The effect of three spray inoculation protocols on Fusarium head blight infection of wheat and *in planta* competition between 3-ADON & 15-ADON chemotypes of *Fusarium graminearum*

BY

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ABSTRACT

Breeding for Fusarium head blight (FHB) resistance in wheat requires accurate and precise phenotyping. In addition, breeding for FHB resistance requires an understanding of the pathogen population to which the host will be subjected. In North America there has been a shift in the population distribution of *Fusarium graminearum* chemotypes. It is important to examine aspects of the competition between *F. graminearum* isolates that produce 3-acetyldeoxynivalenol (3-ADON) or 15-acetyldeoxynivalenol (15-ADON), including how infection by various isolates will affect the expression of resistance in wheat. The first objective of this study was to compare ranking of FHB reactions of ten wheat genotypes using three different macroconidial spray inoculation protocols and to determine if the protocols produce substantially equivalent results in terms of symptom development. The second objective was to examine the outcome of competition between 3-ADON and 15-ADON producing isolates of *F. graminearum* and to examine the effect inoculation with different isolate mixtures on FHB resistance in wheat genotypes 93FHB37 (resistant) and CDC Teal (susceptible). For the first objective three different inoculation protocols were compared over two years using eight genotypes of common wheat (*Triticum aestivum*) and two genotypes of durum wheat (*Triticum turgidum* spp. *durum*). The three inoculation protocols differed in the start and end points for inoculation and received approximately two, five or nine inoculations. Analysis of variance detected significant differences in the development of symptoms across protocols, however, there were few instances of genotype X inoculation protocol interaction. The spearman rank correlations of genotypes for different measures of symptom development and disease progression were very high (ranging from 0.79 to 1) across the inoculation protocols tested. For the second objective, macroconidial inoculum mixtures consisting of equal proportions of different isolates of 3-ADON and 15-ADON chemotype were used to study the effect of isolate mixtures on area under the disease progress curve (AUDPC) for disease incidence, and the ratio of the chemotypes recovered from infected seed on the susceptible cultivar CDC Teal. Over two field seasons and two indoor trials there were no consistent effect of isolate mixture on the

ratio of chemotypes recovered nor was there a significant effect of inoculum mixtures on AUDPC for disease incidence.

FOREWARD

This document is a manuscript style thesis consisting of two manuscripts, they are precluded by a general abstract, general introduction and literature review, and followed by a general discussion, literature cited and appendix. The referencing style of the Canadian Journal of Plant Pathology was used.

1.0 GENERAL INTRODUCTION

In 2008 the total value of wheat exports was 6.8 billion dollars, comprising 16.8% of total agriculture and fisheries trade exports, compared to canola with 3.8 billion dollars and 9.5% of exports (VISTA on the Agri-food Industry and the Farm Community 2011). Wheat was grown on nearly 25 million acres in 2009, nearly double that of canola with approximately 16 million acres (Seeding of principal field crops: Canada). In Manitoba during the period from 1998-2009, the area of wheat grown averaged 3.2 million acres annually (Area of wheat in Manitoba, 1998-2009). Fusarium head blight (FHB) of wheat is a serious disease in nearly all regions of the world where wheat is grown. *Fusarium graminearum* Schwabe is the primary causal agent of FHB of wheat in North America (Wagacha and Muthomi 2007, McMullen *et al*. 1997). Efforts to combat this disease come from a number of different areas of research: breeding for resistance, crop protection, cultural practices and disease forecasting. Two of the major focuses in FHB research are breeding new host genotypes to improve and maintain the level of resistance to FHB, and studying the pathogen and how it interacts with the environment and the host to cause disease.

To select for resistance to FHB, wheat breeders must be able to challenge wheat genotypes with the pathogen in a manner that will closely mimic the natural infection process in the agricultural setting. Arthur (1891) established the critical period of host susceptibility as the period of anthesis. Schroeder and Christensen (1963), Mesterhazy (1978, 1983), Lemmens *et al.* (2004), and Xu *et al.* (2006) compared various sources of inoculum and methods of inoculation. For the majority of FHB screening programs, where macroconidial spray inoculation is used, it is done on a plot-by-plot basis with great care to ensure the correct number of applications at the correct growth stage. This study examined the use of spray inoculation applied to plots starting and ending at various growth stages with all the inoculation treatments proceeding through the critical stage of susceptibility. The aim of this experiment was to determine if the expression of

FHB resistance in ten wheat genotypes was consistent across the different inoculation procedures.

The second part of the study focused on the pathogen *Fusarium graminearum*. The interaction between the host and the pathogen is affected by many external factors. Within the *F. graminearum* species the niche for causing FHB of wheat in North America has been filled by isolates which produce the 15-acetyldeoxynivalenol (15-ADON) derivative of the potent mycotoxin, deoxynivalenol. Most recently there has been recognition that a shift in the structure of *F. graminearum* populations is occurring, with recovery of 15-ADON isolates declining as isolates that produce 3-acetyldeoxynivalenol (3-ADON) increase (Ward *et al.* 2008, Guo *et al.* 2008). This experiment examined the outcome of *in planta* competition between 3-ADON and 15-ADON producing isolates when they were co-inoculated at the same infection site, and examined what effect different isolate mixtures had on the response to infection in a resistant (93FHB37) and susceptible (CDC Teal) host genotype.

2.0 LITERATURE REVIEW

2.1 Importance of Fusarium head blight in Wheat

Fusarium head blight (FHB) is a major disease of wheat (*Triticum aestivum* L.) in North America. In Manitoba, Canada, FHB, caused predominantly by *F. graminearum*, is the most important cereal disease (Gilbert *et al.* 2004). Yield loss can result from reduced seed set due to floral abortion, or development of shrunken, poorly filled kernels due to infection (Snijders 2004). Some lightweight kernels are removed from the grain during harvest (Sutton 1982, Bai and Shaner 1994). Fusarium head blight causes damage to grain, resulting in losses in baking and milling quality (Parry *et al.* 1995, Snijders 2004). In addition, FHB infected grain can be contaminated with potent mycotoxins, such as deoxynivalenol (DON), which are detrimental to plants and animals (Harris and Gleddie 2001, Rocha *et al.* 2005, Desjardins and Hohn 1997). Mycotoxins from infected grain can pose a serious threat to human and livestock health resulting in both acute and chronic effects. (Sutton *et al.* 1982, Parry *et al.* 1995, Pestka 2008). In swine, chronic effects including feed refusal and reduced rate of weight gain are well documented (Pestka and Smolinski 2004). Over the course of the last century there have been a number of documented cases of large-scale contamination of public food supplies with mycotoxins that have resulted in illness and loss of life (Bhat *et al.* 1989, Pestka 2008). Current Canadian Food inspection Agency (CFIA) guidelines for DON contamination are 2 mg/kg for uncleaned soft wheat destined for human consumption. For DON and acetylated derivatives (3-ADON and 15-ADON) a provisional maximum tolerable daily intake (PMTDI) of 1 ug/kg by weight was set in 2010 by the joint FAO/WHO expert committee on food additives (JECFA 2011).

Fusarium head blight is known as a pre-harvest disease but may also infect grain if it is dried too slowly during the post harvest period (Wagacha and Muthomi 2007). *Fusarium graminearum* is the major causal agent of FHB of wheat (*Triticum aestivum*) in

North America (Wagacha and Muthomi 2007, McMullen *et al*. 1997) and worldwide (Nicholson 2003, Osborne and Stein 2007).

2.2 Infection Period and Environmental Conditions

Fusarium graminearum is a facultative parasite of wheat; its lifecycle includes a saprophytic stage in which it over-winters on crop residue, and a parasitic stage causing FHB of wheat (Miedaner *et al.* 2001). *Fusarium graminearum* is the anamorphic stage, which reproduces asexually *via* macroconidia, and *Gibberella zeae* is the teleomorphic stage, reproducing sexually *via* ascospores (Wagacha and Muthomi 2007). The primary source of inoculum under natural conditions is ascospores (Leonard and Bushnell 2003, Trail 2009), produced by the sexual state *G. zeae*. The fruiting body of *G. zeae* is an ostiolate perithecium with a single opening (Leonard and Bushnell 2003). In the natural course of infection, plant debris is the primary source of inoculum (Sutton 1982, Bai and Shaner 1994, Champeil *et al.* 2004). *Fusarium graminearum* grows well over a wide range of temperatures from 15 – 30 degrees Celsius (Gilbert and Fernando 2004, Parry *et al.* 1995, Osborne and Stein 2007). The continued presence of warm temperature, and moisture or high humidity are required for successful production of ascospores and macroconidia (Sutton 1982). Ascospores are dispersed primarily by wind after having been forcibly discharged from the perithecia (Sutton 1982, Leonard and Bushnell 2003, Champeil *et al.* 2004). Splashing or wind driven rain are the primary mechanisms of dispersal for macroconidia *(*Gilbert and Fernando 2004, Leonard and Bushnell 2003, Champeil *et al.* 2004).

The window of susceptibility in wheat to FHB infection is centered around the time of anthesis. Because this window is too small in duration for the pathogen to complete its lifecycle and propagate new spores for subsequent cycles of infection within the lifespan of the crop, we can deduce that FHB of wheat is primarily a monocyclic disease (Sutton 1982, Trail 2009). The monocyclic nature of FHB disease pressure was confirmed in a paper published by Fernando *et al.* (1997). By examining spore dispersal gradients, the dispersal of ascospores from concentrated point loci was described as

having a steep dispersal gradient characteristic of a monocyclic disease. If the initial dispersal had been followed by subsequent repeated dispersals the gradient would be much more gradual in decline as new hosts were infected at the periphery of the gradient (Gilbert and Tekauz 2000).

Arthur (1891) first established that anthesis, grain set and grain filling were the physiological stages associated with FHB infection (Schroeder and Christensen 1963). The physiological stage of susceptibility to FHB is from anthesis through grain filling (Sutton 1982, Osborne and Stein 2007, Del Ponte *et al.* 2007) and may differ between cultivars while still falling in the range of anthesis through grain filling (Schroeder and Christensen 1963). In a study conducted by Sutton (1982), emasculated florets of wheat showed a decreased infection rate compared to non-emasculated florets, indicating that the presence of extruded anthers can greatly increase host infection.

2.3 Infection Process and Host Response

Germination of macroconidia occurs 6-12 hours after inoculation (Pritsch *et al.* 2000, Kang and Buchenauer 1999). Following germination of the spore, dense hyphal growth occurs on the adaxial and abaxial side of the lemma and palea and adaxial side of the glumes (Kang and Buchenauer 2000a). Between 36-48 hours after germination of conidia the hyphae penetrate tissue directly, or occasionally enter *via* stomata. Primary sites of entry are the interior of the floret *via* the lemma and palea, or directly into the ovary (Kang and Buchenauer 2000a). The hyphae grow both inter- and intra-cellularly through the host tissues, down the rachilla toward the rachis, and from the rachis to adjacent spikelets (Schroeder and Christensen 1964, Kang and Buchenauer 2000b). In very susceptible cultivars the hyphae can progress down the peduncle resulting in the death of the entire spike (Schroeder and Christensen 1964). The rate of hyphal growth from the site of infection to the rachis differs considerably between resistant and susceptible genotypes (Schroeder and Christensen 1964, Kang and Buchenauer 2000b).

The initial infection process and early spread of the pathogen is similar in both resistant and susceptible wheat cultivars (Kang and Buchenauer 2000b, Pritsch *et al.* 2000). After initial infection susceptible and resistant genotypes differ in the rate and extent of progress of infection, hyphal density, and quantitative and qualitative alterations of the host cells (Kang and Buchenauer 2000b, Kang *et al* 2008). Kang and Buchenauer (2008) used immuno-labeling of beta 1,3-glucan, and lignin to observe the rate of formation of cell wall appositions and papillae. Following the initial infection process, the authors found that the rate of formation of these structures was much higher for the resistant cultivar Sumai 3 than for the susceptible cultivar Xiaoyan 22. Cell wall thickening and papillae serve as a physical barrier to block the progress of the pathogen and may disrupt the movement of DON into the host cell while also disrupting the movement of nutrients out of the cell (Siranidou *et al.* 2002, Kang and Buchenauer 2008, Boutigny *et al.* 2008). Host cell wall alterations were most intense in host cells directly adjacent to hyphae. The reactions of the host extended in advance of hyphal progress, and were observed to be less intense and less rapid as the distance from hyphae increased (Kang and Buchenauer 2008). Spikes of the resistant cultivar, Frontana, showed a dramatic increase in lignin on the surface of the cell walls when inoculated with *F. culmorum* compared to the uninoculated control tissues (Siranidou *et al.* 2002). In contrast, spikes of the susceptible cultivar, Agent, showed little increase in lignin content when compared to the non-inoculated control. Three days after inoculation, Frontana showed a dramatic increase in electron dense cell wall appositions on the interior of the cell wall opposite the fungal hyphae, compared to Agent, which showed sparse development of appositions that were less dense (Siranidou *et al.* 2002).

2.4 Role of Deoxynivalenol in *Fusarium* spp. Virulence

Fusarium graminearum produces the trichothecene deoxynivalenol (DON), a potent inhibitor of eukaryotic protein synthesis (Desjardins 1997, Harris and Gleddie 2001). Presumably, the role of trichothecene as a pathogenicity factor is related to its inhibitory effect on protein synthesis, thereby delaying the expression of defence-related genes (Harris and Gleddie 2001). Casale and Hart (1988) used a detached leaf disk assay

from the FHB susceptible wheat cultivar Ionia, to observe the effect of DON on protein synthesis by quantifying the *in vitro* incorporation of radiolabelled leucine into protein. Protein synthesis in the leaf disks all but ceased in the presence of 4.5 micromolar solution of DON and resumed when the DON had been rinsed out of solution over a time course of two hours. Deoxynivalenol is produced by the fungus and transported into the host tissues, where it moves in advance of hyphae through the phloem by active transport and the xylem by passive transport (Kang and Buchenauer 1999). Deoxynivalenol can also be translocated from floral tissues into the developing kernels (Snijders and Krechting 1992).

Strong correlations between aggressiveness and trichothecene production of various *Fusarium* isolates on wheat indicate that trichothecenes are virulence factors (Goswami 2005, Mesterhazy 1999). Evidence supporting the hypothesis that trichothecenes are virulence factors for *F. graminearum* causing FHB of wheat, has been further supported with the use of genetically engineered trichothecene knock-out isolates of *Fusarium* which resulted in aggressiveness of the pathogen being severely reduced (Maier *et al.* 2006, Cuzick *et al.* 2008, Eudes *et al.* 2001).

2.5 Resistance to Deoxynivalenol in Wheat

The ability to detoxify DON was first demonstrated in wheat using the cultivars Frontana (resistant to FHB) and Casavant (susceptible to FHB), which varied in ability to degrade DON (Miller and Arnison 1986). Miller and Ewen (1997) demonstrated that ribosomes of Frontana and Casavant exhibited differential tolerance to DON during cell free *in vitro* translation. Also, the two cultivars showed a differential affinity of DON binding to the ribosomes.

The *FHB1 (Qfhs.ndsu-3BS)* QTL for FHB resistance is involved in resistance to trichothecenes and co-localizes with the ability to convert DON to DON-3-glucoside, a detoxification product (Walter *et al.* 2008, Lemmens *et al.* 2005). Though no differential reaction for 3-ADON and 15-ADON detoxification has been found in wheat (*T. aestivum*)

to date, in *Arabidopsis thaliana* it has been demonstrated that the UDP-glycosyltransferase enzyme is able to detoxify DON and 15-ADON by adding a sugar group, but is unable to do so with 3-ADON (Poppenberger *et al.* 2003). Walter *et al.* (2008) demonstrated by way of trichothecene responsive transcriptome analysis in wheat that the *FHB1* QTL plays a role in the very early defence reaction to trichothecenes, and that it is upstream of pathways that result in toxin sequestration and detoxification, and management of oxidative stress resulting in increased cell survival.

Desmond *et al.* (2008) demonstrated that infiltration of leaf segments with DON induced defence gene transcription and intense accumulation of H_2O_2 eventually resulting in a hypersensitive response (HR) and programmed-cell death in wheat (*T. aestivum*). This study demonstrated that DON had an elicitor-like activity which was able to induce a HR in wheat tissues. When cyclohexamide, a potent inhibitor of protein synthesis, was co-infiltrated with DON the defence reaction was greatly reduced. If the elicitation of reactive oxygen species and hypersensitive reaction were due to the inhibition of protein synthesis the co-infiltration of cyclohexamide would have exaggerated the response. In fact there are a number of examples of DON inducing gene transcription in various genetic backgrounds. In the wheat line CM82036, DON induces production of gene products responsible for both production and consumption of reactive oxygen species, including Class III plant peroxidases, and TaGLP2a (Germin-like protein) (Ansari *et al.* 2007). Ponts *et al.* (2003, 2006, 2007) demonstrated that elevated levels of H_2O_2 resulted in the up-regulation of trichothecene pathway gene expression and increased production of DON and ADON trichothecenes. The addition of catalase (an H_2O_2 scavenging enzyme) caused down-regulation of *tri* genes and a decline in DON production. This interaction between DON, H_2O_2 and catalase was reproduced and confirmed by Audenaert *et al.* (2010).

2.6 Cultural Practices Affecting FHB

As *Fusarium* spp. are facultative pathogens of wheat, and can survive as saprophytes on crop residue, care must be taken to mitigate the degree to which infested

crop residues from previous seasons act as a source of inoculum to infect the current crop. Miller *et al.* (1998) used a polymerase chain reaction (PCR) based molecular fingerprint of *F. graminearum* strains to study the effect of tillage on the survival of *F. graminearum* on infested crop residue and subsequent contribution of these introduced strains to inoculum in the following growing season. Comparison of tilled and no-till systems showed that tillage dramatically reduced the survival of stubble borne *F. graminearum* that would otherwise act as a source of local inoculum in the following season. Pereyra and Dill-Macky (2008) found that the significance of the tillage effect on FHB symptoms was largely dependant on the suitability of the environment for establishment of disease. In the years where the environmental conditions were favorable for infection there was no significant effect of tillage system on FHB symptom development, but in years when the environmental conditions were not favorable to establishment of disease, there was a significant effect of tillage on FHB symptom development. The authors concluded that while tillage system and type of crop residue were contributing factors to disease pressure, the effect of environmental conditions was the overriding factor. Guo *et al.* (2010) collected data from 58 producer fields from 2003 to 2006 and used cultural practice data to create models that found that tillage practice had a significant effect on inoculum levels. Tillage may allow for mitigating on-site inoculum but when environmental conditions are favorable the inoculum load in the surrounding area may become overwhelming and mask the benefits of tillage in a particular field (Anita Brûlé-Babel, personal communication, 2011). While controlling the environment at large remains unfeasible, choosing an appropriate tillage system and crop rotation remain viable and practical methods of managing disease pressure.

While tillage can affect the amount of inoculum present in a field, it may also have an impact on pathogen gene flow and sexual recombination in an area. Miedaner *et al.* (2001) conducted a survey for aggressiveness and genetic diversity of *F. graminearum* and *F. culmorum* sampled from infected wheat fields and found that large variation in aggressiveness and molecular marker profiles exists in *F. graminearum* and *F. culmorum* populations both within locations and on a worldwide scale. Aggressiveness in *F. culmorum*, is a quantitative trait with a very high broad sense heritability ($h^2 = 0.92$)

(Miedaner 1997). Miedaner *et al.* (2001) concluded that sexual recombination and balanced selection during the saprophytic stage of the lifecycle might be fostering a wealth of genetic diversity in these *Fusarium* populations. Tillage can affect genetic diversity of *F. graminearum* populations, as light is required for perithecia formation.

Pereyra and Dill-Macky (2008) concluded that wheat and barley residues in no-till and reduced-till management systems could act as a significant source of inoculum for FHB infection of subsequent wheat and barley crops. Comparison of crop residues of wheat, barley, corn, sunflower, fescue and a host of gramineous weeds under no-till and reduced-till systems indicated that *Gibberella zeae* colonized wheat and barley more often than the other crop residues, reinforcing the importance of crop rotation as a measure to mitigate disease pressure.

2.7 A Brief Summary of Breeding for FHB Resistance

The first evidence of differences in wheat cultivar response to FHB came from a report by Arthur (1891). Despite these early observations, the existence of significant genetic resistance in wheat germplasm was still being questioned as recently as 1982 (Martin and Johnston, 1982).

