility of aphid dispersal of *F. graminearum* and *F. langsethiae*. Whilst there was no indication that aphids were capable of transmitting *F. graminearum* between infected and healthy hosts (Chapter 2), when aphids were moved directly from ear to ear *F. langsethiae* was successfully transmitted by aphids (Chapter 3). However attempts to recreate more realistic means of aphid movement between hosts did not demonstrate transmission. While apparently compatible in terms of the size of the pathogen spores, carrying capacity of aphids, temporal overlap on host ears, the association of aphids and *F. langsethiae* – infected hosts appears to be weak and inefficient. The net effect of this mechanism in the field is not known, but may still be relevant in the epidemiology of *F. langsethiae* in increasing the number of primary infections on infected hosts, and in introducing inoculum into fields if aphids can bridge the gap between overwintering reservoirs of inoculum. A survey of grass weeds in wheat field headlands and volunteers was conducted as an accompaniment to the research presented, but no clear trend emerged in the species of weeds from which *F. langsethiae* was detected, and low levels of the pathogen were detected overall. Further study is required to elucidate the overwintering sites of *F. langsethiae,* and the relative importance of aphid dispersal. Further study could also reveal if aphid-dispersal has a more efficient role in oats, which is a more suitable host for *F. langsethiae* (Imathiu *et al*., 2013).

The results presented in this work have revealed that the seedling stage offers another opportunity for aphids to influence the epidemiology of *F. langsethiae* in wheat (Chapter 3). Mirroring the results of the experiments that used wheat ears, pre-treatment of wheat seedlings with aphids led to increased fungal biomass in inoculated seedlings. Whether *F. langsethiae* inoculum would be available to infect seedlings at this stage is not known, but early colonisation of foliar tissues with *F. langsethiae* may lead to ear infection upon emergence through movement of inoculum up the plant. A previous study failed to successfully inoculate wheat seedlings with *F. langsethiae* via application of inoculum onto stembase surfaces with a cotton wrap (Divon *et al*., 2012). Comparisons of inoculation methods and quantities were not investigated in this work, and so it cannot be concluded whether the effect of aphid herbivory could facilitate colonisation by the pathogen using stem-base inoculation.

The relationships between aphids and wheat infected with *F. graminearum* isolates with differing mycotoxin outputs were shown to be diverse (Chapter 4). DON-producing isolates produced a repellent response in aphids, whereas NIV-producing infection led to attraction and in infection devoid of mycotoxin accumulation the same neutral response was observed as with infected hosts as towards healthy hosts. From this, light could be shed on the nature of the volatile chemicals that govern this response. Early results showed a signature blend of six chemicals that were present in volatile emissions from DON-producing *F. graminearum* infection were an effective model of those responsible for eliciting avoidance behaviour in aphids (Chapter 2). Of particular interest was 2-pentadecanone, which itself was repellent without the combined effect of the addition five chemicals in the model. Examination of the abundances of 2-pentadecanone in volatile samples from hosts infected with different chemotypes of *F. graminearum* revealed that 2-pentadecanone is found in greater abundances in infected host volatiles compared to healthy controls regardless of whether DON, NIV or no mycotoxins are produced during the infection (Chapter 4). Thus this chemical appears to be a good marker for *F. graminearum* pathogenesis, and could provide a potential target for crop volatile monitoring as an indicator of FHB disease, in a similar fashion to the use of chemical sensors to detect infection and mycotoxin levels during storage (Eifler *et al*., 2011; Infantino *et al*., 2015).

The production of NIV was attributed to a change in the response of aphids towards infected hosts, but the chemicals responsible for this attraction were not identified in this work. The chemicals responsible are evidently detected by the aphids, and identification of these may provide insight into the differing nature of the phytotoxicity of DON and NIV in wheat. Furthermore, the impacts of these mycotoxins on aphids are not fully understood, and this change in behaviour towards host of different chemotypes may reflect the different levels of suitability for aphid hosts. If NIV producing strains do not reduce host plant quality for aphids as much as DON producing strains there would be less selection pressure for avoidance. Alternatively, the attractiveness of NIV-producing infection may reveal that the two plant attackers are in a different phase in an arms race between pathogen and pest, in which the pest may be exploited for the benefit of the pathogen. Determining the effect of host infection with the different pathogen chemotypes on aphid population dynamics could give the necessary context to these results to draw further conclusions. Recent findings have shown that grain aphids may be able to tolerate DON, in a testament to their co-evolution on cereal hosts (De Zutter *et* *al*., 2015). While DON may not be directly toxic to the aphids, it still may be the factor that facilitates enhanced pathogen colonisation and changes to the host that ultimately causes increased mortality in aphid populations. Whether NIV-producers of the same species are as damaging to aphids remains unknown, as does the impact of T-2 and HT-2 producing *F. langsethiae*. By discovering these relationships, this could be indicative of the relative importance of aphid-FHB interactions in geographic locations with different prevailing *Fusarium* species and chemotypes. If NIV-producing infection is both attractive to aphids and aphid populations do not decline on infected hosts, a prolonged interaction could have more severe consequences post-anthesis than in areas which are dominated by DON-producing populations for which pre-anthesis aphid threat is likely to be of greatest importance to control.

