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PATHOGENICITY, TOXIGENIC POTENTIAL, AND GENOMICS OF *Fusarium graminearum* AND *F. meridionale* CAUSING EAR AND STALK ROT OF MAIZE

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PATHOGENICITY, TOXIGENIC POTENTIAL, AND GENOMICS OF *FUSARIUM GRAMINEARUM* AND *F. MERIDIONALE* CAUSING EAR AND STALK ROT OF MAIZE

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment
at the University of Kentucky

By
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Lexington, Kentucky
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2020

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ABSTRACT OF DISSERTATION

PATHOGENICITY, TOXIGENIC POTENTIAL, AND GENOMICS OF *Fusarium graminearum* AND *F. meridionale* CAUSING EAR AND STALK ROT OF MAIZE

Gibberella ear (GER) and stalk rot (GSR) diseases of maize in Brazil are caused mainly by *Fusarium meridionale*, a species belonging to the *Fusarium graminearum* species complex (FGSC). Another species within this complex, *F. graminearum sensu stricto* (hereafter *F. graminearum*), is second in importance on maize, but is the most common species found causing Fusarium Head Blight disease of wheat in Brazil. The latter species is the predominant cause of GER and GSR in North America, where *F. meridionale* has not been found thus far. In this dissertation I undertook a comparative analysis of pathogenic, saprophytic, toxigenic and genomic traits among a collection of strains representative of the two species and two hosts of origin to address possible explanations for the observed shift in the species dominance between maize and wheat. I initially hypothesized that the shift was due to their differential aggressiveness. To address this hypothesis, four field trials were conducted at different locations in order to study the aggressiveness (percent GER severity) of two *F. meridionale* and two *F. graminearum* strains, all isolated from maize, on maize hybrids with different levels of resistance. Plants were inoculated with single isolates, or with pairs of isolates sequentially and alternately at the silking stage. The results indicated that *F. meridionale* was more aggressive to maize than *F. graminearum*. *Fusarium meridionale* was also more competitive in ears that were co-inoculated with both species. In a second study I used a larger and more representative sample of strains of each species, isolated from both maize and wheat, to inoculate maize ears and stalks in the field. Consistent with my previous study, I found that *F. meridionale* was, on average, more aggressive than *F. graminearum* on maize ears. In contrast, *F. graminearum* was slightly more aggressive on maize stalks than *F. meridionale*. Both species contaminated maize ears with trichothecene mycotoxins, but *F. graminearum* strains produced primarily deoxynivalenol (DON) and its acetylated derivative 15ADON, whereas *F. meridionale* strains produced only nivalenol (NIV). The host of origin made no difference, and there was a lot of intraspecies variation in GER or GSR severity caused by isolates of both species. In a third study, an expanded collection of isolates of the two species was compared for 17 additional saprophytic, pathogenic, and toxigenic traits. Although there was significant intraspecies variation for most of these traits as well, the

strains were strongly structured by species regardless of the host of origin, based on a multivariate analysis. *Fusarium graminearum* was a more aggressive pathogen of wheat, and produced primarily DON in rice cultures or in wheat heads. DON is known to be an important factor driving aggressiveness of *F. graminearum* in wheat. On the other hand, *F. meridionale* grew faster in culture. All *F. meridionale* strains produced mainly NIV both *in vitro* and *in planta*, with the exception of two strains from maize that produced more DON than NIV in wheat heads. In a fourth study, whole genome analysis of selected representatives of both species showed that they were genetically divergent, based on patterns of conservation of single-nucleotide polymorphisms (SNPs) across alignments. There was evidence of frequent outcrossing among strains within both species. The genome analysis also provided clear evidence of recombination between the two phylogenetic species, indicating that they are not genetically isolated, and thus belong to a single biological species. Genetic and phenotypic divergence of *F. meridionale* and *F. graminearum* may indicate adaptive selection to different environmental niches. The results of this study suggest differential aggressiveness and toxigenicity as partial explanations for the predominance of *F. meridionale* on maize and *F. graminearum* on wheat, and they lay a foundation for future studies to explore these associations.

KEYWORDS: *Triticum aestivum*. *Zea mays*. Fusarium head blight. Nivalenol. Deoxynivalenol.