Mechanisms of resistance to FHB can be categorized as active or passive (Mesterhazy 1995). Active resistance includes the production of novel pathogenesis related (PR) proteins (Kang *et al*. 2008, Pritsch *et al.* 2000, Ferreira *et al.* 2007). The speed with which PR-proteins accumulate, the density of the accumulated compounds and the spatial dispersion throughout the plant are all important factors in the resistant reaction of wheat to FHB (Kang and Buchenauer 2000b, Kang *et al* 2008). Passive resistance is based on the morphological characteristics of the host or profile of secondary metabolites, present in the host in a developed state prior to the initial interaction between a pathogen and host, which inhibit germination, infiltration, sporulation or dissemination of the pathogen (Ferreira *et al.* 2007). Plant height (Mesterhazy 1995), presence of awns (Mesterhazy 1995), duration of time the floret

remains open (Schroeder and Christensen 1963), percentage of trapped anthers (Schroeder and Christensen 1963) are examples of passive morphological traits that can influence FHB resistance of wheat under natural conditions.

In a study conducted by Yoshida *et al.* (2005), the authors concluded that near isogenic lines (NIL) of barley differing in cleistogamous (anthers retained in florets at flowering) or chasmogamous (anthers extruded from florets at flowering) flowering habit varied greatly in FHB resistance, with cleistogamous NILs having significantly less severe infection than chasmogamous NILs. Yoshida *et al.* (2005) provided two possible explanations for this phenomenon; 1) Cleistogamous florets remained closed, limiting the probability of spores landing inside the floret. 2) Retained anthers limited the possibility of spores landing directly on the anther. Yoshida *et al.* (2005) further supported this claim by referring to previous supporting research. Cleistogamous flowering habit QTLs in barley (Turuspekov *et al.* 2004) co-localize with a QTL responsible for FHB resistance of barley in the Frederickson/Stander population (Mesfin *et al.* 2003). In wheat it has been demonstrated that extruded anthers can greatly increase the likelihood of infection and that spikes with emasculated florets showed a decreased rate of infection compared to spikes with non-emasculated florets (Sutton 1982). Sutton (1982) showed that compounds produced in anthers, betaine and choline, have shown to increase hyphal growth in vitro. By retaining anthers in the florets the highly conducive environment that anthers provide for germinating spores is limited as a potential site of primary infection.

Inheritance of FHB resistance in wheat is a quantitative trait (Van Eeuwijk *et al.* 1995, Nakagawa 1955, Gilbert and Tekauz 2000, Mesterhazy *et al.* 1999). Resistance to FHB in wheat is conferred by a number of genes each of which contributes to the degree of resistance. The number of genes responsible for quantitative resistance to FHB is estimated to be between two to five (Gilbert and Tekauz 2000), with early estimates putting the number of genes controlling FHB resistance in wheat at three (Nakagawa 1955). Snijders (1990) concluded that additive gene action was the main force for FHB resistance, and that epistasis was not a significant contributor to FHB resistance.

Miedaner (1997) also concluded that additive gene action is the primary mode of action for FHB resistance genes in wheat.

Quantitative trait loci for FHB resistance in spring wheat have been identified on all chromosomes except for 7D (Buerstmayr *et al.* 2009). The QTL located on chromosomes 3BS (*FHB 1*), 6BS (*FHB2*), and 5AS (Qfhs.ifa-5A) are the most readily accepted major QTLs and have been widely validated through marker-assisted selection and field-level screening (Buerstmayr *et al.* 2009). Frontana is a Brazilian spring wheat known for its type I resistance to initial infection (Buerstmayr *et al.* 2009). Type II resistance is commonly obtained from Sumai 3 and derivatives (Rudd *et al.* 2001, Pritsch *et al.* 2000). Sumai 3 was developed in Suzhou, China from the cross, Funo X Taiwanmai (Bai and Shaner 2000). Resistance to FHB can be sub-divided into various types. Type I resistance is resistance to initial infection (Schroeder and Christensen 1963). Type II resistance is resistance to spread from spikelet to spikelet *via* the rachis (Schroeder and Christensen 1963). Type III resistance is characterized as resistance to accumulation of mycotoxin (Miller and Arnison 1986). Type IV resistance is resistance to kernel infection despite a visual confirmation that the spike is symptomatic (Mesterhazy *et al.* 1999). Type V resistance is termed 'tolerance' by Mesterhazy *et al.* (1999) and is characterized by a resistance to yield loss despite the presence of infection in the kernel.

2.8 Artificial Inoculation

The conditions required for establishment of disease must be present in order to evaluate the level of resistance of genotypes, namely an environment conducive to the establishment of the disease, and a strain of the pathogen capable of causing disease. Evaluating wheat genotypes for disease resistance of a quantitative nature requires reproducible inoculation methods with low variance among replications and a quantitative assessment of resistance, both of which must be cost efficient, rapid and adaptable to large scales (Miedaner *et al.* 1997).

In order to breed for FHB resistance in wheat, there is a need for reliable methods of culturing the appropriate *Fusarium* spp., inoculating at the correct physiological growth stage of the host and providing a suitable environment for infection. The cultivation of the asexual stage, *F. graminearum*, and *in vitro* production of inoculum in the form of macroconidia is widespread in FHB screening nurseries. A comparison of effectiveness of ascospores and conidia as sources of inoculum concluded that inoculations conducted using conidia are valid for extrapolation to disease pressure from ascospores (Stack 1989). Bai and Shaner (1996) continued sub-culturing of *F. graminearum* isolates for eight generations and found no effect on the isolates' virulence. No repeatable host by isolate interaction has been demonstrated across environments for FHB of wheat (Miedaner 2001). Resistance to FHB is of a non race-specific nature and therefore any aggressive strain can be used in screening programs (Snijders and Van Eeuwijk 1991, Van Eeuwijk *et al*. 1995, Bai and Shaner 1996, Mesterhazy 1999). As a result of these findings many FHB nurseries rely on the same aggressive strains of *F. graminearum* to provide macroconidia as a source of inoculum for both greenhouse and field-scale inoculations year after year.

Schroeder and Christensen (1963) first recognized that spray inoculation and point inoculation tested different mechanisms of resistance. The relative rankings of FHB resistance of the same wheat cultivars under point inoculation and spray inoculation indicated that type I resistance (resistance to initial infection) was tested by spray inoculation, and type II resistance (resistance to spread of infection) could be visualized by point inoculation. In contrast, Miedaner *et al.* (2003) reported that spray inoculation detected type I and II resistance but could not differentiate between the two forms of resistance. Field plot-scale spray inoculation gave higher heritabilities than point inoculation for both percentage of infected spikelets and relative head weight in a study with 20 genotypes across seven environments. Mesterhazy (1995) concluded that spray inoculation is preferred to point inoculation for determining the level of field resistance because it allows for selection pressure to act on mechanisms of passive resistance such as the presence of awns, the degree to which florets are open, and retention or release of anthers.

Mesterhazy (1983) drew attention to the need to standardize inoculation dates to physiological stages of the host in FHB nurseries in order to minimize unintentional selection for late maturity. Rossi *et al.* (2001) found that increasing duration of post-inoculation incubation in a high humidity environment increased the percentage of infected glumes and paleas in the bread wheat variety 'Centauro'. They also found that the single largest increase in infection was due to presence or absence of surface moisture on the spikes during the incubation period. Xu *et al.* (2003) described an increase in temperature and duration of moisture during the infection process resulting in an increase in disease incidence. In a greenhouse study where inoculated wheat plants were subjected to different lengths of time in a humid environment following inoculation, Hart *et al.* (1984) found that prolonged humidity increased the DON content and decreased the thousand kernel weight in the harvested grain. Prolonging periods of surface moisture on inoculated spikes decreased the length of time to the development of symptoms (incubation period) and increased the incidence of infection (Parry *et al.* 1995). Del Ponte *et al.* (2007) conducted spray inoculations on the susceptible wheat variety Norm at various stages of growth from anthesis through to the late dough stage, and found that percentage Fusarium damaged kernels (FDK) increased from a baseline of 94% when the plants were inoculated at 50% anthesis (Zadoks 65), to 99.6% FDK when inoculation was applied at the late milk stage (Zadoks 77). The percentage FDK decreased sharply if the first inoculation occurred through the early to hard dough growth stages (Zadoks 83-87). They also found that the relationship between time to onset of symptom development and Zadoks growth stage (Zadoks *et al.* 1974) was inversely related based on five different inoculation stages between Zadoks stages 65 and 83. As the growth stage at which plants were inoculated increased (Zadoks 65, 71, 73, 77, 83) the latent period of infection decreased. Del Ponte *et al.* (2007) found that as long as suitable environmental conditions persisted through the flowering and grain-filling period, infection of kernels and contamination of kernels with DON toxin continued.

Epidemic years of FHB of wheat are generally characterized by above average rainfall and humidity during the period of anthesis and grain filling. Moisture and humidity during anthesis are well known to be major contributing factors for disease

incidence and severity under natural conditions (Osborne and Stein 2007, Parry *et al.* 1995, Schroeder and Christensen 1963). A suitable environment is the largest single factor contributing to epidemic FHB outbreaks, therefore attention to environmental conditions is critical when screening cultivars for resistance (Parry *et al.* 1995). Mesterhazy (1988) found that a 24-hour incubation period under high humidity was needed to ensure adequate development of symptoms to differentiate between test and control groups. Snijders and Perkowski (1990) used a misting system for field scale inoculation, 1hr per 24 hrs, in the evening for a period of two weeks following inoculation. Snijders (1990) used an over-head misting system for over a month during anthesis and grain filling. Miller *et al.* (1998) used overhead sprinklers to mist plots once at mid morning and once at dusk, starting at anthesis for wheat, and emergence of silk for corn. Miedaner *et al.* (1993) used two cycles of misting, the day following inoculation and again three days later, two minutes out of every 45 minutes from 6:00 – 11:30 AM. Lemmens *et al.* (2004) used a misting system to administer two pulses of ten seconds each at 15 minutes intervals from 16:00 h to 12:00 h every second day from just prior to anthesis to 26 days post anthesis. Lemmens *et al.* (2004) concluded that over-head misting during anthesis and grain filling lead to greater symptom development and increased yield loss compared to the treatment with no over-head misting. They also found that there was a significant genotype X mist irrigation interaction for DON content in infected wheat grain.

2.9 Assessment and Selection for Resistance

Visual ratings, although vulnerable to subjective bias of the observer, are possibly the best method for quantifying resistance of wheat to FHB (Gilbert and Tekauz 2000). Visual ratings of FHB in wheat are based on incidence and severity. Incidence is defined as the percentage of symptomatic spikes. Severity is the mean percentage of visually infected spikelets per symptomatic spike for the entire area under question. The spike is divided by the number of spikelets present on average so that each spikelet represents a fraction of the spike. After the first few spikelets the remainder of the spike is partitioned into larger fractions such as quarters or thirds; this is the modified Horsfall-Barrett scale described by Stack and McMullen (1998). Snijders and Perkowski (1990) utilized the product of percentage incidence and percentage severity, termed 'head blight rating'. Miedaner *et al.* (1997) suggested that the head blight rating improved differentiation among genotypes differing in level of resistance. Bai and Shaner (1996) established ratings of severity generated from single floret inoculations as a stable and predictive measure of field resistance. Kolb and Boze (2003) developed the incidence, severity, kernel rating index (ISK index) calculated as, 0.3 x (% Incidence) + 0.3 x (% Severity) + 0.4 x (% FDK) or 4 x (kernel rating on 0-9 scale). Kolb and Boze (2003) cited a lack of separation of means for FHB index of moderately and highly resistant cultivars as the main reason for developing a new quantitative incidence of scabby kernels (ISK) index measurement of FHB resistance that includes observation of fusarium damaged kernels (FDK).

Snijders (1990) rated plots at 14, 21 and 28 days after first development of symptoms and used the mean of infected spikelets from three spikes per plant and ten plants per plot as a rating of head blight resistance, concluding that a single rating at 21 days after development of symptoms was as useful as area under the disease progress curve (AUDPC) for measuring resistance in spring wheat. Jeger *et al.* (2001) compared an estimate of AUDPC based on two data points to an AUDPC from ten data points for stripe rust (caused by *Puccinia striiformis*) of wheat on ten different cultivars and concluded that the two methods of assessment were equivalent with spearman rank

correlations of 0.98 and 0.95 at two sites. Engle *et al.* (2003) found that for FHB of wheat, a single rating at 14 days after inoculation (DAI) was equivalent to AUDPC based on three ratings (7, 10 and 14 DAI) for determining FHB resistance in wheat, supporting the previous findings of Snijders (1990).

2.10 Intra and Inter-Specific Competition of *Fusarium spp.*

Until recently, the predominant *F. graminearum* chemotype in North America was 15-ADON, compared to the 3-ADON chemotype that was predominant worldwide (Miedaner *et al.* 1997). Recent surveys of *F. graminearum* isolates preformed by Guo *et al.* (2008) and Ward *et al.* (2008) have exposed rapid changes to the Canadian and North American *F. graminearum* population structure, respectively. A change has occurred in the population structure of *F. graminearum* races in Manitoba and is progressing westward. Isolates possessing the 3-ADON chemotype are becoming more prevalent than the incumbent 15-ADON chemotype isolates in some regions of Manitoba (Guo *et al.* 2008, Ward *et al*. 2008).

The 3-ADON producing chemotypes are thought to cause more accumulation of mycotoxin in the grain (Von der Ohe *et al.* 2010). Ward *et al.* (2008) found no difference in pathogenicity between 15-ADON and 3-ADON isolates of *F. graminearum* when tested individually and under controlled conditions against a susceptible and a resistant wheat cultivar, despite the fact that 3-ADON isolates produced significantly more trichothecenes *in vivo*. The 3-ADON producing chemotypes grew at a greater rate, and produced more conidia than 15-ADON chemotypes in plate growth assays on potato dextrose agar (PDA) (Ward *et al.* 2008). In a recent study conducted by Von der Ohe *et al.* (2010) twelve isolates of 3-ADON and twelve isolates of 15-ADON were used to inoculate a number of wheat cultivars in order to compare the two groups of isolates' ability to create disease symptoms in terms of FHB index and DON production. Von der Ohe *et al* (2010) concluded that while there was no significant difference between 3-ADON and 15-ADON chemotype in terms of FHB index, the 3-ADON chemotype did produce more DON in the grain.

A study by Miedaner *et al.* (2004), conducted using four different isolates of *F. culmorum* to inoculate winter rye, found that aggressiveness as measured by severity, grain weight per spike, and kernel weight was reduced in isolate mixture treatments as opposed to single isolate treatments. Miedaner *et al.* (2004) isolated single spores from infected kernels and found that aggressiveness, level of toxin production, or type of toxin of individual isolates were poor predictors of the outcome of inter-isolate competition in *F. culmorum* causing FHB. Miedaner *et al.* (2004) concluded that competitive ability varies greatly among *F. culmorum* isolates regardless of their aggressiveness. In the study conducted by Xu *et al.* (2007) of inter-specific competition between mixtures of *F. graminearum* and *F. poae*, *F. avenaceum*, and *F. culmorum* spray inoculated onto the wheat cultivar Claire, *F. graminearum* was detected by PCR more frequently than any of the other co-inoculated species over the range of temperatures and durations of humidity tested. Xu *et al.* (2007) found that interspecific competition of DON producing *Fusarium* spp. resulted in significantly increased accumulation of DON toxin in chaff and kernels compared to treatments where the inoculum consisted of only one species. Miedaner *et al.* (2004) found that there was a significant reduction of trichothecene content of grain when the inoculum was a mixture of *F. culmorum* isolates compared to treatments where the inoculum consisted of single isolates. This finding contradicts findings of Xu *et al.* (2007) of interspecific competition in *Fusarium* spp.

3.0 The effect of three spray inoculation protocols on Fusarium head blight infection of cultivars of common wheat (*T. aestivum)* and of durum wheat (*T. turgidum* spp. *durum*)

3.1 ABSTRACT

Fusarium head blight (FHB) caused by *Fusarium graminearum* Schwabe is a serious disease of wheat that can result in severe losses in yield and quality. Reliable measurement of the host reaction is key to successful resistance breeding of cereals. The objective of this study was to compare the effect of three commonly used field macroconidial inoculation protocols on the expression of resistance in eight cultivars of spring wheat and two cultivars of durum wheat that differ in their reaction to FHB. For the three protocols, plots were inoculated at: 1) 50% anthesis and three days later, 2) first anthesis and then at three day intervals until the end of anthesis or 3) the beginning of heading and at three day intervals until the soft dough stage. A non-inoculated control was also included. Disease incidence and severity were measured at three-day intervals from the onset of first symptoms to senescence. Although there were absolute differences in disease incidence and severity among the protocols over two years of testing, the relative ranking of cultivars was consistent and highly correlated across inoculation protocols, suggesting that the number of inoculations can vary greatly and still allow for the unbiased critical comparative evaluation of the level of resistance in a group of genotypes.

3.2.1 INTRODUCTION

Resistance of wheat (*Triticum aestivum* L.) to Fusarium head blight (FHB) is a quantitative trait controlled by a number of genes varying in contribution to resistance (Nakagawa 1955, Gilbert and Tekauz 1999). Reaction to FHB is also highly influenced by environment which is the single largest contributing factor for the successful establishment of disease (Parry *et al.* 1995). The level of disease resistance varies greatly in registered wheat cultivars in Western Canada, with no cultivars exhibiting complete resistance. In an integrated disease management plan there are many options for mitigating the risk of FHB in wheat. These include, but are not limited to, crop rotation, crop residue management, cultivar selection, preventative crop protection products such as fungicides, and management tools such as disease forecasting systems. Cultivar selection still remains the best option and starting point for an integrated disease management strategy.

Developing cultivars with resistance to FHB depends on the ability of plant pathologists and breeders to replicate the conditions under which FHB epidemics take place and then select the genotypes that exhibit a level of resistance equal to or better than currently available cultivars. Breeding material is screened for resistance en masse in field plots, or on a smaller scale in the greenhouse. Point inoculation involves injecting a suspension of macroconidia directly into the floret, and is used to test for type II resistance which is defined as spread of infection within the spike (Schroeder and Christensen 1963). In the field, inoculation may occur through spray application of the inflorescence with laboratory-cultured macroconidia suspended in water, or through dissemination of ascospores from infected spawn spread on the ground. Field inoculation either through spray or infected grain spawn has advantages over point inoculation in that it is more efficient in terms of time taken to inoculate each plant or plot, and that spray method inoculates the plants in a manner that more closely resembles real life conditions. This allows for selection pressure to act on passive means of resistance such as awns, height, and cleistogamous flowers (Mesterhazy 1995).

Parry *et al* (1995) found that an environment conducive to infection is the single largest controlling variable in FHB epidemics. Misting systems are widely used in FHB field nurseries to provide a suitable environment. Stack *et al.* (1989) found that macroconidia could be used to accurately determine FHB resistance in wheat despite the fact that ascospores are the primary inoculum source under natural conditions. Even though screening for field resistance to FHB is an integral part of wheat breeding programs, the growth stages that demark the critical susceptibility for the host remain a subject of debate. Spray inoculation is typically applied for the first time at the point that 50% of the plants in the plot are at anthesis and a second time 2-4 days later. This is done on a plot-by-plot basis, so that each genotype and replicate is inoculated at the same stage of development. In a field containing thousands of plots, each plot requires individual attention to properly time the inoculation. In contrast, where infested grain spawn is the inoculum source, infested grain is spread between and among the plots in the weeks leading up to anthesis and repeated a number of times over the coming weeks to provide a constant source of inoculum for the entire field, irrespective of the timing of anthesis for individual plots (Paulitz 1996). Uniform infection of the field plots is dependent on suitable environmental conditions being conducive for spore production and release. The advantage of this technique is that a larger number of genotypes can be evaluated. However, unless the time of anthesis of each genotype is recorded and disease ratings are done at a consistent time from anthesis, it is difficult to get a valid comparison of genotype response. Lower disease levels in some genotypes may be a function of later maturity or lack of suitable conditions for infection at the critical growth stage. Despite the differences between the two methods, both have been used effectively to select lines with improved FHB resistance. Some of these lines have been registered. There is a need to be able to develop an inoculation protocol that is less time consuming than spray inoculation of individual plots, provides optimum conditions for infection and can account for differences in maturity of genotypes under test. The objective of this study was to compare the effect of three different inoculation protocols and a non-inoculated check on FHB infection in eight common wheat (*Triticum aestivum*) genotypes and two durum wheat (*Triticum turgidum* spp. *durum*) genotypes that differed in response to *F. graminearum*.