The interaction of several different insects has been correlated with increased disease by FHB pathogens across different crops. Knowledge of important ecological interactions is vital to inform farmers and agronomists of the best practice to control particular problems of interest. In the case of FHB, specific weather patterns have been modelled as a means to estimate the level of risk of high mycotoxin contamination (Hooker *et al*., 2002; Bondalapati *et al*., 2012). Adding an insect monitoring component to such a model could offer a way to improve accuracy, and therefore usefulness, of these tools. Knowledge of key times and conditions for heightened FHB risk due to aphid pressure could be used to develop effective monitoring strategies in the lead up to ear emergence, and knowledge of the appropriate conditions for insecticides application so as to decrease the risk of mycotoxin contamination of crops could improve control of the disease. Furthermore, it seems likely that breeding for resistance to aphids would have an added benefit in reducing FHB disease risk.

References

De Zutter N, Audenaert K, De Boevre M, De Saeger S, Stals I, uka V, Haesaert G, Smagghe G. 2015. Tolerance mechanisms of grain aphids *Sitobion avenae* for the *Fusarium* mycotoxin deoxynivalenol [abstract]. *In:* 13th European Fusarium Seminar; 10-14 May 2015; Martina Franca, Italy.

Dhami MK, Weir BS, Taylor MW, Beggs JR. 2013. Diverse honeydew-consuming fungal communities associated with scale insects. *PLoS One* **8**.

Divon HH, Razzaghian J, Udnes-Aamot H, Klemsdal SS. 2012. *Fusarium langsethiae* (Torp and Nirenberg), investigation of alternative infection routes in oats. *European Journal of Plant Pathology* **132:**147-161.

Eifler J, Martinelli E, Santonico M, Capuano R, Schild D, Di Natale C. 2011. Differential detection of potentially hazardous *Fusarium* species in wheat grains by an electronic nose. *Plos One* **6**.

Gagkaeva TY, Shamshev IV, Gavrilova OP, Selitskaya OG. 2014. Biological relationships between *Fusarium* fungi and insects (review). *Sel'skokhozyaistvennaya Biologiya* **3:**13-23.

Imathiu SM, Edwards SG, Ray RV, Back MA. 2013. *Fusarium langsethiae* - a HT-2 and T-2 toxins producer that needs more attention. *Journal of Phytopathology* **161:**1-10.

Infantino A, Aureli G, Costa C, Taiti C, Antonucci F, Menesatti P, Pallottino F, De Felice S, D'Egidio MG, Mancuso S. 2015. Potential application of PTR-TOFMS for the detection of deoxynivalenol (DON) in durum wheat. *Food Control* **57:**96-104.

Kang Z, Buchenauer H. 1999. Immunocytochemical localization of Fusarium toxins in infected wheat spikes by *Fusarium culmorum*. Physiological and *Molecular Plant Pathology* **55:**275-288.

Maier FJ, Miedaner T, Hadeler B, Felk A, Salomon S, Lemmens M, Kassner H, Schaefer W. 2006. Involvement of trichothecenes in fusarioses of wheat, barley and maize evaluated by gene disruption of the trichodiene synthase (Tri5) gene in three field isolates of different chemotype and virulence. *Molecular Plant Pathology* **7:**449-461.

Naessens E, Dubreuil G, Giordanengo P, Baron OL, Minet-Kebdani N, Keller H, Coustau C. 2015. A secreted MIF cytokine enables aphid feeding and represses plant immune responses. *Current Biology* **25:**1898-1903.

Reddy SK, Weng Y, Rudd JC, Akhunova A, Liu S. 2013. Transcriptomics of induced defense responses to greenbug aphid feeding in near isogenic wheat lines. *Plant Science* **212:**26-36.

Thompson GA, Goggin FL. 2006. Transcriptomics and functional genomics of plant defence induction by phloem-feeding insects. *Journal of Experimental Botany* **57:**755-766.

Walling LL. 2008. Avoiding effective defenses: Strategies employed by phloem-feeding insects. *Plant Physiology* **146:**859-866.

Summary of Conclusions

- Aphid herbivory enhances disease progression of *Fusarium graminearum* in wheat
- Aphid-herbivorised hosts have increased mycotoxin accumulation in tissues infected by either *F. graminearum* or *F. langsethiae*
- Aphid herbivory prior to pathogen infection, particularly on ears, induces changes to host biochemistry and/or phytohormones such that host defences are impaired
- *F. langsethiae* may be vectored by *Sitobion avenae*, but on wheat hosts the relationship is weak
- Production of NIV by *F. graminearum* during infection of wheat leads to the production of volatiles that are attractive to aphids
- Infection of wheat with DON-producing isolates leads to production of host volatiles that aphids are repelled by
- Aphid mortality is raised and migrant fecundity impaired after feeding on wheat infected with DON-producing *F. graminearum*

Future Work

- Analysis of the transcriptome or proteome of wheat following *Sitobion avenae* infestation and infection with *Fusarium. graminearum*
- Repetition of experiments of Chapter 5, and mycotoxin extraction
- Exploration of the interactions that occur between *S. avenae* and *F. langsethiae* in oats which are more suitable hosts for the pathogen
- Microscopy of the infection process of *F. langsethiae* through wheat plants
- Elucidation of the chemistry that attracts aphids in the volatiles of NIV-producing *F. graminearum* infection via electroantennography
- Assessment of aphid population dynamics on wheat infected with NIV-producing *F. graminearum* and other related *Fusarium* species