Franklin Jackson Machado

06/22/2020

Date

RESUMO DA DISSERTAÇÃO

PATOGENICIDADE, POTENCIAL TOXIGÊNICO, AND GENÔMICA OF *Fusarium graminearum* E *F. meridionale* CAUSADORES DAS PODRIDÕES DE COLMO E DE ESPIGA EM MILHO

No Brasil, as doenças podridão de Gibberella tanto em espigas (GER) como em colmos (GSR) de milho são causadas principalmente por *Fusarium meridionale*, uma espécie pertencente ao complexo de espécies de *Fusarium graminearum* (FGSC). Outra espécie dentro deste complexo, *F. graminearum* sensu stricto (daqui em diante *F. graminearum*), é a segunda em importância no milho, mas é a espécie mais comum encontrada causando a doença giberela do trigo no Brasil. A última espécie é a causa predominante de GER e GSR na América do Norte, onde *F. meridionale* ainda não foi encontrado. Nesta dissertação, realizei uma análise comparativa de características patogênicas, saprofiticas, toxigênicas e genômicas entre uma coleção de isolados representativa das duas espécies e dos dois hospedeiros de origem para abordar possíveis explicações para a mudança observada na dominância das espécies entre milho e trigo. Inicialmente, minha hipótese era de que a mudança se devia à agressividade diferencial. Para abordar essa hipótese, foram realizados quatro ensaios de campo em locais diferentes, a fim de estudar a agressividade (severidade de GER em porcentagem) de dois isolados de *F. meridionale* e dois de *F. graminearum*, todas isoladas do milho, em híbridos de comerciais de milho com diferentes níveis de resistência. As plantas foram inoculadas com pares de isolados de cada espécie, sequencialmente e alternadamente no estágio de reprodutivo de emissão de estilo-estigmas. Os resultados indicaram que *F. meridionale* foi mais agressivo ao milho que *F. graminearum*. *Fusarium meridionale* também foi mais competitivo em espigas co-inoculadas com ambas as espécies. Em um segundo estudo, usei uma amostra maior e mais representativa de isolados de cada espécie, obtidos de milho ou trigo, para inocular espigas e colmos de milho no campo. Consistente com meu estudo anterior, descobri que *F. meridionale* era, em média, mais agressivo que *F. graminearum* em espigas de milho. Em contraste, *F. graminearum* foi ligeiramente mais agressivo em colmos de milho que *F. meridionale*. Ambas as espécies contaminaram espigas de milho com tricotecenos, mas os isolados de *F. graminearum* produziram principalmente desoxinivalenol (DON) e seu derivado acetilado 15ADON, enquanto os isolados de *F. meridionale* produziram apenas nivalenol (NIV). O hospedeiro de origem não fez diferença, e houve muitas variações intra-espécies na severidade de GER ou GSR, causadas por isolados de ambas as espécies. Em um terceiro estudo, uma coleção expandida de isolados das duas espécies foi comparada acerca de 17 caracteres saprofiticos, patogênicos e toxigênicos adicionais. Embora tenha havido variação significativa intra-espécies para a maioria dessas características, os isolados foram fortemente estruturados por espécie, independentemente do hospedeiro de origem, com base em uma análise multivariada. *Fusarium graminearum* foi um patógeno mais agressivo do trigo, produzido principalmente DON em culturas de arroz ou em espigas de trigo. Sabe-se que o DON é um fator importante que impulsiona a agressividade de *F. graminearum* no trigo. Por outro lado, *F. meridionale* cresceu mais rapidamente em cultura. Todos os isolados de *F.*

meridionale produziram principalmente NIV *in vitro* e *in planta*, com exceção de dois isolados de milho que produziram mais DON que NIV em espigas de trigo. Em um quarto estudo, a análise do genoma completo de representantes selecionados de ambas as espécies mostrou que eles eram geneticamente divergentes, com base em padrões de conservação de polimorfismos de um único nucleotídeo (SNPs) entre alinhamentos. Houve evidências de cruzamentos frequentes entre os isolados de ambas as espécies. A análise do genoma também forneceu evidências claras de recombinação entre as duas espécies filogenéticas, indicando que elas não são geneticamente isoladas e, portanto, pertencem a uma única espécie biológica. A divergência genética e fenotípica de *F. meridionale* e *F. graminearum* pode indicar seleção adaptativa para diferentes nichos ambientais. Os resultados deste estudo sugerem agressividade diferencial e toxigenicidade como explicações parciais para a predominância de *F. meridionale* no milho e *F. graminearum* no trigo, e constituem uma base para estudos futuros para explorar essas associações.

PALAVRAS-CHAVE: *Triticum aestivum*. *Zea mays*. Giberela do trigo. Nivalenol. Desoxinivalenol.

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06/22/2020

Data

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DEDICATÓRIA

Aos meus amados pais, Lena e Olegário, às minhas queridas irmãs, Flávia e Cláudia, aos meus lindos sobrinhos, Daniel e Arthur e à minha companheira de todas as horas, Aline. Muito obrigado!

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CHAPTER 1. LITERATURE REVIEW

1.1 Overview

Fusarium head blight (FHB, a.k.a. wheat scab) and Gibberella ear and stalk rot (GER and GSR) are fungal diseases with major impacts on wheat and maize yields as well as food safety, respectively, worldwide (Goswami and Kistler 2004; Kuhnem et al. 2015; Munkvold 2003a). These diseases cause significant economic losses due to reduction in grain yield, but they also reduce crop value due to contamination of grain with mycotoxins, some of which are regulated for maximum tolerance limits to protect consumers (Goswami and Kistler 2004; Mcmullen et al., 2012; Sutton 1982).