3.3 METHODS & MATERIALS

3.3.1 Experimental design and treatments

A split plot design was used for the layout of the plots in the field. The main plot (MP) effect was the inoculation protocol (four in total) and the sub plot (SP) effect was cultivar (ten in total). The experiment had four replicates, to make a total of 160 plots (4 X 10 X 4). AGROBASE was used to generate the randomization and field plot layout. The experiment was grown at Carman, Manitoba in 2008 and 2009.

Inoculation protocol was the main plot effect. Protocol 1 (MP1) was the control and no inoculum was applied. Protocol 2 (MP2) included two inoculations, the first at 50% anthesis, and the second approximately three days later. Protocol 3 (MP3) involved inoculation at first anthesis, then every three days, until last anthesis (average of six inoculations over approximately three weeks). For protocol 4 (MP4), plots were inoculated at first heading, then every three days, until the soft dough stage (average of nine inoculations over a period of one month).

The genotypes selected for the sub plot effect represented a range of germplasm, including registered cultivars that differ in known reaction to FHB in the field. The susceptible cultivars were; AC Vista, CDC Teal, Amazon, Superb, AC Morse (*T.durum*), and Kyle (*T.durum*). Intermediate cultivars were; AC Cora, AC Barrie. Cultivar 5602HR was considered to be moderately resistant and the experimental line 93FHB37 was classed as resistant.

The plots were four 1 m rows spaced 16 cm apart with a stand density of approximately 280 plants per m^2. The main plots were separated by a 1m wide walkway, with an equally wide walkway between the replicates.

Daily minimum, maximum and mean temperatures and precipitation were monitored at the nearby weather station located on the Ian N. Morrison Research Farm. Figures 3.1 and 3.2 represent conditions in 2008 and 2009 from July 1 to mid-August.

3.3.2 Inoculum Preparation

Four different *F. graminearum* isolates were obtained from Dr. Jeannie Gilbert at the Cereal Research Centre – Agriculture and Agri-Food Canada. The isolates were previously collected from infected wheat in Manitoba, to represent a sample of isolates prevalent in Manitoba. Two of the isolates were 3- ADON producers (M9-04-6, M6-04-4), and two were 15-ADON producers (M1-04-1, M8-04-3). A stock of inoculum was grown out on a plate until sporulation. A 1mm fragment of mycelium was placed in a 500 microlitre eppendorf tube of distilled water and shaken to dislodge spores. The spore suspension was then plated onto water agar and incubated in the dark for 12-18 hours to allow for spore germination. With a dissecting microscope, single spores were excised and transferred to PDA plates to complete the single spore isolation. A single spore culture was generated for each of the isolates and grown out on potato dextrose agar (PDA) for approximately one week. The PDA plates were then used to inoculate the carboxymethyl cellulose (CMC) liquid medium (Appendix 6.2.6). The liquid medium was placed under fluorescent light at room temperature (~22 ^{o}C) while air was pumped constantly into the medium for approximately a week. The medium was then strained through sterile cheesecloth and decanted into sterile glass bottles, then the concentration of macroconidia was determined using a hemacytometer cell counter. The bottled inoculum was stored in the refrigerator at 4 ^{o}C until use, with storage time not exceeding one week.

Figure 3.1 ***Fusarium graminearum*** **macroconidium being cultured in Carboxymethyl cellulose liquid medium.**

3.3.3 Inoculation

Inoculations were conducted at approximately the same time on each inoculation date, shortly after noon. Immediately prior to use, the four isolates were mixed together by adjusting the volumes to obtain an equal concentration of each of the four isolates, The mixture was then diluted with reverse osmosis (RO) water to produce a final total concentration of 50,000 macroconidia/ml. Tween20 (Uniqema Americas LLC) was added to the inoculum at a concentration of 1 ml/litre to act as a wetting agent. The inoculum was applied at a rate of 200 ml per plot, using a backpack-mounted sprayer pressurized to 30 PSI with CO_2 gas using a boom approximately four feet long with 3 flat-fan nozzles at 24 inch spacing. A misting system, used to maintain a humid environment conducive to disease development, was run five minutes per hour from 6 pm until 6 am each night that inoculation had taken place. The spray inoculation protocols

started at different physiological growth stages: first heading, first anthesis, and fifty percent anthesis, and differed in the number of inoculations. Each plot was monitored individually to determine the days on which to begin and end the inoculation treatments.

Figure 3.2 Precipitation, minimum, mean and maximum temperatures during inoculation period for 2008 from July 1stth (183) through August 14th (227) at the Ian N. Morrison Research Farm, Carman, Manitoba.

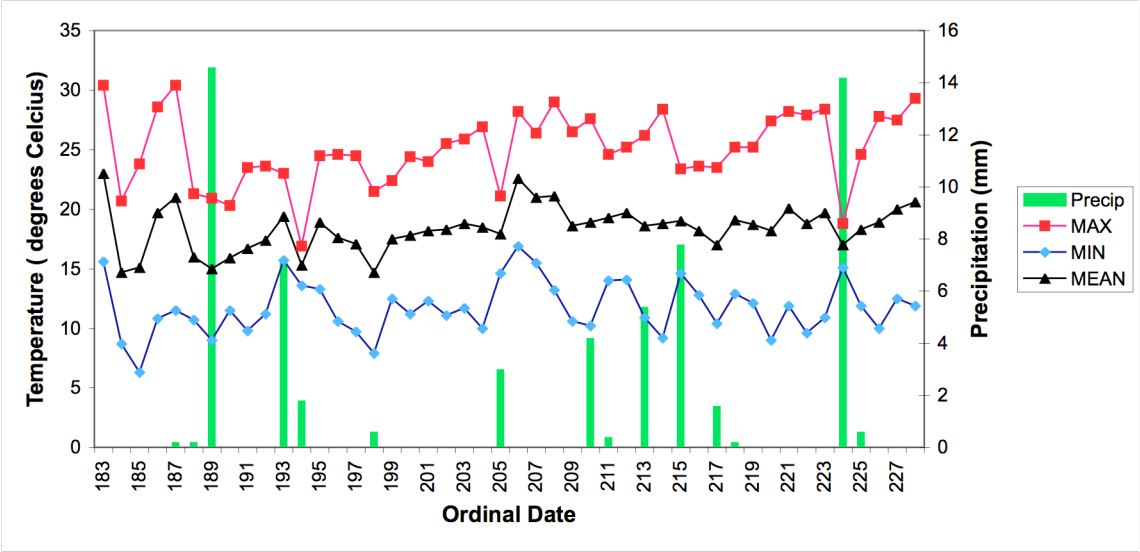


Figure 3.3 Precipitation, minimum, mean and maximum temperatures during inoculation period for 2009 from July 1stth (182) through August 18th (230) at the Ian N. Morrison Research Farm, Carman, Manitoba.

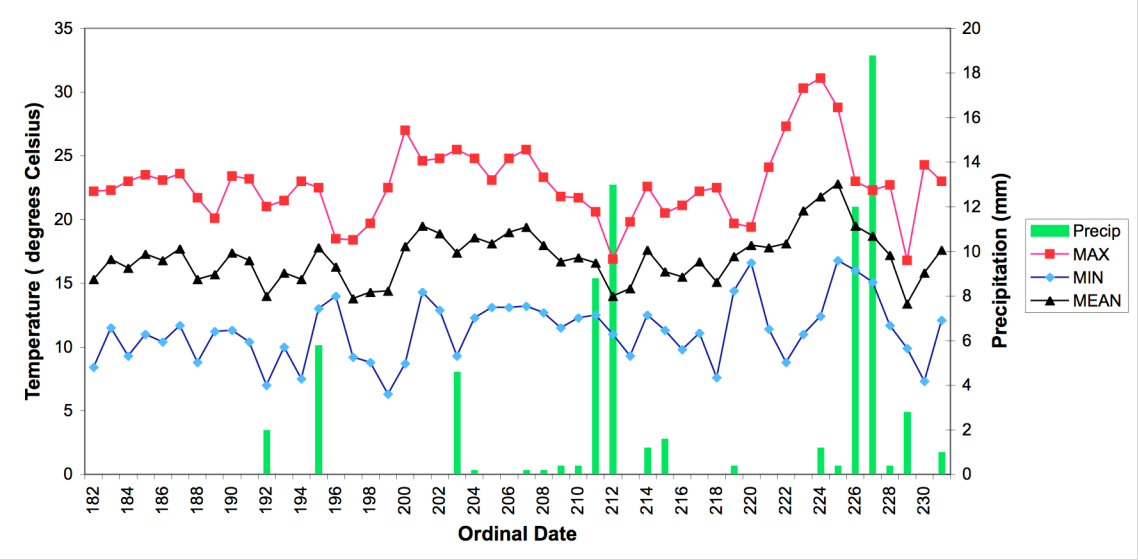
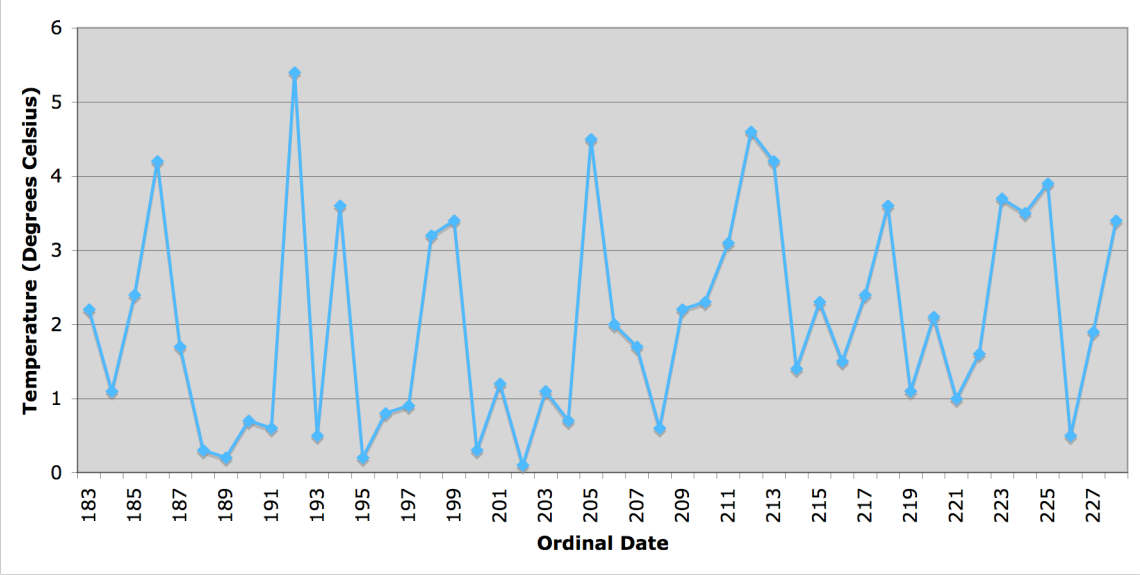


Figure 3.4 Absolute difference in degrees Celsius between mean temperatures for 2008 and 2009 during the period of anthesis to senescence of field plots at the Ian N. Morrison Research Farm, Carman, Manitoba.



3.3.4 Disease measurements

In order to insure a maximum number of observations were obtained, each plot was monitored for the development of symptoms each day after inoculation and visually rated for disease incidence and disease severity every three days from the development of first symptoms until the symptoms were indistinguishable from natural senescence. Symptoms usually became visually noticeable approximately a week after inoculation. Observations were taken every three days in order to collect sufficient data to build an area under the disease progress curve. Disease incidence was the percentage of spikes in the plot that had symptoms, and disease severity was the average percentage of diseased spikelets on symptomatic spikes. The FHB index was calculated from the product of the incidence and severity measurements and standardized to a percentage by dividing by 100. Disease progression was measured by calculating area under the disease progress curve for each plot. The percentage of *Fusarium* damaged kernels (FDK) was determined by taking a random sample of 100 kernels from each plot and counting the number of *Fusarium* damaged kernels based on visual appearance. Samples of 30 g per plot were used to determine DON content in parts per million (ppm) using the ELISA method (see appendix 6.2.4).

In 2008, the accuracy of visual ratings was confirmed by rating the incidence and severity from a single row in each plot, immediately harvesting that row, placing the sample in a cooler with ice and transporting it back to the Department of Plant Science. Samples were frozen and stored at -20^{o}C until the incidence and severity could be hand counted on a sample of fifty spikes randomly taken from the total spikes harvested from that particular row. The visual ratings that took place in the field were then correlated to the hand counts of incidence and severity. The hand counts were carried out for the first replication only because the correlation proved to be very high. The correlations of visual rating to hand count for the first replication are presented as Table 3.1 below.

Table 3.1. Correlation of hand counted and visually assessed Fusarium head blight incidence, severity and FHB index on a single replicate of the 2008 field study.

Hand counted		Visually rated	
	Incidence	Severity	FHB Index
Incidence	0.93		
Severity		0.88	
FHB Index			0.93

3.3.5 Single rating at 21 days after 50% anthesis

From a breeding perspective, it is impractical to conduct multiple measurements of FHB reaction for thousands of lines in a FHB screening nursery in order to establish an AUDPC for each line. In most cases, lines may be evaluated as few as one or two times per replicate. There are a number of reasons for this: 1) disease rating is time consuming, 2) disease ratings will change as time progresses and requires enough labor to complete the necessary ratings in a short period of time, and 3) studies have demonstrated that one or two ratings at the right time may be equivalent to an AUDPC (Snijders 1990). In order to compare the inoculation protocols based on a single rating as would be done in a standard screening nursery, the point of 21 days after 50% anthesis was chosen. The 21 day period following 50% anthesis is commonly used by breeders and pathologists and allows for sufficient time for the disease to develop and to easily differentiate among genotypes with different reactions to FHB. Both the host and the pathogen respond to changes in the environment, and differences in environmental conditions from one year to the next can result in quite different rates of disease development or host response over the same period of time in different years. Although 21 days after 50% anthesis is commonly used for nursery evaluation cool conditions in 2009 (Figure 1.2) significantly delayed disease progression. As a result, growing degree days (GDD) (See appendix 6.2.5) were used to standardize and approximate the correct day to take and compare the ratings for 2009. Twenty-one days after 50% anthesis was determined on a plot-by-plot basis for 2008, the GDD accumulated between 50% anthesis and 21 days later, were

calculated. The accumulated GDD for 2008 was then used to determine the date of best fit in 2009 on which to take the rating. This was accomplished by adding the days required to accumulate these GDD onto the date of 50% anthesis. The adjustment for GDD resulted in a much better comparison of the data than if the arbitrary 21st day after 50% anthesis was used.

3.3.6 Data analysis

Data were analyzed using ANOVA in PROC GLM, T-Tests, and spearman rank correlations in the SAS version 9.1 software. For the ANOVA model statement the dependent variables were incidence, severity, FHB index, FDK and DON. The main-plot effect (MP) was the inoculation protocol, and the sub-plot effect (SP) was the ten wheat genotypes. The independent variables were Year, Block (Year), MP, MP*Year, Block*MP*Year, SP, SP*MP, SP*Year, SP*MP*Year and Year*MP. Random variables included Block (Year) and Block*MP*Year, the remaining variables were treated as fixed effects. The error term used to test the MP and MP*Year effect was Block*MP*Year, the error term used to test Year effect was Block (Year), and the error term used to test SP was SP*Year.

The decision to combine the two years data was made by examining the results of the Proc univariate and plot of residual by predicted values for FHB index, FDK and DON content. Despite finding significant differences between the years in ANOVA the distribution of residual by predicted values was sufficiently normal to warrant combining the data for analysis (see appendix 6.2.1 through 6.2.3).

3.4 RESULTS

3.4.1 Two-year combined analysis of AUDPC FHB index.

Table 3.2 shows that there was a significant difference among inoculation protocols in terms of AUDPC for FHB index ($P \leq 0.001$) even when the control group was removed. The difference between the control group and the inoculation protocols accounted for the majority of the main plot variation. There was also a significant protocol by genotype interaction ($P \leq 0.001$) for AUDPC FHB index (Table 3.2). The spearman rank correlations for AUDPC FHB index of the genotypes in Table 3.3 shows that the lowest correlation was 0.9 while the highest was 0.98, indicating nearly perfect ranking of genotypes across inoculation protocols for the two years combined. Table 3.4 shows the two most resistant genotypes rank as the most resistant across all treatments. The two most resistant genotypes also retain distinct LSD t-test letter groups across the three different inoculation protocols. While the ANOVA indicates the protocols are significantly different and that protocol by genotype interactions exist ($P \leq 0.001$) the spearman rank correlations and the LSD T-tests both suggest that these differences, while they do exist, may be of little biological importance in selecting the most resistant genotypes. It is worth noting that the non-inoculated control group, which can be assumed to have either been infected by natural inoculum or to some extent, drift from the inoculated treatments, produced spearman rank correlations with other protocols that were very high (0.90, 0.94, 0.94 for MP3, MP2, MP4 respectively).

Similar results were seen with AUDPC incidence and severity, where despite significant differences among treatments ($P < 0.001$), the spearman rank correlations indicated that all inoculation protocols produced similar results and LSD t-tests easily differentiated the most resistant genotypes (data not shown).

Table 3.2. Analysis of variance of combined data from 2008 and 2009 for area under the disease progress curve of Fusarium head blight index of ten spring wheat genotypes inoculated under different inoculation protocols.

Source of variance	df	mean squares
Year	1	4128929.77 ***
Block(Year)	6	1307.34 NS
Protocol	3	5249939.63 ***
Protocol Vs. Control contrast	1	15452411.08 ***
Among innoculated protocols	2	148703.9 ***
Year*Protocol	3	11725.67 NS
Year*Block*Protocol	18	13353.38 *
Genotype	9	1699260.05 ***
Protocol*Genotype	27	87759.16 ***
Year*Genotype	9	214870.74 ***
Year*Protocol*Genotype	27	18737.73 ***
Error	216	7067.31

NS = not significant at p = 0.05, * = significant at p = 0.05, ** = significant at p = 0.01, *** = significant at p = 0.001.

Table 3.3. Spearman rank correlations for the rank of genotypes across inoculation protocols in terms of area under the disease progress curve of Fusarium head blight index of both years (2008, 2009) combined.

	(1)	(2)	(3)	(4)
(1) MP1	1	0.94 <.0001	0.9 0.0003	0.94 <.0001
(2) MP2		1	0.98 <.0001	1 <.0001
(3) MP3			1	0.98 <.0001
(4) MP4				1

The top number is the spearman rank correlation coefficient, and the bottom number the probability of observing a correlation equal to (or closer to |1|) under the null hypothesis that the true correlation is zero. MP1 - uninoculated control group, MP2 - inoculated at 50% anthesis and three days later, MP3 - inoculated from beginning through to end of anthesis, MP4 - inoculated from heading through to soft dough stage

Table 3.4. Genotype mean comparison under different inoculation protocols using the least significant difference T-test of data for area under the disease progress curve of Fusarium head blight index. Genotypes with the same least significant difference t-test letter grouping in a column are not significantly different. Data are for 2008 and 2009 combined. Alpha error rate of 0.05.

	MP1	MP2	MP3	MP4
CDC Teal	220.17 ab	945.19 a	995.54 a	991.54 a
AC Vista	265.70 a	945.64 a	930.21 ab	1041.55 a
AC Morse	190.03 b	862.16 a	876.45 b	905.26 b
AC Cora	89.09 cd	563.78 b	757.77 c	689.60 c
Superb	112.63 c	637.61 b	702.29 cd	737.11 c
Amazon	47.89 de	548.07 b	613.52 de	682.31 c
Kyle	105.97 c	432.63 c	516.65 ef	499.58 d
AC Barrie	51.59 de	428.41 c	482.27 f	465.17 d
5602HR	17.18 e	213.47 d	303.10 g	318.41 e
93FHB37	8.07 e	118.03 e	164.89 h	181.29 f
LSD (0.05)	47.49	92.66	103.79	82.43

MP1 - uninoculated control group
MP2 - inoculated at 50% anthesis and three days later
MP3 - inoculated from beginning through to end of anthesis
MP4 - inoculated from heading through to soft dough stage

3.4.2 The effect of inoculation protocol on DON ppm in grain samples

There was no significant effect of inoculation protocol on DON accumulation ($P \leq 0.05$) in the analysis of combined years once the effect of the control group was partitioned out (Table 3.5). Also there was no significant protocol by genotype interaction. Spearman rank correlations for DON concentration (ppm) among genotypes under different inoculation protocols resulted in correlation coefficients ranging from 0.79 to 0.93 for the combined years (Table 3.6). In the LSD t-test (Table 3.7) there were

no distinct LSD t-test letter groupings for either the two most resistant or the two most susceptible genotypes This is most likely due to a high error variance, as indicated by the large LSD values.