In Brazil, a handful of species within the *Fusarium graminearum* species complex (FGSC) cause all three diseases, but the dominant species varies according to the host (Astolfi et al., 2011; Del Ponte et al., 2015; Kuhnem et al., 2016). In maize, *F. meridionale* is found most often causing GER and GSR, and it is also recovered most frequently among perithecia produced on maize stubble (Kuhnem et al., 2016). On the other hand, FHB in wheat and barley is caused mainly by *F. graminearum* (Astolfi et al., 2011; Del Ponte et al., 2015). Among the toxins produced by FGSC, B-trichothecenes and zearalenone are produced in the largest quantities. The B-trichothecenes include deoxynivalenol (DON) and nivalenol (NIV), and their respective acetylated forms 3ADON, 15ADON, and 4ANIV (Miller and Greenhalgh 1991). While *F. graminearum* produces either deoxynivalenol DON or NIV, depending on the region, *F. meridionale* is a consistent NIV-producing species (Ward et al., 2002; Del Ponte et al., 2015). An individual FGSC strain can be assigned to a B-trichothecene chemotype based on the combination of B-trichothecene and

acetylate produced in the highest amount (Desjardins 2008; Ward et al. 2008). The most common chemotypes are 3ADON or 15ADON (strains produce DON and a smaller amount of 3ADON or 15ADON), or NIV. *Fusarium graminearum* strains affecting cereals in Brazil are thus far only of the 15ADON chemotype (Del Ponte et al., 2015; Kuhnem et al., 2016). While DON has been confirmed as an important aggressiveness factor for *F. graminearum* spread within wheat heads in several studies (Bai et al. 2002; Desjardins et al. 1996; Harris et al. 1999; Maier et al. 2006), the role of NIV in the infection and spread of disease in wheat or maize remains unclear (Maier et al. 2006).

In this dissertation, I tested the hypothesis that host dominance of *F. meridionale* versus *F. graminearum* in Brazil was related to differential aggressiveness of the two species on maize versus wheat. To address this hypothesis, I used a combination of field and laboratory studies, and characterized the phenotypes and genotypes of a representative group of strains. Results of the study supported the hypothesis: thus, *F. meridionale* was more aggressive and more competitive on maize ears than *F. graminearum*, whereas *F. graminearum* was more aggressive on point-inoculated wheat heads. However, the high degree of intraspecies variation that I observed for aggressiveness and other phenotypes; evidence for relationships between host dominance and other factors including fertility, toxigenicity, and growth rate; and evidence for recombination among and between the two phylogenetic species; suggested that additional factors are also important in structuring the populations of *F. meridionale* and *F. graminearum* on maize and wheat in Brazil.

1.2 Economic impact and management of GER and GSR

Maize is typically grown in rotation with one or two other crops with little overall crop diversity. In Brazil, early-maturing soybean is sown in the beginning of the summer cropping season, followed by maize. In southern Brazil, wheat is planted as a winter crop following either soybean or maize. Wheat and other small grains are typically cultivated in a no-tillage system and FHB is of common occurrence, thus GER and GSR are more prevalent in these southern subtropical climates (Del Ponte et al. 2009). Both maize diseases are increasing in importance as the application of improved technologies, and agronomic practices including irrigation and double-cropping, have expanded (Costa et al. 2019).

Multiple species of the FGSC can cause FHB and GER (Goswami and Kistler 2004; Kuhnem et al. 2015; Munkvold 2003a). FGSC members produce zearalenone (ZON) in addition to the type-B trichothecenes DON and NIV, and all of these compounds are extremely harmful for human and animal health (Chen et al. 2019). There are mycotoxin limits for maize-based food and feed produced in Brazil, but these include only DON and ZON, and not NIV among the FGSG mycotoxins (ANVISA 2011). Although NIV-producing species are also found associated with wheat, NIV has not been regulated by any country so far (Ferrigo et al. 2016; Park et al. 2018). Fungicides are used for management of FHB and GER in Brazil, however the unpredictability of fungicide performance for disease control (Andriolli et al. 2016), means that breeding for host resistance is a priority (Mesterházy et al. 2012). Resistance to GSR and GER in maize is a complex trait that is influenced by genetic background and environmental factors, as well as by the pathogen population (Mesterházy et al. 2012; Yang et al. 2010). Hybrids differ significantly in

resistance to both diseases (Reid and Zhu, 2005). To date, there are no reports of complete resistance to GER or GSR, and the mechanisms of quantitative resistance are not well understood (Mesterházy et al. 2012). In addition to fungicides and selection of resistant cultivars, cultural practices are also recommended to minimize the stresses that increase plant susceptibility to fungal invasion (Gatch et al., 2002; Munkvold, 2003b).

1.3 Disease cycle of GER and GSR

Members of FGSC survive in residues of graminaceous crops including wheat, barley, rye and maize (Leplat et al., 2013; Pereyra & Dill-Macky, 2008). Most FGSC members have a broad spectrum of hosts among these graminaceous crops, and also weeds, which can contribute to the primary inoculum (Leplat et al. 2013). Non-graminaceous weeds and crops such as soybean, sunflower and alfalfa have also been reported as hosts for FGSC members (Pereyra and Dill-Macky, 2008). The fungi can overwinter as mycelia or as sexual structures called perithecia, from which sexual spores (ascospores) are produced and forcibly ejected into the atmosphere (Dufault et al., 2006; Leplat et al., 2013; Trail et al., 2005). Asexual spores (macroconidia) are produced in crop debris and in infected plant tissues, and are released and dispersed mainly by water splash during rainfall or overhead irrigation (Leplat et al. 2013). Infection occurs in high relative humidity (>80%) and temperatures ranging from 20º to 30º C (Del Ponte et al., 2004). The primary pathway for infection of maize kernels by *F. graminearum* is via the silks, which are highly susceptible during the first 6 days after silk emergence, becoming less susceptible thereafter (Munkvold 2003a; Reid and Zhu, 2005). Spores reach maize silks by splashing, wind dispersal, or insect vectors (Munkvold 2003a). Some infections can be initiated by lepidopteran insect injury to the kernels, but this is a less important pathway than silk

infection for GER, in contrast to other maize ear rots (Munkvold 2003a). Gibberella ear rot is favored by high levels of moisture around silking, followed by moderate temperatures and high rainfall during the maturation period (Munkvold 2003a; Sutton, 1982). FGSC members overwintering in infected plant residues can infect maize stalks through natural entry points (i.e. nodes), wounds caused by insects or mechanical damage, or by direct penetration of the root and stalk (Gatch et al. 2002). Plants are predisposed to stalk rot by any stress that reduces the photosynthetic capacity of the plant following anthesis, when the developing ear competes with the stalk for carbohydrates (Gatch and Munkvold, 2002; Dodd, 1980).

1.4 Distribution and ecology of FGSC

Different FGSC members dominate as the cause of FHB, GER, and GSR in different regions of the world (Boutigny et al. 2011; Carter et al. 2000; Castañares et al. 2016; Del Ponte et al. 2015; Gomes et al. 2015, 2016; Kuhnem et al. 2016; Sampietro et al. 2011; Umpiérrez-Failache et al. 2013). *Fusarium graminearum* is the most common species causing FHB in wheat and barley worldwide (Del Ponte et al. 2015; Kelly and Ward 2018) and is the most frequent cause of GER and GSR in North America. However in South America, including Brazil, and in Nepal, *F. meridionale* is more important as the causal agent of GER and GSR (Kuhnem et al. 2016; Sampietro et al. 2011; Desjardins and Proctor 2011). *Fusarium meridionale* is also the second most common species causing FHB in wheat in Brazil after *F. graminearum*, and it is increasing in importance in regions where maize is a major crop (Del Ponte et al. 2015). Other *Fusarium* species, including FGSG members (*F. graminearum*, *F. asiaticum*, *F. boothii, F. cortaderiae,* and *F. austroamericanum*) and non-members (*F. culmorum*, *F. cerealis* and *F. poae*), cause GER

and GSR disease in other regions of the world (Basler 2016; Kuhnem et al. 2016; Lee et al. 2012; Ndoye et al. 2012). It is unknown why one species tends to dominate among isolations from specific cereals in some regions. Possibilities could include differences in climate; variation in the microbiome that may incorporate more or different competitors; or differences in the cropping systems that may affect opportunities for cross-inoculation. Another possibility could be variations in saprophytic fitness in different regions or on alternate substrates. For example, *F. asiaticum* from rice was recovered in higher frequencies from rice straw than other FGSC species (Lee et al. 2009), and similarly, *F. meridionale* was the species that was recovered most frequently from maize stubble and stalks in Brazil (Kuhnem et al. 2016). It could also reflect competition among species that are most prevalent in the various locations (Carter et al., 2002). For example, the most common species causing FHB, *F. graminearum* 15ADON, has been shown to be more fertile and more aggressive on wheat compared with other species (Liu et al. 2017; Nicolli et al. 2018; Zhang et al. 2016). In South Africa, *F. boothii* was the only species infecting maize ears, while *F. graminearum* dominated in wheat spikes, which led the authors to hypothesize a selective disadvantage for *F. graminearum* on maize ears relative to other FGSC (Boutigny et al. 2011). *Fusarium boothii* has only recently been reported in North America, on wheat (Valverde-Bogantes et al., 2019), and it was shown to be less aggressive and toxigenic on wheat than *F. graminearum*. *Fusarium meridionale*, which dominates on maize in Brazil, has never been found in North America.