Table 3.5. Analysis of variance of the 2008 and 2009 combined data for deoxynivalenol (DON) measured in parts per million in grain samples of ten spring wheat genotypes that were inoculated under using different inoculation protocols.

Source of variance	df	mean squares
Year	1	4165.94 ***
Block(Year)	6	273.40 ***
Protocol	3	3313.36 ***
Protocol Vs. Control contrast	1	9882.95 ***
Among innoculated protocols	2	28.57 NS
Year*Protocol	3	731.98 ***
Year*Block*Protocol	18	79.10 NS
Genotype	9	607.18 ***
Protocol*Genotype	27	59.76 NS
Year*Genotype	9	50.23 NS
Year*Protocol*Genotype	27	26.70 NS
Error	216	67.01

NS = not significant at p = 0.05, * = significant at p = 0.05, ** = significant at p = 0.01, *** = significant at p = 0.001.

Table 3.6. The effect of inoculation protocol on deoxynivalenol parts per million in grain samples represented by the Spearman rank correlations of genotypes across inoculation protocols. Data are for both years (2008 and 2009) combined.

	(1)	(2)	(3)	(4)
(1) MP1	1	0.73 0.0158	0.68 0.0289	0.88 0.0008
(2) MP2		1	0.79 0.0061	0.93 0.0001
(3) MP3			1	0.84 0.0022
(4) MP4				1

The top number is the spearman rank correlation coefficient, and the bottom number the probability of observing a correlation equal to (or closer to |1|) under the null hypothesis that the true correlation is zero. MP1 - uninoculated control group, MP2 - inoculated at 50% anthesis and three days later, MP3 - inoculated from beginning through to end of anthesis, MP4 - inoculated from heading through to soft dough stage

Table 3.7. Least significant difference T-test of data for deoxynivalenol in grain samples indicating the grouping of the genotypes under different inoculation protocols. Data are for both years (2008 and 2009) combined.

	MP1	MP2	MP3	MP4
AC Vista	6.913 b	30.263 a	22.75 ab	25.475 a
Superb	4.338 bcd	20.913 ab	26.2 a	22.775 ab
AC Morse	11.763 a	19.538 b	21.613 ab	25.525 a
Amazon	3.125 cd	17.425 b	14.625 b	17.288 bcd
CDC Teal	5.125 bc	17.013 bc	21.138 ab	20.038 abc
Kyle	9.888 a	16.95 bc	18.688 ab	18.113 abcd
5602HR	2.55 cd	15.963 bc	14.813 b	14.175 dc
AC Barrie	1.963 d	14.088 bc	13.25 b	13.463 dc
AC Cora	1.788 d	11.9 bc	15.663 b	12.888 dc
93FHB37	2.063 d	7.025 c	13.625 b	10.388 d
LSD (0.05)	2.8823	10.205	9.7914	7.8143

MP1 - uninoculated control group
MP2 - inoculated at 50% anthesis and three days later
MP3 - inoculated from beginning through to end of anthesis
MP4 - inoculated from heading through to soft dough stage

3.4.3 The effect of inoculation protocol on FDK

There was a significant effect of inoculation protocol on percentage FDK in the combined years analysis ($P \leq 0.01$) (Table 3.8). Protocol*genotype was also ($P \leq 0.001$). Despite the highly significant protocol*genotype interactions the Spearman rank correlations remained high, ranging from 0.87 to 0.89 (Table 3.9). The LSD T-test shows the absence of distinct LSD t-test letter groupings for the two most resistant genotypes across all protocols and the uninoculated control group (MP1). Again as with DON, relatively large LSD values seen in Table 3.10 indicated a high degree of variance, which made detecting significant differences in FDK difficult.

Table 3.8. Analysis of variance for percentage of Fusarium damaged kernels in grain samples from ten spring wheat genotypes under different inoculation protocols. Data are for both years combined (2008 and 2009).

Source of variance	df	mean squares
Year	1	25543.37 ***
Block(Year)	6	299.88 **
Protocol	3	24851.54 ***
Protocol Vs. Control contrast	1	71501.28 ***
Among innoculated protocols	2	1526.68 **
Year*Protocol	3	5261.21 ***
Year*Block*Protocol	18	177.64 **
Genotype	9	6121.30 ***
Protocol*Genotype	27	430.89 ***
Year*Genotype	9	382.12 ***
Year*Protocol*Genotype	27	181.95 ***
Error	216	78.66

NS = not significant at p = 0.05, * = significant at p = 0.05, ** = significant at p = 0.01, *** = significant at p = 0.001.

Table 3.9. The effect of inoculation protocol on Fusarium damaged kernels in grain samples represented by the Spearman rank correlations of genotypes across inoculation protocols combined over two years (2008 and 2009).

	(1)	(2)	(3)	(4)
(1) MP1	1	0.96 <.0001	0.92 0.0002	0.90 0.0003
(2) MP2		1	0.89 0.0005	0.87 0.0012
(3) MP3			1	0.89 0.0005
(4) MP4				1

The top number is the spearman rank correlation coefficient, and the bottom number the probability of observing a correlation equal to (or closer to |1|) under the null hypothesis that the true correlation is zero. MP1 - uninoculated control group, MP2 - inoculated at 50% anthesis and three days later, MP3 - inoculated from beginning through to end of anthesis, MP4 - inoculated from heading through to soft dough stage

Table 3.10. Least significant difference (LSD) T-test of data for Fusarium damaged kernels in grain samples indicating the LSD t-test letter grouping of the genotypes under different inoculation protocols. Data are for both years (2008, 2009) combined. Alpha error rate of $P \leq 0.05$.

	MP1	MP2	MP3	MP4
AC Vista	14.63 b	63.88 a	73.00 a	68.13 a
AC Morse	18.38 a	66.00 a	65.25 ab	66.88 a
CDC Teal	10.88 c	43.75 bc	62.13 b	60.63 ab
Kyle	13.38 bc	48.25 b	50.13 c	40.25 de
AC Cora	4.63 ef	26.38 de	46.75 c	38.50 ef
Amazon	7.00 de	45.25 b	46.50 c	48.75 cd
Superb	9.88 cd	33.88 cd	44.50 c	54.63 bc
5602HR	3.50 ef	19.75 e	26.13 d	29.50 fg
AC Barrie	4.00 ef	20.63 e	24.50 d	25.00 g
93FHB37	3.25 f	16.63 e	23.88 d	24.75 g
LSD (0.05)	3.63	11.07	9.85	9.14

MP1 - uninoculated control group
MP2 - inoculated at 50% anthesis and three days later
MP3 - inoculated from beginning through to end of anthesis
MP4 - inoculated from heading through to soft dough stage

3.4.4 Two year combined analysis of FHB index for a single rating approximately 21 days after 50% anthesis.

Table 3.11 shows a significant effect ($P \leq 0.01$) of inoculation protocols on FHB index at a single rating approximately 21 days after 50% anthesis. Despite the significant effect of inoculation protocol, when the Spearman rank correlations are examined there is nearly perfect correlation ranging from 0.96 to 1 (Table 3.12). Table 3.13 shows the LSD t-test for a single rating of both years combined and indicates that the two most resistant genotypes remain stable, sharing the same LSD t-test letter grouping across all three inoculation protocols, distinct from all other genotypes. Table 3.11 also indicates no

significant three-way interaction occurred between year, inoculation protocol and genotype, nor any significant interaction between year and protocol. This suggests that the protocols, despite interacting with the genotypes to some degree produce consistent results across years and that the spearman rank correlation and the LSD T-test reveal a high degree of similarity in the ranking of the genotypes in terms of FHB index.

Table 3.11. Analysis of variance for Fusarium head blight index for ten spring wheat genotypes under different inoculation protocols using a single rating approximately 21 days after 50% anthesis in both 2008 and 2009.

Source of variance	df	mean squares
Year	1	878.57 ***
Block(Year)	6	39.47 NS
Protocol	3	31293.51 ***
Protocol Vs. Control contrast	1	91658.82 ***
Among innoculated protocols	2	1110.22 **
Year*Protocol	3	86.15 NS
Year*Block*Protocol	18	167.50 ***
Genotype	9	9855.53 ***
Protocol*Genotype	27	784.78 ***
Year*Genotype	9	1181.05 ***
Year*Protocol*Genotype	27	85.06 NS
Error	216	59.44

NS = not significant at p = 0.05, * = significant at p = 0.05, ** = significant at p = 0.01, *** = significant at p = 0.001.

Table 3.12. The effect of inoculation protocol on Fusarium head blight index of ten genotypes of spring wheat using a single rating approximately 21 days after 50% anthesis represented by the Spearman rank correlations. Data are for 2008 and 2009 combined.

	(1)	(2)	(3)	(4)
(1) MP1	1	0.96	1	1
		<.0001	<.0001	<.0001
(2) MP2		1	0.96	0.96
			<.0001	<.0001
(3) MP3			1	1
				<.0001
(4) MP4				1

The top number is the spearman rank correlation coefficient, and the bottom number the probability of observing a correlation equal to (or closer to |1|) under the null hypothesis that the true correlation is zero. MP1 - uninoculated control group, MP2 - inoculated at 50% anthesis and three days later, MP3 - inoculated from beginning through to end of anthesis, MP4 - inoculated from heading through to soft dough stage

Table 3.13. LSD T-test of data for FHB index from a single rating approximately 21 days after 50% anthesis of ten spring wheat genotypes following different inoculation protocols at Carman, MB in 2008 and 2009. Alpha error rate of $P \leq 0.05$.

	MP1	MP2	MP3	MP4
AC Vista	10.96 a	69.11 a	70.5 a	83.22 a
AC Morse	7.36 a	66.16 a	62.59 ab	65.31 b
CDC Teal	7.77 a	64.87 a	67.57 a	66.17 b
Amazon	2.93 b	49.86 b	53.94 bc	61.27 b
Superb	1.73 b	35.36 c	42.18 d	43.19 c
AC Cora	2.02 b	33.82 cd	51.72 c	47.79 c
AC Barrie	0.99 b	25.01 d	28.53 e	28.24 d
Kyle	1.21 b	24.82 d	31.77 e	31.38 d
5602HR	0.23 b	8.79 e	14.96 f	18.38 e
93FHB37	0.16 b	7.81 e	10.39 f	13.88 e
LSD (0.05)	4.01	9.51	8.93	7.26

MP1 - uninoculated control group

MP2 - inoculated at 50% anthesis and three days later

MP3 - inoculated from beginning through to end of anthesis

MP4 - inoculated from heading through to soft dough stage

3.5 DISCUSSION

This two-year study showed that the relative ranking for resistant and susceptible wheat genotypes were stable across the different inoculation protocols. Despite this, there were absolute differences in terms of symptom development and post harvest measurements among the different inoculation protocols, and significant inoculation protocol by genotype interactions for FHB index for both AUDPC and the single rating analysis.

There were statistically significant protocol by genotype interactions for all response variables except DON content, but when the LSD t-tests are examined the most resistant cultivars remain in a stable rank while the genotypes of intermediate resistance frequently change rank. Therefore, change in ranking of intermediate genotypes was the main reason for the statistically significant inoculation protocol by genotype interaction.

Del Ponte *et al.* (2007) conducted a greenhouse study with the susceptible cultivar, Norm, to examine the effect of growth stage during inoculation on symptom development and found that the fraction of visually scabby kernels was very stable for inoculations occurring from Zadoks stage 65 – 77, or 50% anthesis through late milk, with a range of 94% to 99%. In our study the two-year treatment means for FDK were 38%, 46% and 45% for treatments MP2, MP3 and MP4 respectively. These results were reflected in the ANOVA that depicted a significant effect of treatment on FDK ($P \leq 0.01$). From these ANOVA results we can extrapolate that as the number of inoculations applied increases, the occurrence of FDK increases. However, as the greatest difference between the protocol means is only 7% (MP4 and MP2), and the spearman rank correlations among protocols are strong (ranging from 0.87 – 0.89), it is fair to conclude that despite absolute differences among the protocols the resistance rankings of the genotypes tested remained stable across protocols.

Del Ponte *et al.* (2007) observed that DON (mg/kg) increased dramatically from inoculations occurring at 50% anthesis to inoculations occurring at kernel watery ripe

stage, but then started to decline towards its lowest levels found in kernels from plants that were inoculated at hard dough. In a field study using ten winter wheats, Lemmens *et al.* (2004) found that two applications of inoculum (Zadoks 65 and again 2 days later) versus a single application (Zadoks 65) had a significant effect ($P \leq 0.05$) on AUDPC incidence. Lemmens *et al.* (2004) also found that two applications of inoculum had a significant effect on DON content ($P \leq 0.05$), in the non-misted treatments, while in the misted treatments there was no significant effect of additional applications of inoculum on DON content ($P \leq 0.05$). In contrast to the findings of Del Ponte *et al.* (2007) this study found no significant difference of inoculation protocol on DON content in grain for the two years combined. These results agreed with those of Lemmens *et al.* (2004) where no significant effect ($P \leq 0.05$) of inoculation protocol on DON content was observed in the combined years. This may be due to the inoculation protocols themselves, where Del Ponte *et al.* (2007) used single applications of spray inoculum at discrete growth stages, the protocols in this study and in Lemmens *et al.* (2004) all overlapped the period of Zadoks 65 (50% anthesis), which is generally regarded as the critical period for disease susceptibility.

Xue *et al.* (2006) compared the effect of source of inoculum and number of applications on development of symptoms in five wheat genotypes in field plots over three years using spray inoculation, infested barley and corn kernels and infested wheat debris as sources of inoculum. The study monitored disease severity, percent infected spikelets and FHB index at soft dough, and post harvest AUDPC, FDK and DON content. For the spray inoculation component, the study compared the effect of two applications of inoculum (Zadoks 65 and again three to four days later) versus three applications (Zadoks 65 and two additional applications three days apart) of macroconidial spray inoculum. The inoculation protocols using infested kernels and infested debris consisted of two applications three and two weeks before anthesis, or three applications three, two and one week before anthesis. When the three years were combined, the only significant genotype X inoculation protocol interaction was for percentage of infected spikelets ($P \leq 0.01$). The comparison of two versus three applications of inoculum resulted in no significant difference ($P \geq 0.15$) for any measure of

symptoms in any of the individual years or all years combined for all sources of inoculum. Yang *et al.* (1999) found that Spearman rank correlations for 250 F_5 lines inoculated with either single floret inoculation or infested grain spawn was 0.74, 0.79 and 0.77 for infected spikelet rate, reaction index, and disease index, respectively. Paulitz (1996) found when infested corn kernels were used as a source of inoculum that ascospores were released over a period of between two and three weeks. Considering this observation by Paulitz (1996), the studies of Xue *et al.* (2006) and Yang *et al.* (1999) serve to compare the effects of application of inoculum over a wide range of plant growth stages. Their results are relevant to this study despite the differences in the methods of inoculation.

In conclusion, this study has demonstrated that although there was a significant effect of inoculation protocol on symptom development, and that there was significant inoculation protocol X host genotype interaction, these effects were largely localized to the genotypes of intermediate resistance and as a whole the Spearman rank correlations remained high. As a result, the most resistant genotypes remained stable across inoculation protocols. Results from the current study demonstrate that inoculum applied over a wide range of growth stages between heading and soft dough (Zadoks 59 through 85) will produce substantially equivalent results so long as inoculation includes the period of 50% anthesis. As the most resistant genotypes are the ones to be advanced through the selection process, the ranking and stability of genotypes with susceptible and intermediate reactions to FHB are of lesser importance and the application of these findings could greatly contribute to the efficiency of FHB screening on a field scale. An application of these findings is that the inoculation protocols used in FHB screening programs can be adapted to have a wider window of physiological susceptibility of the host and spray inoculation could be carried out en masse instead of on each individual row. If these findings were used to adapt the inoculation protocol from individual rows to mass inoculations, backpack sprayers used to apply inoculum could be replaced with boom-type sprayers mounted to an all terrain vehicle or a three-point hitch of a tractor. These adaptations would dramatically increase the efficiency and capacity of the screening program, thereby increasing the probability of finding FHB resistant genotypes.

4.0 *In planta* competition between 3-ADON & 15-ADON chemotypes of *Fusarium graminearum* on wheat (*Triticum aestivum*)

4.1 ABSTRACT

Currently there is a population shift occurring between chemotypes of *Fusarium graminearum*, the causal agent of Fusarium head blight (FHB) of wheat. Although the reasons for the population change are not fully understood, differences in competition between isolates of different chemotype may be one explanation. The primary objective of this experiment was to examine the outcome of *in planta* competition between isolates of 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) chemotype. This was done by conducting two studies, one in the field and another under controlled conditions. Two isolates of each chemotype were selected. Previous work showed that these isolates were similar for aggressiveness. The isolates were cultured separately and macroconidial concentrations were determined. For the controlled environment study, isolates were mixed in four different pairs of combinations of 3-ADON and 15-ADON, a treatment of all four isolates combined and a control treatment of water, for a total of six treatments. If the inoculum mixtures are comprised of equal parts of the isolates, the outcome of the chemotype specific PCR will indicate how *in planta* competition may be contributing to the overall population shift that has been observed. The mixtures served as inoculum for dual floret inoculation of a highly susceptible line (CDC Teal) and a highly resistant line (93FHB37) of wheat (*Triticum aestivum*). After harvest, kernels from the susceptible line were used to generate single spore cultures of *Fusarium graminearum*. Polymerase chain reaction (PCR) was used to determine the chemotype of approximately 100 single spore isolations per treatment. No significant differences were found among treatments for AUDPC for incidence for either greenhouse study or growth room study. Only two treatments had consistent results across both the greenhouse and growth chamber, with the re-isolation ratios differing significantly from a 1:1 ratio in favor the 15-ADON isolate M8-04-3. Each time the chemotype re-isolations failed to fit a 1:1 ratio it was in favor of the 15-ADON isolates, which is opposite to the phenomenon that is being observed in the natural environment.

The field study consisted of the wheat genotypes CDC Teal, AC Barrie, Superb, 5602HR and 93FHB37. The treatments were three different inoculation protocols that

differed in the number of inoculations and the growth stages of the plots at inoculation, plus a control group that was inoculated with water. The inoculum was comprised of a four-way mixture of equal parts of 2 isolates of 3-ADON and 2 isolates of 15-ADON *F. graminearum.* The seed from these plots provided the samples for chemotyping. In all instances where the chemotype re-isolations differed significantly from a 1:1 ratio it was in favor of the 3-ADON isolates. There were no inoculation protocols for which significant variation from the 1:1 ratio was consistent over both years. AC Barrie was the only cultivar for which the re-isolations ratios were consistently different than a 1:1 ratio for both years of the study.

4.2 INTRODUCTION

In North America, *Fusarium graminearum* Schwabe is the primary causal agent of Fusarium head blight (FHB) of wheat (*T.aestivum* L.) (Wagacha and Muthomi 2007, McMullen *et al*. 1997). *Fusarium* spp. causing FHB produce potent mycotoxins called trichothecenes. These mycotoxins play an active role in pathogen virulence. In studies where the ability of the pathogen to produce trichothecenes has been disrupted, the pathogen's virulence has been dramatically reduced (Maier *et al.* 2006, Cuzick *et al.* 2008, Eudes *et al.* 2001). Inhibition of protein synthesis in eukaryotes is one of the major effects of exposure to trichothecenes. Though not all trichothecenes possess this trait, deoxynivalenol which is produced by *F. graminearum* does inhibit protein synthesis. This mode of action affects infected wheat plants and humans and livestock that consume trichothecene contaminated grain (Harris and Gleddie 2001, Rocha *et al.* 2005, Desjardins and Hohn 1997). In addition, infection results in yield loss in small grains from shrunken, low-density kernels, floral abortion resulting in poor seed set (Snijders 2004) and reduction in baking and milling quality (Parry *et al.* 1995, Snijders 2004).

Ward et al (2007) concluded that a dramatic shift across North America from 15-acetyldeoxynivalenol (15-ADON) to 3-acetyldeoxynivalenol (3-ADON) chemotypes is occurring. The appearance of the 3-ADON chemotype of *F. graminearum* in North America is a recent event, with little record of its presence before 1998 (Ward et al 2007). Waalwijk et al (2003) reported that changes in environmental conditions or cultural factors such as increased corn acreage could account for a similar chemotype shift witnessed in the Netherlands. Until recently, FHB in Manitoba was predominately caused by the 15-ADON chemotype of *F. graminearum,* but this has been changing rapidly as isolates of the 3-ADON chemotype are increasing in range and displacing the 15-ADON chemotype (Guo *et al.* 2008).