1.5 Genetics and genomics of FGSC

Another possibility for geographic differences in host dominance could be genetic variability among strains of the same species across regions, perhaps as a result of

hybridization or divergence due to localized selection. Members of FGSG are homothallic, but they can also outcross. Among *F. graminearum* strains in the laboratory, the rate of outcrossing was as high as 35% (Bowden and Leslie 1999) and in China, *F. graminearum* exhibited outcrossing rates of 6 to 20% in the field (Chen and Zhou 2009). High genotype diversity among some populations of *F. graminearum* in North America and Europe has led some to suggest that outcrossing is frequent in the field (Walker at al., 2001; Zeller et al., 2004; Talas et al., 2012; 2015). Stability among some subpopulations in North America has also been observed, suggesting that rates of outcrossing may vary in different locations (Goswami and Kistler 2004). The existence of subpopulations that are genotypically and phenotypically distinct (aka. genetic drift) could indicate the presence of diversifying selection during adaptation to different ecological niches (Kelly and Ward, 2018; Valverde-Bogantes et al., 2019).

Fusarium graminearum can also outcross with other FGSC members in the laboratory, e.g. *F. asiaticum* and *F. meridionale*, but generally fertility levels are low and segregation ratios are skewed (J. F. Leslie, personal communication). To date, only a few natural hybrids have been found (O'Donnell et al., 2000; Boutigny et al., 2011). Isolates of FGSC are simultaneously assigned to species and trichothecene chemotype by using a well-established multi-locus genotyping (MLGT) assay (Ward et al. 2008). Phylogenetic studies based on sequencing of multiple genes, and stability of species identified by the MLGT assay, suggest that the frequency of interspecies hybridization events has been insufficient to oppose isolation by genetic drift (O'Donnell et al., 2008; Starkey et al., 2007; Ward et al., 2008; Yli-Mattila et al., 2009). However, given the existence of skewed segregation ratios, the identification of hybrids will be more efficiently achieved with the

ability to investigate large numbers of markers, i.e. single nucleotide polymorphism (SNP) markers, across whole genomes. Until these methods can be applied widely, we may be underestimating the degree of interspecies hybridization among members of the FGSC.

1.6 Hypothesis and objectives of this dissertation

In spite of significant research attention over the years, diseases caused by members of FGSC remain as one of the most serious problems on cereal grains worldwide. Skewed host-species associations are common when multiple FGSC species and cereal hosts co-occur. The case of *F. meridionale* and *F. graminearum* on maize and wheat in Brazil is a typical example of this (Castañares et al. 2016; Del Ponte et al. 2015; Gomes et al. 2015; Kuhnem et al. 2016; Sampietro et al. 2011). It is not clear why these host preferences exist, when both species can cause both diseases. It suggests that *F. meridionale* and *F. graminearum* have distinguishable phenotypes relevant to selection or competition on these two hosts. An improved understanding of the host-specific differences in FGSC composition is critical for development of more effective genetic or chemical control strategies targeting disease and mycotoxin reduction. In this dissertation, I focused on the hypothesis that relative dominance of *F. meridionale* on maize and of *F. graminearum* on wheat in Brazil is due to differing levels of aggressiveness on these hosts. Several studies have reported that *F. graminearum* is more aggressive to wheat compared with other FGSC members (Goswami and Kistler 2005; Nicolli et al. 2015, 2018; Spolti et al., 2012; Tóth et al. 2005). In my dissertation, I compared aggressiveness to maize, together with 17 other pathogenicity and fitness-related traits, among a large collection of strains representing the two species and hosts of origin. I tested whether members of the two species were distinguishable for many or most of these traits, which would demonstrate biological

relevance of the phylogenetic species. I also conducted a preliminary genomic comparison of representatives of the two species to determine the degree of genetic divergence, and to evaluate the likelihood of admixture due to outcrossing within and between the species.

CHAPTER 2. GIBBERELLA EAR ROT IN MAIZE EARS CAUSED BY *FUSARIUM MERIDIONALE* AND *F. GRAMINEARUM*: A SINGLE VS. SEQUENTIAL ALTERNATING SPECIES INOCULATION