In vitro analysis revealed that 3-ADON chemotypes have faster rates of growth than 15-ADON chemotypes, and also produced significantly more macroconidia ($P <$

0.005), and a higher trichothecene concentration in growth media ($P < 0.001$) (Ward *et al* 2007). Ward *et al* (2007) found that significantly higher deoxynivalenol (DON) content accumulated in grain of the FHB susceptible wheat cultivar Roblin when inoculated with 3-ADON isolates than when inoculated with15-ADON isolates ($P > 0.05$). This finding was supported by a field study where 3-ADON and 15-ADON chemotypes of *F. graminearum* were used to inoculate a number of wheat cultivars (Von der Ohe *et al*. 2010). The increase in toxin concentration produced by the 3-ADON chemotype poses a significant threat to the quality of grain marketed by Canadian wheat producers, as infection by 3-ADON chemotypes produce more DON toxin in grain. Currently, the Canadian Food Inspection Agency (CFIA) has set DON content in soft wheat destined for human consumption at 2 ppm, and in the United States the maximum is 1 ppm for finished wheat products. The joint FAO/WHO expert committee on food additives set a provisional maximum tolerable daily intake (PMTDI) at 1 ug/kg by weight for DON and acetylated derivatives (JECFA 2011). The Canadian grain commission has set the maximum allowable FDK limit for No.1 Canada Western Red Spring (CWRS) and Hard White (CWHW) wheat at 0.25% by weight. The objective of this study was to examine the outcome of *in planta* competition between *F. graminearum* isolates of 3-ADON and 15-ADON chemotype, the effect of different inoculum mixtures on development of symptoms in spring wheat cultivar CDC Teal, and the effect of wheat genotype and inoculation protocol on *in planta* competition between 3-ADON and 15-ADON isolates. This study may give insight into the *F. graminearum* population shift that is occurring in the wheat growing areas of the Red River valley.

4.3 MATERIALS & METHODS

Samples for this set of experiments were collected from both field and greenhouse studies.

4.3.1 Field Study

Seed harvested from FHB-infected wheat plots from the inoculation protocol study conducted in 2008 and 2009 was used to examine the outcome of competition between the 15-ADON and 3-ADON chemotypes of *F. graminearum*. The description of the materials and methods for this experiment are detailed in Chapter 3 of this document, pages 32 - 33. Field plots were inoculated with a mixture of equal parts of two 3-ADON isolates (M9-04-6 and M6-04-4) and two 15-ADON isolates (M1-04-1 and M8-04-3). Plots were inoculated under three different inoculation protocols that differed in the number of inoculations applied, and an uninoculated check was included. Five spring wheat genotypes representing a range of resistance to FHB were selected to draw samples for the field component of the chemotype study; CDC Teal, Superb, AC Barrie, 5602HR and 93FHB37. The experiment was replicated four times. The plots were harvested and the seed was saved. The seed samples were used as the source of inoculum to make single spore cultures. Fifty grams of seed from each of the four replications were combined, after which fourteen kernels were randomly chosen for each of the five genotypes under the four treatments. The lab of Mr. Randall Clear at the Canadian Grain Commission, Winnipeg, Manitoba, Canada, carried out the culture of colonies from infected seed, single spore isolations and chemotyping *via* polymerase chain reaction (PCR). The protocol used to generate the single spore isolations is described below in section 4.3.6. The chemotype of each single spore isolate was determined using PCR, the protocol used is described below in sections 4.3.7 and 4.3.9.

4.3.2 Controlled Environment Study

The controlled environment study was conducted in two locations: A walk-in growth room at the Department of Plant Science, and the greenhouse at the Crop Technology Centre, University of Manitoba. For both of the controlled environment studies the Cornell mix nutrient blend with Sunshine LA4 was used (Appendice 6.2.7). The plants were watered by hand as required, and supplementary nutrients were provided as 15 ml of 20-20-20 per gallon of water, every two weeks. For both the growth room and the greenhouse the light cycle was set for 16 h day, 8 h night. In the greenhouse the temperature settings were 18-22 ^{o}C daytime and 14-18 ^{o}C nighttime, in the growth room the temperature was set to 21 ^{o}C daytime and 19 ^{o}C nighttime. The same four *F. graminearum* isolates that were used for the field study were used in the controlled environment study. The protocol used to generate the single spore isolations is described below in section 4.3.6. A completely randomized design with two genotypes, five replications and six treatments were used for a total of sixty pots per study. The two genotypes used in this study were CDC Teal and 93FHB37. CDC Teal is a hard red spring wheat cultivar that is adapted to western Canada and was registered in 1991. It has a FHB rating of 'very poor' (Seed Manitoba 2011). The experimental line 93FHB37 produced by the Cereal Research Centre in 1993 from a cross of Ning8331/Hy611, it has a high degree of resistance and was used as the negative control. Depending on the number of tillers produced per plant, a maximum of six tillers were rated for each plant. Area under the disease progress curve (AUDPC) for severity was determined by monitoring symptom development and rating every three days from the first sign of symptoms until senescence, ratings were based on the total number of spikelets below the point of inoculation, the rating did not include the two inoculated spikelets or the spikelets above the point of inoculation. The seed was harvested at maturity. Five inoculation mixtures served as the treatments in this study along with a water control, a detailed description of the treatments are described below in section 4.3.5. The seed harvested from the controlled environment study was used to generate single spore cultures of *F. graminearum* for chemotyping by PCR, described below in sections 4.3.8

and 4.3.9. The single spore isolations, DNA extraction and PCR were carried out at the facilities of the Department of Plant Science, University of Manitoba.

4.3.3 Dual Floret Inoculation

The spike was inoculated when anthesis had progressed along the majority of the spike and fresh yellow anthers were extruded from spikelets in the middle of the spike. The dual floret inoculation method was used (Cuthbert *et al.* 2006). The number of spikelets were counted on each spike and the spikelets on opposite sides of the spike one-third (1/3) of the way from the top of the spike were inoculated. For each spikelet, viewed from left to right, the outer floret on the left side of the spikelet was inoculated by injecting 10 ul of inoculum between the lemma and palea using a micropipette. See section 4.3.5 for a description of the inoculum. The inoculated florets were marked with a black felt marker on the outer glume so that they could be identified later. Following inoculation, the spikes were covered with a glassine bag for 48 hours to provide a humid environment conducive to the pathogen. After 48 hours the bags were removed for the remainder of experiment.

4.3.4 Rating of Severity

Once the glassine bag was removed from the inoculated spikes they were checked regularly for the development of symptoms, this was done to insure that symptoms were detected early and a maximum number of observations were collected. The spikelets that were inoculated were not counted in the rating, nor were any spikelets above this point. Only spikelets below the point of inoculation were used for rating. Spikes were evaluated every three days for the number of visibly infected spikelets over the total number of spikelets below the inoculation point. The floret inoculation method results in the death of the spikelets above the point of inoculation due to blockage of vascular tissue by the pathogen.

4.3.5 *Fusarium graminearum* Isolates & Treatments

The treatments consisted of mixtures of 15-ADON and 3-ADON isolates of *Fusarium graminearum* obtained from the lab of Dr. Gilbert at Agriculture Canada Cereal Research Centre, Winnipeg, Manitoba. The 15-ADON isolates were M1-04-1 and M8-04-3 and the 3-ADON isolates were M9-04-6 and M6-04-4. Isolate mixtures comprised the treatments as follows: M1-04-1 and M9-04-6 (treatment 1), M1-04-1 and M6-04-4 (treatment 2), M8-04-3 and M9-04-6 (treatment 3), M8-04-3 and M6-04-4 (treatment 4), and a mixture of all four isolates in equal proportions (treatment 5). Tween 20 was added to the inoculum at 1ml/L and water with Tween 20 served as a control. Inoculum of each isolate was produced separately using the methods described in section 3.3.5 of chapter 3 of this document. The macroconidial concentration of each isolate was determined using a hemacytometer. Inoculum treatments were generated by ensuring that each isolate was equally represented and adjusted to a final concentration of 50,000 macroconidia/ml. The diluted inoculum was kept on ice during transport to the greenhouse and/or growth room and while being sampled. During the inoculation process the diluted inoculum was periodically shaken lightly in order to ensure the macroconidia were in suspension.

4.3.6 Single Spore cultures

For the controlled environment study the infected spikes for each pot were hand harvested and the seed combined from all spikes on the same plant. For single spore cultures, twenty kernels per plant were taken at random from the controlled environment samples and for the field study the sample size arbitrarily chosen at 14 kernels.
The following protocol was used to obtain single spore cultures of samples from the field and controlled environment studies:

Kernels were surfaced sterilized by submerging in solution of 5% No Name® bleach (5% Sodium hypochlorite NaOCl) (5 ml bleach : 95 ml sterile distilled water) for 60 seconds. The samples were then placed on sterile filter paper and left to dry in the

laminar flow-hood. Once dry, the kernels were plated onto potato dextrose agar (PDA) with five kernels to a plate and put under continuous fluorescent light for five days or until sufficient aerial mycelium had developed, but not so long that the colonies coalesced. Plated kernels were then stored in a refrigerator at 4^{o}C until the single spore isolations were conducted. Sterile dissecting probes were used to sample aerial mycelium from infected kernels and suspend the mycelium in water so that the mycelium could be diluted out by swabbing and allowed to germinate; a 1 mm piece of aerial mycelium was put into a 1.5 ml eppendorf tube with 500 ul sterile distilled water. The eppendorf tube was then held against the vortex shaker for 5 seconds. The 500 ul spore suspension was evacuated from the pipette near the edge of a PDA plate and a portion of the suspension was streaked across the plate, and the excess poured off. The plates were allowed to surface dry in the laminar flow-hood and then incubated for no more than 24hrs in the dark at 20 ^{o}C. Following incubation, a dissecting microscope was used to find a single germinated macroconidium that was not touching the mycelium of other germinated spores. One germinating spore was cut from each plate and plated onto fresh PDA, thereby completing the single spore isolation. Therefore, for each kernel plated out, a single germinating macroconidium was taken and grown in pure culture, providing a random sample of the *F. graminearum* isolates infesting the seed samples. PDA plates were grown in the dark at 20 ^{o}C for approximately a week until they had filled the plates and then stored in the refrigerator until sampled for DNA extraction.

4.3.7 DNA Isolation Protocol for Field Study

The following DNA isolation protocol was provided by Sung Lee at the Canadian Grain Commission, Grain Research Lab, Winnipeg, Manitoba and used for all DNA isolations from field samples.

Centrifuge tubes with a single large ceramic bead were chilled on ice. Mycelium from one half of the single spore plates was scraped off into the chilled centrifuge tube. The tubes were placed in the freezer overnight. On the day of extraction, 20 ml of dry seed extraction buffer (see Appendix 6.2.8) and 80 ul Roche RNase A were added to a 25 ml falcon tube, and mixed gently to avoid creating excess bubbles due to the presence of

sodium dodecyl sulfate (SDS) in the extraction buffer. 1 ml of the dry seed extraction buffer master mix was added to each of the frozen samples from the previous day. The tubes were then shaken in a high-speed bead shaker mill for two cycles of 1 minute. The shaken samples were kept at room temperature for 5 minutes, after which tubes were centrifuged for 5 min at 6200 rpm. Immediately following centrifugation, 600ul of supernatant was transferred to a 1.5 ml tube while taking care to avoid aspirating the mycelial debris on the top and bottom of the sample. In the new tube with the supernatant, 600 ul of room temperature isopropanol was added, the isopropanol and the supernatant were mixed by manually inverting the tube approximately 20 times. Tubes were centrifuged for 1 minute at 13,000 rpm and the supernatant was discarded. To remove the excess supernatant, the samples were spun for an additional 30 seconds at 13,000 rpm and the remaining supernatant was removed by pipette. The samples were allowed to air dry on the bench with the lids open for 5 minutes. 100ul TE (pH 8.0) was added to dissolve the pellet, to aid the dissolving of the pellet the tube was put back into the mixer mill with no bead and shaken twice for 15 seconds at a time. The DNA was spun in the centrifuge for 15 seconds at 14,000 rpm and frozen immediately until ready for use. When the DNA was taken out of the freezer it was allowed to sit on the bench top for a few minutes until melted and then, centrifuged for 10 minutes at 14,000 rpm prior to use.

4.3.8 DNA isolation protocol for greenhouse and growth room study

Each single spore culture plate was divided in half. The two halves were scraped into separate 1.5 ml microcentrifuge tubes, one portion was to be used for DNA isolation and chemotype identification while the other tube was kept as a backup copy as a precaution. The tubes were put in the freeze drier at 10 microns Hg and -40 ^{o}C for a period of 24 hours to make the samples easier to grind. The samples were then transferred to a 96 deep-well plate for grinding. Liquid nitrogen was poured over the plate and into the wells to make the samples brittle and facilitate grinding the tissue by hand. Following grinding the samples 400 ul of dry seed extraction buffer was added to each well using a multi-channel pipette. The 96 well plate was incubated at 60^{o}C for 30 minutes, and shaken

briefly on the plate shaker every ten minutes. Following incubation the plate was kept at room temperature for 5 minutes. In the fume-hood 400 ul chloroform:isoamyl alcohol (24:1) was added to each well using the multi-channel pipette. The plate was put on the plate shaker for 30 minutes, and then spun on the centrifuge for 15 minutes at 3750 rpm.

In fume hood, 100 ul of supernatant was removed with a pipette from the top layer, taking care to avoid any debris, and transferred to new deep well plate. To each well of the new plate, 2 ul RNAse A [1 mg/ml] was added and left at room temperature for five minutes for RNAse to work. Then, 200 ul of isopropanol was added to each well and the plate was put on the plate shaker for 5 minutes to precipitate the DNA. The plate was then spun on the centrifuge for ten minutes at 3750 rpm and then quickly inverted over the sink to pour off the supernatant followed by an additional five minutes in the centrifuge at 3750 rpm to force off remaining liquid by evaporation. With the multi-channel pipette 100 ul TE buffer was added to each well, and placed on the plate shaker for five minutes or until the pellet dissolved and the DNA was suspended. The DNA was then transferred to a PCR plate, spun for 1 minute at 3750 rpm and used or frozen immediately.

4.3.9 Primer Sequences, PCR master mix and Thermocycler Program

The primer sequence and base pair size of bands published by Ward *et al.* (2008) were used to determine the chemotype of the *F. graminearum* isolates For the 670 bp 15-ADON band the forward primer was 5`- CATGAGCATGGTGATGTC-3`, and the reverse primer was 5'-TACAGCGGTCGCAACTTC-3', and for the 410 bp 3-ADON band the forward primer was 5`- CATGAGCATGGTGATGTC-3` and the reverse primer was 5'- CTTTGGCAAGCCCGTGCA-3'.

The PCR master mix recipe for multiplex PCR to determine the chemotype of *F. graminearum* Isolates consisted of the following:

DDH_2O 14.3 ul, AmpliTaq buffer II 2.5 ul, dNTP's (2 mM) 2ul, Ampli $MgCl_2$ (25 mM) 2ul, 12CON primer (10 uM) 0.5 ul, 12NF primer (10 uM) 0.5 ul, 12-15F (10

uM) 0.5 ul, 12-3F (10 uM) 0.5 ul, Ampli Taq 0.2 ul. The total master mix volume was 23 ul plus 2 ul DNA for a total reaction volume of 25 ul. For the controlled environment studies 11.3 ul of DDH_2O and 5 ul DNA were used. The thermocycler program for the PCR reaction was: Denaturation at 94˚C for 2 minutes, followed by 30 cycles of 94˚C for 30 seconds, 52˚C for 30 seconds and 72˚C for 1 minute, followed by a final extension at 72˚C for 7 minutes, and then the sample was held at 20˚C until removed from the thermocycler.

4.3.10 Data Analysis

Data analysis was done using SAS version 9.1 software. Analysis included ANOVA in PROC GLM, and Chi-square. For the controlled environment studies ANOVA model, the dependent variable was AUDPC of incidence, the independent variables were site, genotype, genotype*treatment, site*genotype, site*treatment, plants (site), site*genotype*treatment and plants (site*genotype*treatment). The random variables were site and plants (site), all other variables were considered fixed. Plants (site*genotype*treatment) was used as an error term for site, site*genotype, site*treatment, and site*genotype*treatment. Site*genotype was used as the error term for genotype. Site*treatment was used as the error term for treatment. Site*genotype*treatment was used as the error term for genotype*treatment.

Analysis of variance for the field study was not conducted due to incomplete sampling. As a result, for the field study the data used to examine the effect of inoculation protocol was combined across genotypes and the data for examining the effect of wheat genotype was combined across inoculation protocols.

4.4 RESULTS

4.4.1 Combined analysis of controlled environment studies

Table 4.1 shows the ANOVA for AUDPC of disease incidence combined for the growth room and greenhouse study. As there were highly significant differences between sites (greenhouse or growth chamber) and site*genotype interactions, the results from the two controlled environments are presented separately below.

Table 4.1. ANOVA for area under the disease progress curve of disease severity for studies conducted under two controlled environment conditions.

Source	df	Mean Square
Site[A]	1	676971.38 ***
Genotype[B]	1	57842307.89 ***
Treatment[C]	5	2786902.67 ***
Genotype*Treatment[D]	5	2483606.84 ***
site*Genotype[A]	1	216647.61 *
Site*Treatment[A]	5	83374.74 ns
Plants(Site)	8	30119.87 ns
Site*Genotype*Treatment[A]	5	33332.07 ns
Plants(Site*Genotype*Treatment)	88	36884.46 ns
Error	569	41959

[A] tests of hypothesis using Plants(Site*Genotype*Treatment) MS as an error term
[B] tests of hypothesis using Site*Genotype MS as an error term
[C] tests of hypothesis using Site*Treatment MS as an error term
[D] tests of hypothesis using Site*Genotype*Treatment MS as an error term

4.4.2 Greenhouse study

Analysis of variance for area under the disease progress curve (AUDPC) of disease incidence revealed that there were no significant differences between pots that had been inoculated with different mixtures of 3-ADON and 15-ADON isolates of *F. graminearum* (Table 4.2). As expected, AUDPC differed significantly between cultivars ($P \leq 0.001$), with CDC Teal being heavily infected compared to 93FHB37. When the control was partitioned out of the treatment effect, there were no significant differences among treatments for AUDPC incidence ($P > 0.05$) (Table 4.2), although there was a significant genotype*treatment interaction ($P \leq 0.001$).

Chi-square analysis revealed that only the treatment #2 inoculation mixture resulted in single spore cultures that were not significantly different from a 1:1 ratio for 3-ADON:15-ADON isolates (Table 3 and 4). Treatments #1, 3 and 4 inoculation mixtures differed significantly from a 1:1 ratio ($P \leq 0.01$), and treatment #5 inoculation mixture was very different from a 1:1 ratio ($P \leq 0.001$) (Table 4.3). In all of these instances the 15-ADON chemotype was more prevalent. These observations are depicted graphically in Figure 4.1.

Table 4.2. For the CTC greenhouse, analysis of variance with control group removed revealed no significant differences among treatments in terms of area under the disease progress curve for disease severity

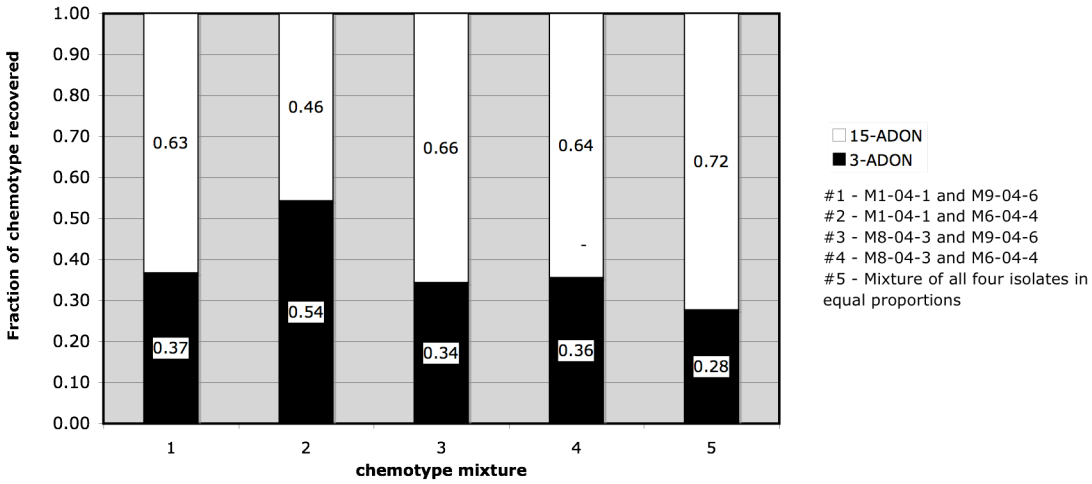
Source	DF	Mean Square
Genotype	1	34903593.08 ***
Treatment	5	1772859.52 ***
Among treatments	4	88922.58 ns
Control vs. treatment contrast	1	8508607.269 ***
Genotype*Treatment	5	1447336.85 ***
Plants(Genotype*Treatment)	48	62061.75 ns
Error	300	66365.88

Table 4.3. Chi-square goodness of fit table for single spore cultures of *F. graminearum* for different inoculum mixtures, tested for a 1:1 3-ADON:15-ADON chemotype ratio present in the original inoculum for the greenhouse experiments.

	#1	#2	#3	#4	#5
15 A observed	67	47	63	74	65
15 A expected	53	51.5	48	57.5	45
(obs-exp)2/exp	3.698	0.393	4.688	4.735	8.889
3A observed	39	56	33	41	25
3A expected	53	51.5	48	57.5	45
(obs-exp)2/exp	3.698	0.393	4.688	4.735	8.889
X^2	7.396	0.786	9.375	9.47	17.778
P> X^2	0.0065	0.3752	0.0022	0.0021	<.0001

#1 - M1-04-1 and M9-04-6 , #2 - M1-04-1 and M6-04-4 , #3 - M8-04-3 and M9-04-6 , #4 - M8-04-3 and M6-04-4 , #5 - Mixture of all four isolates in equal proportions

Figure 4.1. The fraction of each *F. graminearum* chemotype recovered from infected seeds of CDC Teal for the different inoculum mixtures, where the inoculum was comprised of 1:1 ratio of 3-ADON and 15-ADON isolates. Data are from the greenhouse study.



4.4.3 Growth Room Study

Table 4.4 shows the results of ANOVA conducted on AUDPC for disease incidence data collected from the trial carried out in the growth room at the Department of Plant Science. The success of the inoculation procedure is indicated in the significant difference ($P \leq 0.001$) between genotypes in terms of AUDPC disease incidence. There was no significant difference ($P \leq 0.05$) among the five inoculation mixtures in terms of their ability to affect the progress of infection as measured by AUDPC disease incidence. Table 4.5 shows the chi-square analysis testing the data for a fit to a 1:1 ratio. Three of the five inoculation mixtures resulted in isolations of 3-ADON:15-ADON that were not significantly different from the ratios used in the inoculations. The remaining two inoculation mixtures (treatments #3 & #4) resulted in 3-ADON:15-ADON re-isolation ratios that were significantly different ($P \leq 0.001$) from the 1:1 ratio in which they were applied, with the 15-ADON isolates being recovered in higher proportion than the 3-ADON isolates These observations are depicted graphically in Figure 4.2.

Table 4.4. Analysis of variance with control group removed revealed no significant differences between treatments in terms of area under the disease progress curve for disease severity. Data are for the experiment in the growth room.

Source	DF	Mean Square
Genotype	1	23891767.56 ***
Treatment	5	1125714.41 ***
Among treatments	4	18987.54 ns
Control vs. treatment contrast	1	5366164.72 ***
Genotype*Treatment	5	1078617.1 ***
Plants(Genotype*Treatment)	48	10368.96 ns
	269	14739.36

Figure 4.2. The fraction of each *F. graminearum* chemotype recovered from infected seeds of CDC Teal for the different inoculum mixtures, where the inoculum was comprised of 1:1 ratio of 3-ADON and 15-ADON isolates. Data are from the growth room study.

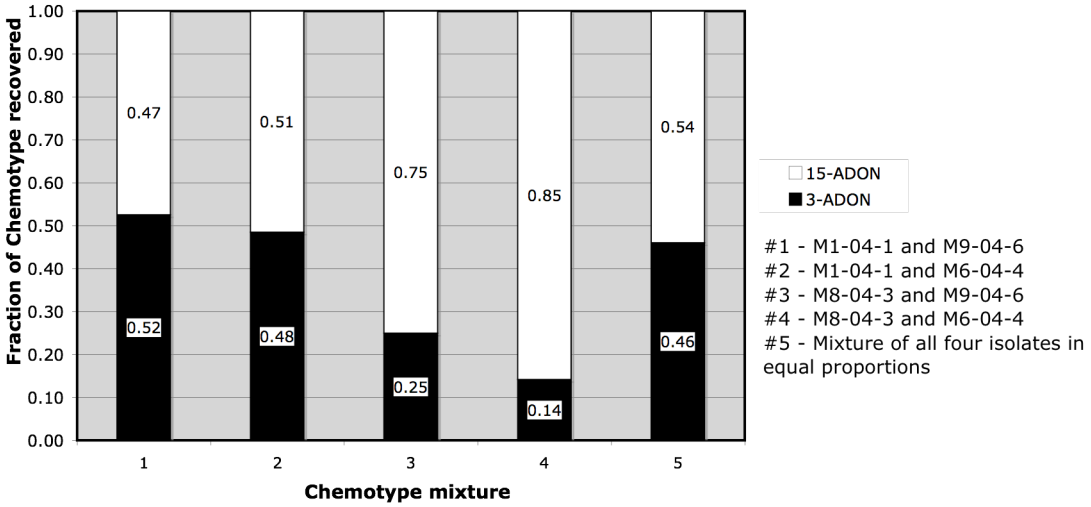


Table 4.5. Chi-square goodness of fit table for different inoculum mixtures of 3-ADON and 15-ADON chemotypes of *F. graminearum*, depicting the fit of single spore culture chemotypes to the 1:1 chemotype ratio that was present in the original inoculum. Single spores were recovered from seed of CDC Teal from the growth room study.

	#1	#2	#3	#4	#5
15 A observed	41	52	69	92	54
15 A expected	43	50.5	46	53.5	50
(obs-exp)2/exp	0.093	0.045	11.5	27.706	0.32
3A observed	45	49	23	15	46
3A expected	43	50.5	46	53.5	50
(obs-exp)2/exp	0.093	0.045	11.5	27.706	0.32
X^2	0.186	0.089	23	55.411	0.64
$P > X^2$	0.6662	0.7653	<.0001	<.0001	0.4237

#1 - M1-04-1 and M9-04-6 , #2 - M1-04-1 and M6-04-4 , #3 - M8-04-3 and M9-04-6 , #4 - M8-04-3 and M6-04-4 , #5 - Mixture of all four isolates in equal proportions

4.4.4 2008 Field Study

Chi-square tests conducted by genotype of kernels from which the *F. graminearum* chemotypes were recovered resulted in no significant variation from a 1:1 ratio in three of the five genotypes tested. *F. graminearum* single spore cultures recovered from AC Barrie and 93FHB37 were in a ratio significantly different from 1:1 ($P \leq 0.01$) (Table 4.6). In these genotypes it was the 3-ADON chemotype that was more prevalent than 15-ADON chemotype.

The inoculation protocols used to generate the seed for chemotype sampling were as follows; MP1 was the uninoculated control (infection resulting from either spray drift or natural inoculum), MP2 was inoculated once at 50% anthesis and again three days later, MP3 was inoculated at three day intervals from first through last anthesis (approximately 5 inoculations), and MP4 was inoculated at three day intervals from heading through soft dough (approximately 10 inoculations) (see Chapter 3 for details). When the 2008 field study data were analyzed by inoculation protocol: MP1 was not significant, MP2 was significant ($P \leq 0.01$), MP3 was not significant, and MP4 was significant ($P \leq 0.05$) (Table 4.7). The instances of statistical significance for MP2 and MP4 indicated that the 3-ADON chemotypes were more prevalent than 15-ADON chemotypes. The results are presented graphically in Figures 4.3 and 4.4.

Figure 4.3. The fraction of 15-ADON and 3-ADON chemotypes of *F. graminearum* recovered from infected seeds across five wheat genotypes collected from the 2008 field study.

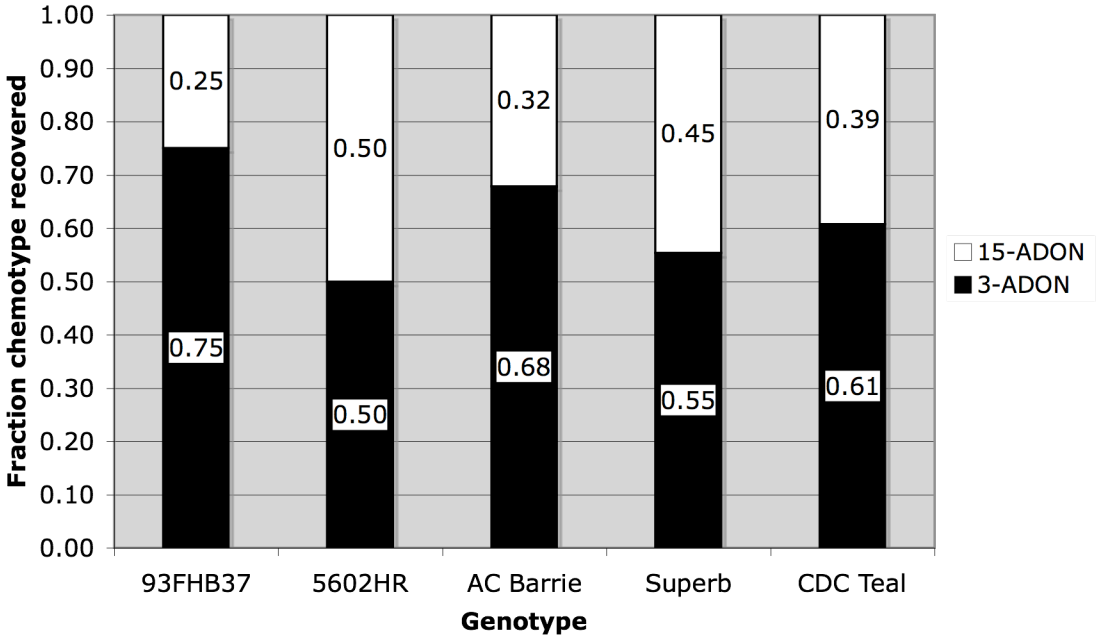


Table 4.6. Chi-square goodness of fit table depicting the fit of single spore isolation of 3-ADON and 15-ADON chemotypes of *F. graminearum* to the 1:1 chemotype ratio that was present in original inoculum applied to five cultivars in the 2008 field season.

	AC Barrie	93FHB37	Superb	5602HR	CDC Teal
15 A observed	18	14	25	28	22
15 A expected	28	28	28	28	28
(obs-exp)2/exp	3.571	7	0.321	0	1.286
3A observed	38	42	31	28	34
3A expected	28	28	28	28	28
(obs-exp)2/exp	3.571	7	0.321	0	1.286
X^2	7.143	14	0.643	0	2.571
$P > X^2$	0.0075	0.0002	0.4227	1	0.1088

Figure 4.4. The fraction of 15-ADON and 3-ADON *F. graminearum* chemotypes recovered from infected seeds across the uninoculated control and the three inoculation protocols in the 2008 field study.

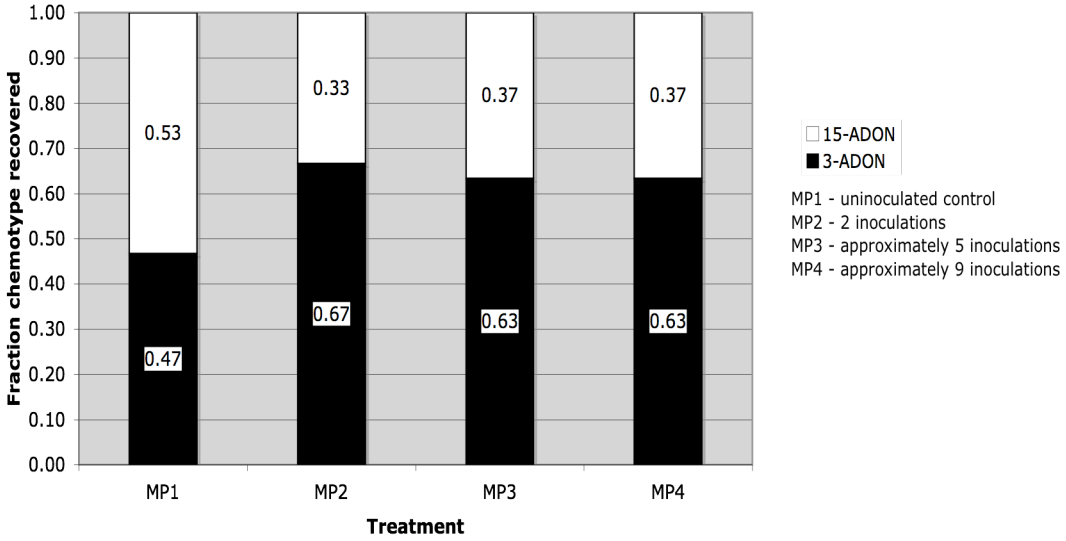


Table 4.7. Chi-square goodness of fit table depicting the fit of single spore isolation of 3-ADON and 15-ADON chemotypes of *F. graminearum* to the 1:1 chemotype ratio that was present in original inoculum for all inoculation protocols for the 2008 field study.

	MP1	MP2	MP3	MP4
15 A observed	31	23	27	26
15 A expected	35	35	35	35
(obs-exp)2/exp	0.457	4.114	1.829	2.314
3A observed	39	47	43	44
3A expected	35	35	35	35
(obs-exp)2/exp	0.457	4.114	1.829	2.314
X^2	0.914	8.229	3.657	4.629
$P > X^2$	0.339	0.0041	0.0558	0.0314

MP1 - uninoculated control, MP2 - 2 inoculations, MP3 - approximately 5 inoculations, MP4 - approximately 9 inoculations

4.4.5 2009 Field Study

Chi-squares conducted by genotype resulted in one of five genotypes differing significantly from a 1:1 ratio for 3-ADON:15-ADON isolates recovered from infected seed (Figure 4.5 and Table 4.8). Isolates recovered from seed of AC Barrie wheat were significantly different ($P \leq 0.05$) from a 1:1 ratio with 3-ADON being the more prevalent chemotype (Table 4.8). The *F. graminearum* isolates recovered from the remaining four genotypes were did not vary to a significant degree from the 1;1 3-ADON: 15-ADON ratio with which the plants were inoculated. When the data were analyzed in terms of inoculation protocol, only seed from the uninoculated control plots yielded a 3-ADON: 15-ADON ratio that significantly ($P \leq 0.05$) varied from a 1:1 ratio (Table 4.9 and Figure 4.6) with the 3-ADON chemotype being more prevalent than the 15-ADON chemotype.

Figure 4.5. The fraction of 15-ADON and 3-ADON chemotypes of *F. graminearum* recovered from infected seeds across five wheat genotypes collected from the 2009 field study.

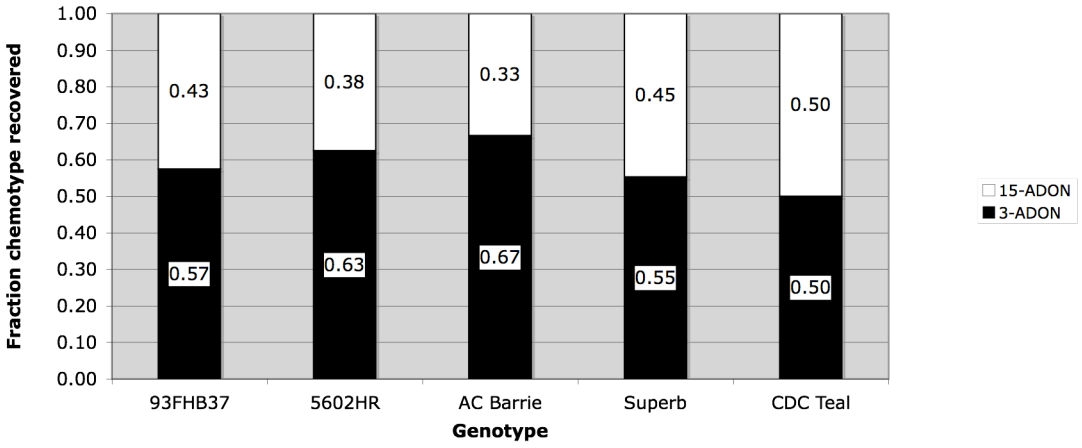


Table 4.8. Chi-square goodness of fit table depicting the fit of single spore isolation of *F. graminearum* chemotypes to the 1:1 ratio that was present in original inoculum for five cultivars in the 2009 field study.

	AC Barrie	93FHB37	Superb	5602HR	CDC Teal
15 A observed	18	20	25	21	26
15 A expected	27	23.5	28	28	26
(obs-exp)2/exp	3	0.521	0.321	1.75	0
3A observed	36	27	31	35	26
3A expected	27	23.5	28	28	26
(obs-exp)2/exp	3	0.521	0.321	1.75	0
X^2	6	1.043	0.643	3.5	0
$P > X^2$	0.0143	0.3072	0.4227	0.0614	1

Figure 4.6. The fraction of each *F. graminearum* chemotype recovered from infected seeds across the uninoculated control and the three inoculation protocols in the 2009 field study.

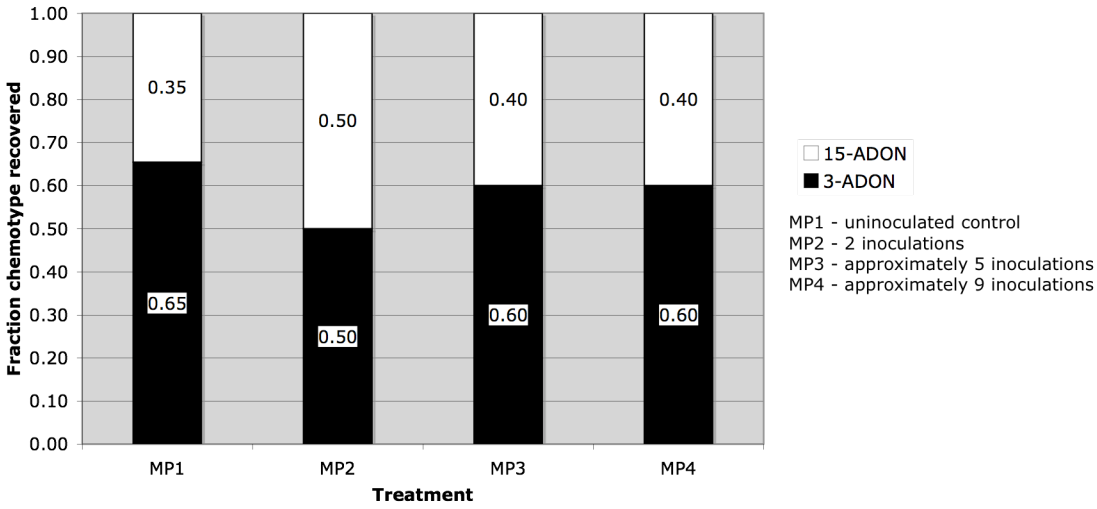
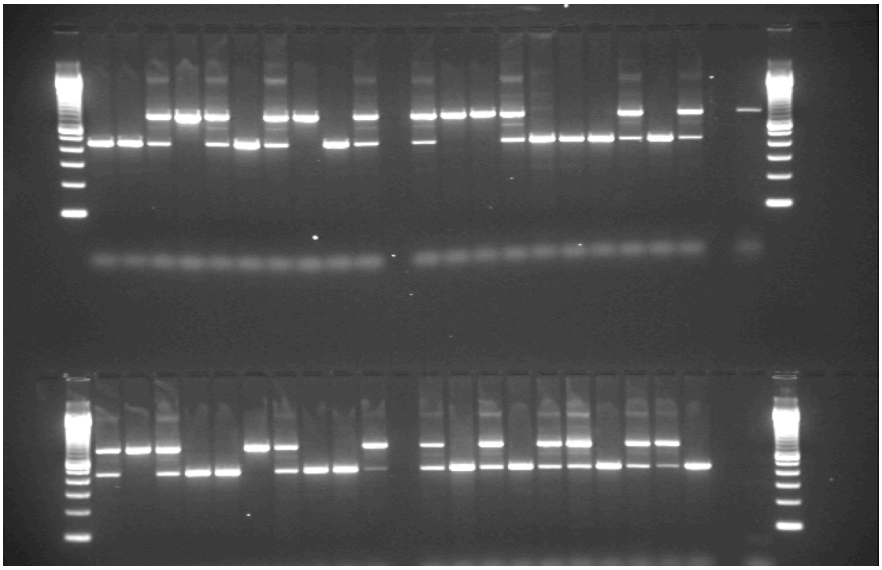


Table 4.9. Chi-square goodness of fit table depicting the fit of single spore cultures of *F. graminearum* chemotypes to the 1:1 chemotype ratio that was present in original inoculum for all inoculation protocols for the 2009 field study

	MP1	MP2	MP3	MP4
15 A observed	19	35	28	28
15 A expected	27.5	35	35	35
(obs-exp)2/exp	2.627	0	1.4	1.4
3A observed	36	35	42	42
3A expected	27.5	35	35	35
(obs-exp)2/exp	2.627	0	1.4	1.4
X^2	5.255	0	2.8	2.8
$P > X^2$	0.0219	1	0.0943	0.0943

MP1 - uninoculated control, MP2 - 2 inoculations, MP3 - approximately 5 inoculations, MP4 - approximately 9 inoculations

Figure 4.7 Image of multiplex PCR products identifying chemotypes of *F. graminearum* single spore cultures recovered from CDC teal in 2009 field study.