Abstract

In Brazil, a handful of species within the *Fusarium graminearum* species complex (FGSC) infect cereal crops at relative frequencies that vary according to the host. In maize, *F. meridionale* is the most prevalent FGSC species causing both ear and stalk rots (GER and GSR) as well as producing perithecial inoculum on maize stubble. In contrast, another species in the FGSC, *F. graminearum*, is the most common species causing Fusarium head blight (FHB) in wheat in the same region. The cause of this difference in dominance is unknown but may be related to differences in aggressiveness between the two species on the two hosts. A four location-hybrid study was conducted to compare the aggressiveness (measure of GER severity) of *F. meridionale* (Fmer) and *F. graminearum* (Fgra) strains, both isolated from maize. These were inoculated singly or sequentially alternated at the silking stage, totaling four treatments: Fgra and Fmer (each inoculated alone, four days after silk emergence), and Fgra→Fmer and Fmer→Fgra (sequentially inoculated, six days apart). The mean GER severity was highest in Fmer (52.1%), intermediate in Fmer→Fgra (40.3%) and Fgra→Fmer (38.3%) and lowest in Fgra (23.8%). The production of mycotoxins deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZON) was assessed in one experiment. Only NIV was detected in kernels after inoculation of Fmer alone, and DON was the only toxin found after inoculation of Fgra. Both NIV and DON (1.2:1 ratio), together with ZON, were found in grains harvested from the Fmer→Fgra sequential treatment. In contrast, only NIV was found in the Fgra→Fmer treatment. These results suggest that *F. meridionale* is more aggressive to maize ears than *F. graminearum*. These results also demonstrate the need to focus attention on the presence of NIV in maize grains, which is neglected in the current regulatory legislation.

KEYWORDS: *Zea mays*, *Fusarium graminearum* species complex, nivalenol, interaction, FGSC.

2.1 Introduction

Several species within the *Fusarium graminearum* species complex (FGSC) are the cause of major diseases of winter and summer cereal crops, including Fusarium head blight (FHB) in wheat (McMullen et al. 2012) and Gibberella ear rot (GER) and stalk rot (GSR) in maize (Goswami and Kistler 2004; Munkvold 2003a). FGSC members are particularly common as cereal pathogens in the subtropical climate of southern Brazil where wheat is grown during the winter-spring and maize during the summer-fall seasons. GER is favored by increased levels of moisture around silking, followed by moderate temperatures and high rainfall during the maturation period (Munkvold 2003a; Sutton 1982). Above-normal rainfall during El Nino years in the south of Brazil, and an increasing use of irrigation, has increased GER risk. As a result, trichothecene mycotoxins typically produced by GER pathogens have been found contaminating commercial maize grain (Oliveira et al. 2017).

Both FHB in wheat and barley and GER of maize are known to reduce yield (Duffeck et al. 2019; Munkvold 2003a) but the presence of mycotoxins that accumulate in the kernels and reduce product value is of increasing concern to producers (Munkvold et al. 2019). FGSC species are known to produce several mycotoxins, but the most important ones belong to the B-trichothecene group, including nivalenol (NIV) and deoxynivalenol (DON) (Miller and Greenhalgh 1991). These mycotoxins can accumulate in grain at levels considered unsafe for both livestock and human consumption, posing a serious threat to food safety (Pestka 2010; Rocha et al. 2005). To mitigate the impact on animal and human health, maximum tolerated limits have been established for *Fusarium* mycotoxins in wheat and maize grain and byproducts in Brazil and worldwide (ANVISA, 2011; van Egmond et

al. 2007). In Brazil, DON, ZON and fumonisins (B1 and B2) are the only mycotoxins that are currently regulated for maize (ANVISA, 2011, 2017).

Host shifts in dominance among different FGSC members have been reported in South America (Castañares et al. 2016; Del Ponte et al. 2015; Gomes et al. 2015, 201; Kuhnem et al. 2016; Sampietro et al. 2011). For example, *F. graminearum* of the 15ADON genotype is the most prevalent species causing FHB in wheat and barley in South America and worldwide (Del Ponte et al. 2015; Kelly and Ward 2018). In Brazil, *F. meridionale*, a NIV-producing species, is the dominant FGSC species in maize, and is also increasing in importance in wheat in regions where maize is a major crop (Del Ponte et al. 2015). A few other NIV-producing FGSC species have been found in association with GER in maize in other regions of the world, usually at minor frequency, including *F. asiaticum*, *F. cortaderiae* and *F. austroamericanum*, and also some non-NIV, non-FGSC species (*F. culmorum*, *F. cerealis* and *F. poae*) (Basler 2016; Desjardins and Proctor 2011; Kuhnem et al. 2016; Lee et al. 2012; Ndoye et al. 2012).

A recent survey of mycotoxins contaminating maize grains in southern Brazil was consistent with previous work suggesting that *F. meridionale* was the primary maize pathogen. The survey found that NIV mycotoxin, which is produced mainly by *F. meridionale*, and rarely by *F. graminearum*, was present in more samples (76%) than the DON mycotoxin (48%), though levels for both were lower than the Brazilian limits of contamination for DON (Oliveira et al. 2017).

Management of GER aims to reduce infection by toxigenic fungi in order to suppress mycotoxin production (Munkvold 2003a). The most effective control for GER is the use of host genetic resistance. However, breeding for resistance is complicated by the

diversity of pathogenic species (Mesterházy et al. 2012; Munkvold 2003a). Complete resistance to GER has not been reported and the mechanisms underlying resistance are not completely understood (Mesterházy et al. 2012). In fact, when each of the 14-most planted maize hybrids in southern Brazil was challenged with *F. meridionale*, none was resistant to GER (Nerbass et al. 2015). The use of foliar fungicides to control maize diseases (Esker et al. 2018), including ear rots, has increased in recent years (Andriolli et al. 2016; Anderson et al. 2017; Luna and Wise 2015; Fingstag et al. 2019).