Two DNA samples per well, lanes with two bands indicate one sample of each chemotype and lanes with a single band indicate both samples were of the same chemotype. The top band (670 bp) indicates 15-ADON chemotype and the lower band (410 bp) indicates 3-ADON chemotype.

4.5 DISCUSSION

In the field study there was no inoculation protocol for which there was a consistently significant difference from a 1:1 (3ADON:15ADON) chemotype ratio in either year. The results were inconsistent over cultivar and inoculation protocols over both years of the field study, but in all instances in which the chi-square test resulted in statistically significant variation from a 1:1 ratio, the 3-ADON chemotype was more prevalent. In contrast, instances of statistically significant variation from a 1:1 ratio in the chi-square tests for the controlled environment studies indicated that the 15-ADON was the more prevalent chemotype. This would appear to put the results of the field and controlled environment studies at odds, but little is known about the effects of differences in environment (temperature, humidity, precipitation, etc) have on *in planta* competition between 3-ADON and 15-ADON isolates. Also, the controlled environment studies only recovered isolates from one wheat genotype (CDC Teal) as opposed to five wheat genotypes in the field study. The field study was done using spray inoculation, and the controlled environment study was done using point inoculation, which may have an unknown effect on the outcome of competition between the isolates of different chemotypes. In the field study a misting system was used in both years throughout most of the infection susceptibility period, whereas in the controlled environment study there was no misting and the only attempt to provide a humid environment was to place a glassine bag on the inoculated spike for 48 hours following inoculation. Therefore, there were many environmental differences that could have contributed to the differences in chemotype ratios seen between the field and controlled environment studies.

For both the greenhouse and the growth room experiments, there was no significant effect of inoculum mixture on AUDPC disease incidence. These findings are supported by the previous findings of Ward *et al.* (2008) who concluded that no significant differences in aggressiveness were present between 3-ADON and 15-ADON isolates when cultivars Roblin and 5602HR were tested using spray inoculation. Also in support of results from the present study, Von der Ohe *et al.* (2010) found no significant

difference between 3-ADON and 15-ADON chemotypes for aggressiveness measured as FHB index against resistant and susceptible wheat genotypes in a multi-site, multi-year study. Miedaner *et al.* (2004) used four *F. culmorum* isolates either alone or in pairs, to inoculate two winter rye genotypes in order to study the effects of competition between *F. culmorum* isolates on measures of disease resistance. The seed harvested from the mixed inoculum treatments was used to re-isolate cultures for RAPD marker profiling to see if the re-isolations fit the same 1:1 ratio as the original inoculum mixtures. Miedaner *et al.* (2004) found that inoculum of isolate mixtures was less aggressive than single isolate inoculum. They also found isolate competitive ability was independent of aggressiveness.

In the field study, only AC Barrie had a 3-ADON:15-ADON ratio that was significantly different from 1:1 over both years . Guo *et al.* (2008) examined the population distribution and of 15-ADON and 3-ADON chemotypes across Manitoba and conducted 291 isolations to determine chemotype distribution at different locations, the average across locations and cultivars across locations. The average over all cultivars tested was 66.3% 15-ADON and 33.7% 3-ADON, whereas the average for the cultivar Superb was 72.2% 15-ADON and 27.8% 3-ADON, AC Barrie had the largest fraction of 3-ADON isolates recovered with an average of 61.8% 3-ADON and 38.2% 15-ADON.

Between 1998 and 2006, AC Barrie was the number one wheat cultivar across the prairies (Grenier 2007). While there is a substantial body of evidence that resistance to FHB in wheat is not race specific (Snijders and Van Eeuwijk 1991, Van Eeuwijk *et al.* 1995, Bai and Shaner 1996, Mesterhazy 1999) none of these studies examined the outcome of *in planta* competition between *F. graminearum* isolates or chemotypes. It could be possible that both this study and that of Guo *et al.* (2008) reported a preference for 3-ADON isolates over 15-ADON isolates infecting AC Barrie, although further in depth studies would have to be conducted before any conclusions could be drawn.

This thesis is the first to use PCR to examine the effect of chemotype on competition between isolates of *F. graminearum*. The results of this study, while not

always definitive, did serve to demonstrate that changes in the relative abundance of *F. graminearum* isolates of different chemotypes can occur *in planta*. Ward et al (2008) found that 3-ADON chemotypes caused significantly ($P < 0.05$) greater accumulation of DON toxin *in vitro* than 15-ADON chemotypes. Increased DON content in grain could pose a real threat to Canadian wheat exports. As the 3-ADON chemotype continues to encroach on and out-compete the incumbent 15-ADON chemotype across Manitoba and into the western provinces more research is required in order to understand what role agricultural practices have in this population shift and what, if anything, can be done to mitigate it.

5.0 GENERAL DISCUSSION & CONCLUSION

The first objective of this thesis was to examine the effects of three different spray inoculation protocols on the development of FHB symptoms in spring wheat, and answer the question: Do the three inoculation protocols produce evaluations of the ten genotypes that are substantially equivalent?

Previous studies have demonstrated that wheat is generally insensitive to differences in number of applications of inoculum in field plot trials so long as inoculation occurs during the critical period of susceptibility surrounding anthesis (Xue *et al.* 2006, Yang *et al*. 1999). Results from this study support these observations, as most measurements of resistance had no significant interactions between the inoculation protocols and the genotypes; the genotypes responded in kind to each inoculation protocol according to their level of resistance to FHB. Though there were some absolute differences between the protocols in terms of disease symptoms, it is the stability of the group of genotypes being tested that is most important. In addition, these results validate screening methods that evaluate FHB on a large number of genotypes using *F. graminearum* infested kernels to mimic natural inoculum. When natural sources of inoculum are relied on, or infected grain spawn is used for inoculation, there is no way to ensure equal timing of inoculum application, or even equal rates of application, yet results that accurately reflect the true levels of field resistance were generated en masse. Previous studies have also demonstrated high degrees of similarity between resistance ratings generated using point inoculation and infested kernels (Yang *et al*. 1999), or point inoculation and spray inoculation (Xue *et al*. 2006), even though it was concluded by Schroeder and Christensen (1963) that point inoculation tests for type II resistance and spray inoculation tests for type I resistance. The current study demonstrates that for either AUDPC or a single rating approximately 21 days after 50% anthesis, that the three inoculation protocols tested produce substantially equivalent ranking of cultivars in terms of resistance, with little or no statistically significant interaction between protocols and genotypes. These results indicate that the plant breeding community could significantly

reduce labour costs by using large-scale macroconidial spray inoculation for FHB screening nurseries.

In conclusion, to effectively evaluate the level of resistance to FHB a spray inoculation protocol should consist of at least two applications of inoculum during the period including the date of 50% anthesis, and additional applications starting prior to anthesis or extending past anthesis should not substantially effect the ranking of a group of genotypes in terms of resistance to FHB.

The second objective was to examine the outcome of *in planta* competition between isolates of 3-ADON and 15-ADON chemotype of *F. graminearum* causing FHB in wheat, and to examine the effect of inoculum consisting of different isolate mixtures on AUDPC disease incidence in the susceptible wheat cultivar CDC Teal. The effect of number of applications of inoculum, and different wheat genotypes, on *in planta* competition between *F. graminearum* isolates of 3-ADON and 15-ADON chemotype were also examined in a field study over two years.

A substantial body of work exists which examines the variation for aggressiveness in *F. graminearum* populations, and members of the *Fusarium* genus known to cause FHB of wheat. The use of inoculum comprised of mixtures of aggressive isolates is widespread. In this study there were no differences in aggressiveness for five different inoculum mixtures of 3-ADON and 15-ADON chemotypes in terms of AUDPC disease incidence in controlled environment studies. It has been concluded numerous times previously that resistance to FHB in wheat is not race specific and that any aggressive isolate can be used for screening for resistance (Snijders and Van Eeuwijk 1991, Van Eeuwijk *et al*. 1995, Bai and Shaner 1996, Mesterhazy 1999). The current study is the first to examine the outcome of *in planta* competition between 3-ADON and 15-ADON chemotypes of *F. graminearum* on wheat. Though there were conflicting results between the controlled environment and field studies in terms of the chemotype isolation ratios, but differences in environmental conditions and low sample size could reasonably explain these discrepancies. In addition, differences seen between the field

and controlled environment studies could be accounted for by differences in the inoculation method, the presence of the misting system in the field study, or the number of genotypes tested. The results of the 2008 and 2009 field season, though they do show the 3-ADON to be more competitive than 15-ADON in a number of instances, are not as overwhelming as we would be led to believe from our observations of the natural system. There were no consistent patterns between the field and controlled environment studies in terms of *in planta* competition between 3-ADON and 15-ADON isolates of *F. graminearum*. Caution must be exercised when comparing the results of this study to the changes being seen in the environment at large without knowing historical chemotype distribution of an area in question. It is possible that a large degree of the population shift we are witnessing is taking place in other portions of the pathogen's lifecycle such the saprophytic stage on crop residues, or due to environmental factors such as overwintering success of the two chemotypes.

To truly understand the mechanisms by which 3-ADON is becoming the more prevalent chemotype, studies must be conducted in a controlled manner to create scenarios that are representative of the agricultural system in which this population shift is taking place. An example of such a study would be to use infected wheat kernels and stubble, cultured with mixtures of 3-ADON and 15-ADON separately as inoculum and monitor over the whole season. Spore traps could sample the air to determine the frequency of each chemotype actually acting as airborne inoculum. At harvest the chaff and kernels could be used to generate single spore isolates, and the following season the crop residue could be sampled to determine what fraction of each chemotype is present in crop residue, followed by another cycle of sampling airborne spores with spore traps. The results of the isolates recovered and grown as single spore cultures at different stages can be compared to determine the source of greatest pressure on the population structure.

In closing, it is possible to determine resistance in wheat to FHB by inoculating over a wide physiological growth stage range as long as inoculation includes the period of 50% anthesis. This is valuable for its implications towards comparing breeding material inoculated under different protocols, or in cases where precision application and

row-by-row inoculation is not feasible. With regards to the second study, while the results from the controlled environment study may have been inconclusive in explaining the population shift between *F. graminearum* chemotypes being witnessed in Manitoba and largely in Canada, the field study was able to "recreate" this population shift. The concept of recreating the conditions and scenarios around the struggle between 3-ADON and 15-ADON chemotypes of *F. graminearum* in order to study this population shift bears further examination as it could potentially yield valuable information on how to mitigate or even reverse this trend.

6.1 LITERATURE CITED

Ansari, K.I., Walter, S., Brennan, J.M., Lemmens, M., Kessans, S., McGahern, A., Egan, D., and Doohan, F.M. 2007. Retrotransposon and gene activation in wheat in response to mycotoxigenic and non-mycotoxigenic-associated *Fusarium* stress. Theor. Appl. Genet.114:927-937.

"Area of wheat in Manitoba, 1998-2009" Industry intelligence section, MAFRI. Accessed: December 2nd, 2010. Retrieved from http://www.gov.mb.ca/agriculture/news/markets/all_graphs_pdf/crops/wheat_area.pdf

Arthur, J.C. 1891. Wheat scab. Indiana Agric. Exp. Stn Bull. 36:129-132.

Audenaert, K., Callewaert, E., Hofte, M., De Saeger, S. and Haesaert, G. 2010. Hydrogen peroxide induced by the fungicide prothioconazole triggers deoxynivalenol (DON) production by *Fusarium graminearum.* BMC Microbiology 10:112.

Bai, G.H., and Shaner, G. 1994. Scab of wheat: Prospects for control. Plant Dis. 78:760-766.

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

Bai, G.H., Shaner, G. and Ohm, H. 2000. Inheritance of resistance *to Fusarium graminearum* in wheat. Theor Appl Genet 100:1-8.

Bhat, R.V., Beedu, S.R., Ramakrishna, Y. and Munshi, K.L. 1989. Outbreak of trichothecene mycotoxicosis associated with consumption of mould-damaged wheat production in Kashmir Valley, India. Lancet 8628:35-37.

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

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

Casale, W. L., and Hart, L. P. 1988. Inhibition of 3H-leucine incorporation by trichothecene mycotoxins in maize and wheat tissue. Phytopathology 78:1673-1677.

Champeil, A., Dore, T. and Fourbet, J.F. 2004. Fusarium head blight: epidemiological origin of the effects of cultural practices on head blight attacks and the production of mycotoxins by Fusarium in wheat grains. Plant Science 166:1389-1415.

Cuthbert, P.A., Somers, D.J., Thomas, J., Cloutier, S. and Brûlé-Babel, A. 2006. Fine mapping *Fhb1*, a major gene controlling fusarium head blight resistance in bread wheat (Triticum aestivum L.). Theo Appl Genet 112:1465-1472.

Cuzick, A., Urban, M., and Hammond-Kosack, K. 2008. Fusarium graminearum gene deletion mutants *map1* and *tri5* reveal similarities and differences in the pathogenicity requirements to cause disease on Arabidopsis and wheat floral tissue. New Phytologist 177: 990-1000.

Engle, J. S., Madden, L. V., and Lipps, P. E. 2003. Evaluation of inoculation methods to determine resistance reactions of wheat to Fusarium graminearum. Plant Dis. 87:1530-1535.

Eudes, F., Comeau, A., Rioux, S., and Collin, J. 2001. Impact of trichothecenes on Fusarium head blight [Fusarium graminearum] development in spring wheat (Triticum aestivum). Can. J. Plant Pathol. 23:318-322.

Del Ponte, E. M., Fernandes, J. M. C. and Bergstrom, G. C. 2007. Influence of Growth Stage on Fusarium Head Blight and Deoxynivalenol Production in Wheat. J. Phytopathology 155:577-581.

Desjardins, A.E. and Hohn, T.M. 1997. Current Review: Mycotoxins in Plant Pathogenesis. Molecular Plant-Microbe Interactions 2:147-152.

Desmond, O. J., Manners, J. M., Stephens, A. E., Maclean, D. J., Schenk, P. M., Gardiner, D. M., Munn, A. L., and Kazan, K. 2008. The Fusarium mycotoxin deoxynivalenol elicits hydrogen peroxide production, programmed cell death and defence responses in wheat. Molec. Plant Pathol. 9:435-445.

Fernando, W. G. D., Paulitz, T. C., Seaman, W. L., Dutilleul, P., and Miller, J. D. 1997. Head blight gradients caused by *Gibberella zeae* from area sources of inoculum in wheat field plots. Phytopathology 87:414-421.

Ferreira, RB, Monteiro, S. Freitas, R. Santos, C.N. Chen, Z. Batista, L.M. Duarte, J. Borges, A. and Teixeira, A.R. The role of plant defence proteins in fungal pathogenesis. Molecular Plant Pathology 5:677-700.

Gilbert, J. and Tekauz, A. 2000. Review: Recent developments in research on fusarium head blight of wheat in Canada. Can. J. Plant Pathol. 22:1-8.

Gilbert, J., Fernando, W. G. D., Ahmed, H. U., and Brûlé-Babel, A. 2004. "The role of crop stubble in production of inoculum of Fusarium graminearum." In: Canty, S.M., Boring, T., Wardwell, J. and Ward, R.W (Eds.), Proceedings of the 2nd International Symposium on Fusarium Head Blight; incorporating the 8th European Fusarium Seminar; 2004, 11-15 December ; Orlando, FL, USA. Michigan State University, East Lansing, MI. pp. 452.

Gilbert, J. and Fernando, W.G.D. 2004. Epidemiology and biological control of *Gibberella zeae / Fusarium graminearum.* Can. J. Plant Pathol. 26: 464-472.

Goswami, R.S., and Kistler, H.C. 2005. Pathogenicity and in planta mycotoxin accumulation among members of the Fusarium graminearum species complex on wheat and rice. Phytopathology 95:1397-1404.

Guo X.W., Fernando, W.G.D., Bullock, P. and Sapirstein, H. 2010. Quantifying cropping practices in relation to inoculum levels of Fusarium graminearum on crop stubble. Plant Path. 59:1107-1113.

Guo X.W., Fernando, W.G.D., and Seow-Brock, H.Y. 2008. Population Structure, Chemotype Diversity, and Potential Chemotype Shifting of Fusarium graminearum in Wheat Fields of Manitoba. Plant Dis. 92:756-762.

Harris, L.J., and Gleddie, S.C. 2001. A modified RPL3 gene from rice confers tolerance of the Fusarium graminearum mycotoxin deoxynivalenol to transgenic tobacco. Physiological and molecular plant pathology 58:173-181.

Hart, L.P., Pestka, J.J. and Liu, M.T. 1984. Effect of kernel development and wet periods on production of deoxynivalenol in wheat infected with *Gibberella zeae*. Phytopathology 74:1415-1418.

Joint FAO/WHO Expert Committee on Food Additives (JECFA). Available online at: http://www.fao.org/ag/agn/agns/jecfa/JECFA72%20Summary%20Version%2016%20%20Mar%20FINAL%20rev%20(2).pdf accessed on August 23, 2011.

Jeger, M.J. and Viljanen-Rollinson, S.L.H. 2001. The use of the area under the disease-progress curve (AUDPC) to assess quantitative disease resistance in crop cultivars. Theor Appl Genet 102:32-40.

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

Kang, Z., and Buchenauer, H. 2000a. Cytology and the ultrastucture of the infection of wheat spikes by *Fusarium culmorum.* Mycol. Res. 9:1083-1093.

Kang, Z., and Buchenauer, H. 2000b. Ultrastructural and immunocytochemical investigation of pathogen development and host responses in resistant and susceptible wheat spikes infected by *Fusarium culmorum.* Physiological and Molecular Plant Pathology. 57:255-268.

Kang, Z., Buchenauer, H., Huang, L., Han, Q. and 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.* Eur J Plant Pathol 120:383-396.

Kolb, F.L. and Boze, L.K. 2003. An alternative to the FHB index: incidence, severity, kernel rating (ISK) index. In: Description of proceedings of the National Fusarium Head Blight Forum, 2003, Dec 13-15; Bloomington MN. East Lansing: Michigan State University P. 259.

Lemmens, M., Buerstmayr, H., Krska, R., Schuhmacher, R., Grausgruber, H., Ruckenbauer, P. 2004. The effect of inoculation treatment and long-term application of moisture on Fusarium head blight symptoms and deoxynivalenol contamination in wheat grains. European Journal of Plant Pathology 110: 299-308.

Lemmens, M., Scholz, U., Berthiller, F., Dall'Asta, C., Koutnik, A., Schuhmacher,R., Adam, G., Buerstmayr, H., Mesterhazy, A., Krska, R., Ruckenbauer, P. 2005. The ability to detoxify the mycotoxin deoxynivalenol co-localizes with a major QTL for Fusarium head blight resistance in wheat. Mol. Plant Microbe Interact. 18:1318-1324.

Leonard, K. J., and Bushnell, W. R. 2003: Fusarium Head Blight of Wheat and Barley American Phytopathological Society. APS Press, St Paul, MN, USA.

Maier F.J., Miedaner T., Hadeler B., Felk A., Salomon S., Lemmens M., Kassner H., and Schafer 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.

Martin, R. A. and Johnston, H. W. 1982. Effects and control of fusarium diseases of cereal grains in the Atlantic Provinces. Canadian Journal of Plant Pathology 4: 2, 210-216.

McMullen M., Jones R. and Gallenberg, D. 1997. Scab of wheat and barley: a reemerging disease of devastating impact. Plant Dis 81: 1008-1021.