In North America, GER is caused mainly by *F. graminearum* of the 15ADON genotype although the 3ADON genotype can also be an important contributor depending on the region (Burlakoti et al. 2017; Kuhnem et al. 2015). The toxin profile of a *Fusarium* species has been considered to influence its pathogenesis (Ward et al. 2002). While DON has been confirmed as an aggressiveness factor that facilitates fungus spread within wheat heads and, to a lesser extent, in maize ears in several studies (Bai et al. 2002; Desjardins et al. 1996; Harris et al. 1999; Maier et al. 2006), the role of NIV in the infection and spread of the disease within the maize ear remains unclear (Maier et al. 2006). There are very few studies that investigate differential pathogenicity among members of the FGSC with different toxin profiles that cause GER. In South Africa, *F. boothii*, a 15ADON-producing species that was dominant on maize (Boutigny et al. 2011), produced more severe symptoms and higher levels of mycotoxins compared with DON-producing *F. graminearum* isolates that were highly aggressive to wheat (Beukes et al. 2018). In that study, only the *F. boothii* isolates were obtained from maize, while the *F. graminearum* isolates had been recovered from wheat. Multiple members of the FGSC and of other species complexes can co-occur in the same field or even in the same maize ears (Logrieco

et al. 2002; Oldenburg et al. 2017; Picot et al. 2012), but the effects of any interspecific interaction on disease development are unknown. In this study I tested the hypothesis that *F. meridionale* from maize is more aggressive to maize ears, and produces significant levels of NIV trichothecenes, when compared with *F. graminearum* also obtained from maize. Isolates were inoculated singly, and they were also co-inoculated sequentially and alternately in order to study any interspecific interactions.

2.2 Materials and Methods

2.2.1 Fungal isolates and inoculum preparation

Two *F. graminearum* (DON-producing) isolates and two *F. meridionale* (NIV-producing) isolates were selected from a collection obtained from symptomatic maize kernels (Stumpf et al. 2013). These isolates had been identified to species as part of a previous study that showed the dominance of *F. meridionale* in Brazilian maize (Kuhnem et al. 2016).

A spore suspension was prepared by growing isolates individually on potato dextrose agar (PDA) for 10 days with a 12-h dark/light cycle. The macroconidia were filtered through two layers of cheesecloth to reduce the number of mycelial fragments present in the inoculum. The macroconidial suspensions of each isolate were quantified by using a hemocytometer. Spore suspensions of the two isolates of each species were diluted in sterile water and then mixed in a 1:1 ratio (v/v) to achieve the desired macroconidia concentrations (5×10^5 macroconidia/ml) (Reid et al. 1995).

2.2.2 Field trial in Southern Brazil (subtropics)

Field experiments were carried out in a no-till area located in the municipality of Eldorado do Sul, Rio Grande do Sul (RS) state, during the 2012 and 2013 growing seasons, and in the municipality of Lages, Santa Catarina (SC) state, during the 2012-growing season. The locations are ~ 400 km apart. In each location and year, two field trials, sown on different dates, were arranged in a 2x5 factorial experiment in a split-plot design with four replications. Two ear-rot-susceptible commercial maize hybrids, P30F53 HR® and STATUS TL®, were randomly assigned to the main plots, each of which consisted of five rows (6-m-long row with 50 cm between rows). The subplot units consisted of five plots of one row each, for which five inoculation treatments were randomized. The ten central plants of each row were inoculated. All plots were bordered by non-inoculated rows of the same maize hybrid (Fig. S2.5).

2.2.3 Field trials in Southeastern Brazil (Tropics)

One field experiment was conducted at the experimental station at the Universidade Federal de Viçosa (20º44'44" S, 42º50'59" W, 661 m above sea level) during the 2017 winter growing season. Seeds of the maize hybrid RB9004 PRO2® were sown in April that year. The field trials were arranged in a randomized complete block design with four replications, and each of the treatments was randomly assigned to the experimental units (Table 2.1). Each block consisted of an individual row. Each plot consisted of a 5-m-long row with 60 cm between rows. The central ten plants in each plot were inoculated. All plots were bordered by non-inoculated rows of the same maize hybrid. Plots were fertilized

following chemical soil analyses, and sprinkle-irrigated as needed. Weather variables (precipitation - rain plus irrigation; maximum, average and minimum relative humidity - RH; and maximum, average and minimum temperature) were recorded hourly by an automatic meteorological station (Squitter, Squitter Soluções em Monitoramento Ambiental, São José dos Campos, São Paulo, Brazil). The station was located within the field and data were collected from sowing to maize harvest. Average weather variables are presented in Figure S2.6.