Mesfin A., Smith, K.P., Dill-Macky, R., Evans, C.K., Waugh, R., Gustus, C.D. and Muehlbauer, G.J. 2003. Quantitative trait loci for Fusarium head blight resistance in barley detected in a two-rowed by six-rowed population. Crop Sci 43: 307-318.

Mesterhazy, A. 1978. Comparative analysis of artificial inoculation methods with Fusarium spp. on winter wheat varieties. Phytopathologische Zeitschrift 93:12-25.

Mesterhazy, A. 1983. Breeding wheat for resistance to *Fusarium graminearum* and *F. culmorum*. Z. Pflanzenzucht. 91:295-311.

Mesterhazy, A. 1988. Expression of resistance to Fusarium graminearum and F. culmorum under various experimental conditions. J.Phytopathol 12:293-209.

Mesterhazy, A. 1995. Types and components of resistance to Fusarium head blight of wheat. Plant Breeding 114:377-386.

Mesterhazy, A., Bartok, T., Mirocha, C.G., and Komoroczy, R. 1999. Nature of wheat resistance to *Fusarium* head blight and the rol of deoxynivalenol for breeding. Plant Breeding 118:97-110.

Miedaner, T., Borehardt D. C. and Geiger H. H. 1993. Genetic analysis of inbred lines and their crosses for resistance to head blight *(Fusarium culmorum, F. graminearum)* in winter rye. Euphytica 65: 123-133.

Miedaner, T. 1997. Review: Breeding wheat and rye for resistance to Fusarium diseases Plant Breeding 116, 201-220.

Miedaner, T., Reinbrecht, C., Lauber, U., Schollenberger, M., and Geiger, H.H. 2001. Effects of genotype and genotype x environment interaction on deoxynivalenol accumulation and resistance to Fusarium head blight in rye, triticale, and wheat. Plant Breeding 120:97-105.

Miedaner, T., Moldovan, M., and Ittu, M. 2003. Comparison of spray and point inoculation to assess resistance to Fusarium head blight in a multienvironment wheat trial. Phytopathology 93:1068-1072.

Miedaner,T., Schilling, A.G., and Geiger, H.H. 2004. Competition effects among isolates of Fusarium culmorum differing in aggressiveness and mycotoxin production on heads of winter rye. European Journal of Plant Pathology 110: 63-70.

Miller, J.D. and Arnison, P.G. 1986. Degradation of deoxynivalenol by suspension cultures of the fusarium head blight resistant wheat cultivar Frontana. Can. J. plant Pathol. 8: 147-150.

Miller J.D., and Ewen, M.A. 1997. Toxic Effects of Deoxynivalenol on Ribosomes and Tissues of the Spring Wheat Cultivars Frontana and Casavant. Natural Toxins 5:234-237.

Miller, D., Culley, J., Fraser, K., Hubbard, S., Meloche, F., Ouellet, T., Seaman, W.L., Seifert, K.A., Turkington, K. and Voldeng, H. 1998. Effect of tillage practice on fusarium head blight of wheat. Can. Journal of Plant Pathol. 20:95-103.

Nicholson P., Chandler E., Draeger R.C., Gosman N.E., Simpson D.R., Thomsett M. and Wilson, A.H. 2003. Molecular tools to study epidemiology and toxicology of fusarium head blight of cereals. European Journal of Plant Pathology 109: 691-703.

Osborne, L.E. and Stein, J.M. 2007. Epidemiology of fusarium head blight on small-grain cereals. International Journal of Foood Microbiology 119:103-108.

Pereyra, S.A., and Dill-Macky, R. 2008. Colonization of the residues of diverse plant species by *Gibberella zeae* and their contribution to Fusarium head blight inoculum. Plant Dis. 92:800-807.

Parry D.W., Jenkinson P. and McLeod L. 1995. Fusarium ear blight (scab) in small grains

– a review. Plant Pathology 44:207-38.

Paulitz, T.C. 1996. Diurnal release of ascospores by *Gibberella zeae* in inoculated wheat plots. Plant Dis. 80:674-678.

Pestka, J.J. 2008. Mechanisms of deoxynivalenol-induced gene expression and apoptosis, Food Additives & Contaminants: Part A, 25: 1128-1140.

Pestka, J.J. and Smolinski, A.T. 2004. Deoxynivalenol: Toxicology and Potential Effects on Humans. Journal of Toxicology and Environmental Health, Part B, 8: 1, 39 -69.

Pritsch, C., Muehlbauer, G.J., Bushnell, W.R., Somers, D.A. and Vance, C.P. 2000. Fungal development and induction of defence response genes during early infection of wheat spikes by *Fusarium graminearum.* Molecular Plant Microbe Interactions 2:159-169.

Pritsch, C., Vance, C. P., Bushnell, W. R., Somers, D. A., Hohn, T. M., and Muehlbauer, G. J. 2001. Systemic expression of defence response genes in wheat spikes as a response to Fusarium graminearum infection. Physiol. Mol. Plant Pathol. 58:1-12.

Poppenberger, B., Berthiller, F., Lucyshyn, D., Sieberer, T., Schuhmacher, R., Krska, R., Kuchler, K., Glossl, J., Luschnig, C. and Adam, G., 2003. Detoxification of the Fusarium Mycotoxin Deoxynivalenol by a UDP-glucosyltransferase from Arabidopsis thaliana. The Journal of Biological Chemistry 48:47905-47914.

Rocha, O., Ansari, K. and Doohan, F. M. 2005. Effects of trichothecene mycotoxins on eukaryotic cells: A review. Food Additives & Contaminants 22:369-378.

Rossi,V. Ravanetti, A., Pattori, E. and Giosue, S. 2001. Influence of temperature and humidity on the infection of wheat spikes by some fungi causing fusarium head blight. Journal of Plant Pathology 83:189-198.

Rudd, J.C., Horsley, R.D., McKendry, A.L., and Elias, E.M. 2001. Host Plant Resistance Genes for Fusarium head blight: Sources, Mechanisms, and Utility in Conventional Breeding Systems. Crop Sci. 41, 620-627.

Schroeder, H.W., and Christensen, J.J. 1963. Factors affecting resistance of wheat to scab caused by *Gibberella zeae*. Phytopathology. 53: 831-838.

Seed Manitoba 2011. http://seedmb.ca/

"Seeding of principal field crops: Canada" Statistics Canada. June 23rd, 2009. Web. Accessed: December 2nd, 2010. Retrieved from http://www.statcan.gc.ca/daily-quotidien/090623/t090623b1-eng.htm

Snijders, C.H.A. and Perkowski, J. 1990. Effects of head blight caused by *Fusarium*

culmorum on toxin content and weight of wheat kernels. Phytopathology 80:566-570

Snijders, C.H.A. 1990. Diallel analysis of resistance to head blight caused by *Fusarium culmorum* in winter wheat. Euphytica 50: 1-9.

Snijders, C.H.A. and Van Eeuwijk, E.A. 1991. Genotype x strain interactions for resistance to Fusarium head blight caused by Fusarium culmorum in winter wheat. Theor Appl Genet 81:239-244.

Snijders, C.H.A. 2004. Resistance in wheat to Fusarium infection and trichothecene formation. Toxicology Letters 153 37-46.

Stack, R.W. 1989. A comparison of the inoculum potential of ascospores and conidia of *Gibberella zeae*. Can. J. Plant Pathol. 11:137-142.

Stack, R.W., and McMullen, M.P. 1998. A visual scale to estimate the severity of Fusarium head blight. Bull. PP-1095. North Dakota State Univ. Ext. Ser., Fargo.

Stein, J.M., Osborne, L.E., Bondalapati, K.D., Glover, K.D. and Nelson, C.A. 2009. Fusarium head blight severity and deoxynivalenol concentration in wheat in response to *Gibberella zeae* inoculum concentration. Phytopathology 99:759-764.

Siranidou, E., Kang, Z. and Buchenauer, H. 2002. Studies on symptom development, Phenolic compounds and morphological defence responses in wheat cultivars differing in resistance to *Fusarium* head blight. J. Phytopathology 150:200-208.

Sutton, J.C. 1982. Epidemiology of wheat head blight and maize ear rot caused by *Fusarium graminearum*. Canadian Journal of Plant Pathology 4:195-209.

Snijders, C.H.A. and Krechting, C.F. 1992. Inhibition of deoxynivalenol translocation and fungal colonization in Fusarium head blight resistant wheat. Can. J. Bot. 70:1570-1576.

Trail, F. 2009. For blighted waves of grain: Fusarium graminearum in the post genomics era. Plant physiology 149: 103-110.

Turuspekov, Y., Mano, Y., Honda, I., Kawada, N., Watanabe, Y. and Komatsuda, T. 2004. Identification and mapping of cleistogamy genes in barley. Theor Appl Genet 109: 480-487.

"Vista on the Agri-food Industry and the Farm Community" Statistics Canada. November 10th, 2009. Web Accessed: March 7, 2011. Retreived from http://www.statcan.gc.ca/pub/21-004-x/21-004-x2009002-eng.pdf

Van Eeuwijk, E.A., Mesterhazy, A., Ruckenbauer, P., Keizer, L. C. R, Kling, C.H.I., Saur, L., Burstmayer, H., Lemmens, M., Maurin, N., and Snijders, C. H. A. 1995. Assessing non-specificity of resistance in wheat to head blight caused by inoculation with

European strains of Fusarium culmorum, *F. graminearum* and *F. nivale* using a multiplicative model for interaction Theor Appl Genet 90: 221-228.

Von Der Ohe, C., Gauthier, V., Tamburic-Ilincic, L., Brûlé-Babel, A., Fernando, D.W., Clear, R., Ward, T.J. and Miedaner, T. 2010. A comparison of aggressiveness and deoxynivalenol production between Canadian *Fusarium graminearum* isolates with 3-acetyl and 15-acetyldeoxynivalenol chemotypes in field-grown spring wheat. European Journal of Plant Pathology. 127:407-417.

Waalwijk, C., Kastelein, P., Vries, I.D., Kerenyi, Z., Lee, T.V.D., Hesselink, T., Kohl, J. and Kema, G., 2003. Major changes in *Fusarium* spp. in wheat in the Netherlands. Eur. J. Plant Pathol. 109:743-754.

Wagacha, J.M., and Muthomi, J.W. 2007. Review - *Fusarium culmorum*: Infection process, mechanisms of mycotoxin production and their role in pathogenesis in wheat. Crop Protection 26:877-885.

Walter, S., Brennan, J.M., Arunachalam, C., Ansari, K.I., Hu, X., Khan, M.R., Trognitz, F., Trognitz, B., Leonard, G., Egan, D. and Doohan, F.M. 2008. Components of the gene network associated with genotype-dependent response of wheat to the Fusarium mycotoxin deoxynivalenol. Funct. Integr. Genomics 8:421-427.

Ward, T.J., Clear, R.M., Rooney, A.P., O'Donnell, K., Gaba, D., Patrick, S., Starkey, D.E., Gilbert, J., Geiser D.M. and Nowicki, T.W. 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 45:473-484.

Xu, X. 2003. Effects of environmental conditions on the development of Fusarium ear blight. European Journal of Plant Pathology 109:683-689.

Xu, X.M., Monger, W., Ritieni, A. and Nicholson, P. 2007. Effect of temperature and duration of wetness during infection periods on disease development, fungal biomass and mycotoxin concentration on wheat inoculated with single, or combinations of, *Fusarium* species. Plant Pathology 56:943-956.

Xue, A.G., Butler G., Voldeng, H.D., Fedak, G. and Savard, M.E. 2006. Comparison of the influence of inoculum sources on the development of fusarium head blight and the deoxynivalenol content in spring wheat in a disease nursery. Can. J. Plant Pathol. 28:152-159.

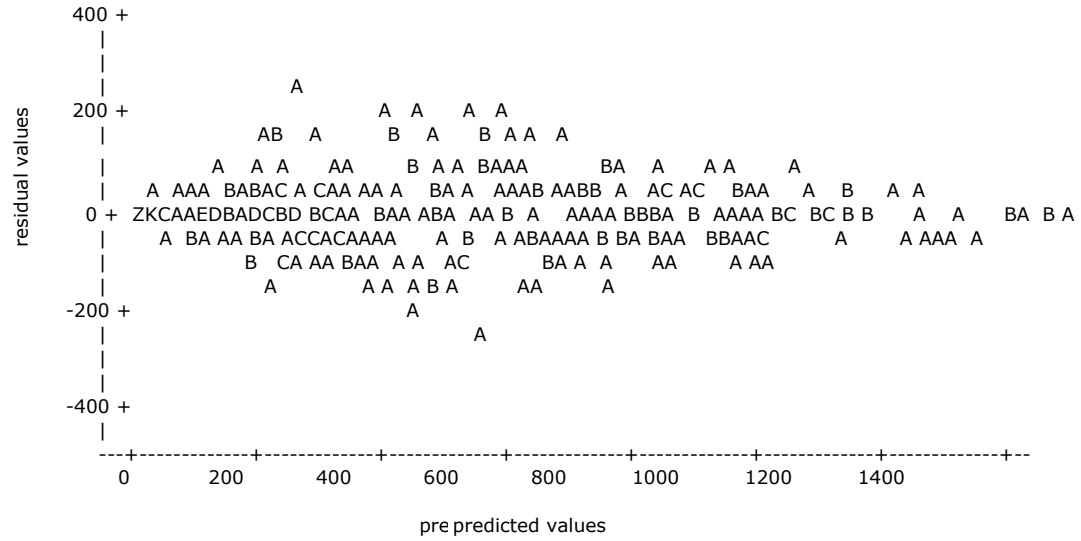
Yang, Z.P., Yang, X.Y. and Huang D.C. 1999. Comparison of evaluation methods for selection of resistance to Fusarium head blight in a recurrent selection programme in wheat (*Triticum aestivum* L.). Plant Breeding 118:289-292.

Yoshida, M., Kawada, N., and Tohnooka, T. 2005. Effect of row type, flowering type and several other spike characters on resistance to Fusarium head blight in barley. Euphytica 141: 217-227.

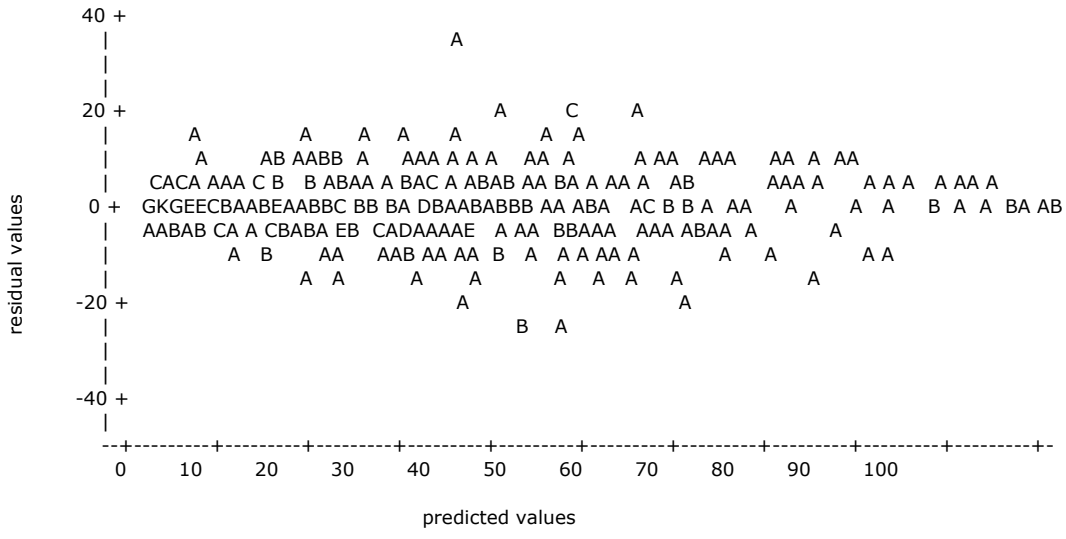
Zadoks, J.C., Chang, T.T., and Konzak, C.F. 1974. A decimal code for the growth stages of cereals. Weed Research 14:415-421.

6.2 APPENDICES

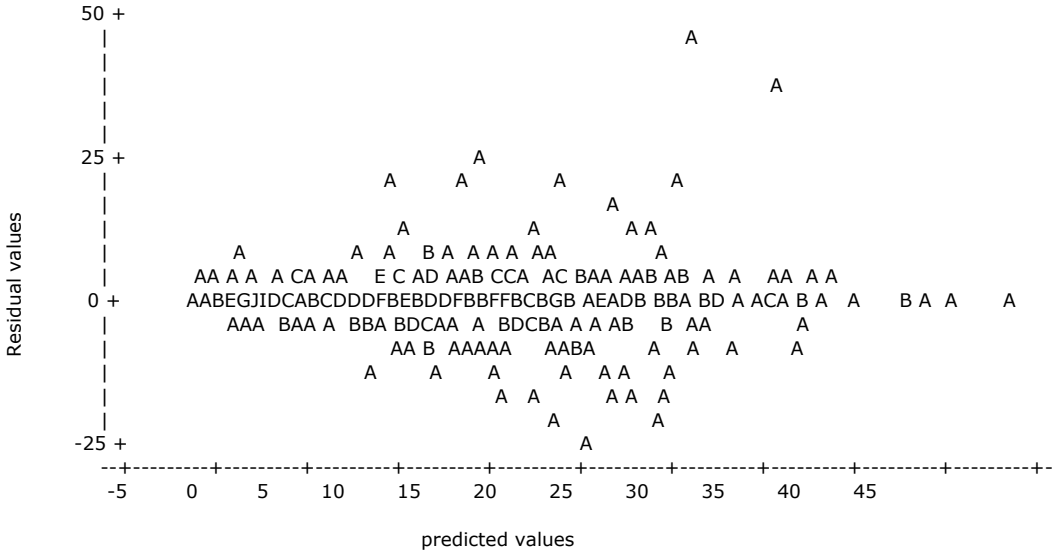
Appendix 6.2.1 Homogeneity of Variance test for the two individual years of data for AUDPC FHB index indicates a normal distribution on the plot of residual and predicted values.



Appendix 6.2.2 Homogeneity of Variance test for the two individual years of data for FDK indicates a normal distribution on the plot of residual and predicted values.



Appendix 6.2.3 Homogeneity of Variance test for the two individual years of data for DON content indicates a normal distribution on the plot of residual and predicted values.



Appendix 6.2.4 ELISA procedure, from Dr. Victor Limay Rios, Department of Plant Agriculture, Ridgetown Campus, University of Guelph.

The entire sample was finely ground using a ROMER grinding/sub-sampling mill to a texture that would pass through a 20 mesh (Model 2A, Romer Labs, Inc., Union, MO). For DON analysis, a 10-g subsample was extracted using ionized water. Toxin content was quantified using competitive direct enzyme-linked immonosorbent assay (EZ-Quant DON ELISA Plate Kit (Diagnostix, Thermo Fisher Scientific, Mississauga, ON)]) following the manufacturer's instructions. The DON detection limit was 0.5 ppm.

Appendix 6.2.5 Growing degree day formula.

$(T_{max} + T_{min} / 2) - T_{base}$

T_{max} = daily maximum temperature in degrees celsius
T_{min} = daily minimum temperature in degrees celsius
T_{base} = Base temperature of 5 ^{o}C

Appendix 6.2.6 CMC Media recipe

- NH4NO3 Ammonium Nitrate 1.50 g
- KH2PO4 Potassium phosphate 1.50 g

- MgSO4-7H2O Magnesium Sulfate 0.75 g
- Yeast Extract 1.50 g
- Carboxylmetyl-cellulose 22.50 g
- dH2O 1500 ml
- Streptomycin sulfate 0.38 g

Appendix 6.2.7 Cornell mix fertilizer for soiless mix

- 150 g 14-14-14
- 0.70g Chelated Zinc (14%)
- 2.0 g fritted trace elements
- 1.5 g chelated iron (13.2%)
- 100 g Caclium Carbonate
- 120 g 0-45-0
- 2 x 79L Sungro Sunshine LA4 soiless mix

Appendix 6.2.8 Dry Seed Extraction Buffer for use in DNA extraction, from the lab of Randy Clear, Canadian Grain Commission, Winnipeg, MB, Canada.

- to make 1L:
    - 200 ml 1M Tris-HCl (pH 7.5)
    - 57.6 ml 5M NaCl
    - 50 ml of 500 mM EDTA
    - 5g SDS

- 1. Add SDS to a large beaker containing 600 ml nanopure water. Cover top with foil and stir using stir bar and low heat.
- 2. Pour dissolved SDS and water into 1L flask
- 3. Add remaining solutions.
- 4. Bring up to 1L with nanopure water
- 5. Store in container with lid.