2.3 Inoculation procedures

The five inoculation treatments are summarized in Table 2.1. Single inoculation treatments were made four days after silking (Reid et al. 2002). Plants were individually inoculated by injecting the suspensions (2 ml of a 5×10^5 macroconidia/ml solution) into the silk channel of the primary ear using a syringe with an obtuse needle (Anderson et al. 2016; Reid et al. 1995). In order to determine whether there was a positive or negative interaction of the two species in co-inoculations, sequential, alternated inoculations were made six days apart. A mock inoculation (sterile distilled water) was included as a negative control. Inoculations were followed by three consecutive days of irrigation. When grain moisture content reached an estimated 22%, the ten inoculated ears for each treatment were handpicked, husked, and the GER severity score on each ear was rated as the percentage area of the ear that was symptomatic (Reid et al. 2002).

2.3.1 Mycotoxin analysis

Mycotoxins were measured for the field experiment conducted in Viçosa in 2017. Harvested maize ears were dried at 60 °C for five consecutive days, shelled, and grains were stored dry at -20 °C until analysis. Mycotoxin in grains was determined by bulking the individual ears from each block, which was considered as a replicate. A 10-g sub-sample of each pooled replicate was ground by using a coffee grinder and then homogenized. The ground samples were sent to the Virginia Tech Deoxynivalenol (DON) Testing Lab, Blacksburg (Virginia). The amount of DON and each of its acetylated forms (15ADON and 3ADON), NIV, and ZON were quantified using a gas chromatography–mass spectrometry method as described previously (Fuentes et al. 2005; Mirocha et al. 1998).

2.3.2 Data analysis

The main and interaction effects of the treatment factors on the response variable were evaluated under a linear mixed modelling framework at 5% significance. Trials, maize hybrids, and replicates were treated as random effects in our model. The model was expanded to account for the main and interaction effects of the species from each host of origin. The mixed models were fitted using the *lmer* function of ‘lme4’ package (Bates et al. 2015) of R (R Core Team 2019). The 'emmeans' package (Lenth 2019) was used to estimate the back-transformed lsmeans and respective confidence intervals. The function *cld* from ‘multcomp’ R package (Hothorn et al. 2008) was used for multiple comparison of treatment means at 5% significance.

2.4 Results

Mean GER severity differed among the treatments ($P < 0.001$); it ranged from 1% to 100% (median = 20%) being lowest and highest in Fgra and Fmer, respectively, compared with the other treatments ($P < 0.05$) (Fig. 2.1). Severity induced by Fmer (52.1%) was two times higher than by Fgra (23.8%) (Fig. 2.2). Severity in Fgra→Fmer and Fmer→Fgra treatments did not differ from one another ($P = 0.793$) but differed from each of the single inoculations (Table 2.2). No visible GER symptoms were found in the non-inoculated controls of any experiment, suggesting no influence of background inoculum.

Trichothecene levels in kernels from Fmer and Fgra treatments ranged from 0.50 to 2.05 μg/g (ppm) respectively (Fig. 2.2B). For the sequential inoculation treatments, DON and NIV levels ranged from 0.35 to 3.15 μg/g in the Fgra→Fmer treatment. DON was detected in only one sample of Fmer→Fgra at similar levels with NIV and ZON (0.70 μg/g, 0.60 μg/g and 0.40 μg/g, respectively). Only NIV was detected in kernels from ears inoculated with Fmer, whereas DON was the only mycotoxin detected in kernels from the Fgra treatment. In contrast, NIV was more abundant in kernels from ears of the Fgra→Fmer treatment. Finally, in the Fmer→Fgra treatment NIV, DON, and a smaller amount of ZON were detected (Fig. 2.3A). Neither 15ADON nor 3ADON were detected in any sample (Fig. 3A). There was a positive correlation between NIV production and GER severity (Fig. 2.3B).

2.5 Discussion

In this study, I tested the hypothesis that *F. meridionale* from maize is more aggressive to maize ears than maize isolates of *F. graminearum*. Evidence of potential host preference among members of the FGSC has been reported from different parts of the world where multiple FGSC species infect different cereal crops (Boutigny et al. 2011; Carter et al. 2000; Del Ponte et al. 2015; Umpiérrez-Failache et al. 2013; van der Lee et al. 2015). For example, differential aggressiveness among FGSC species has been reported for FHB in wheat (Goswami and Kistler 2005; Nicolli et al. 2015; Spolti et al. 2012; Tóth et al. 2005). In contrast, colonization did not differ between *F. meridionale* and *F. graminearum* inoculated onto soybean pods in the field (Chiotta et al. 2016). In most of the previous studies of GER, *F. boothii*, the dominant species associated with the disease in South Africa and China, produced the same amount of disease as *F. graminearum* in field experiments (Beukes et al. 2018; Gai et al. 2017). One study from China reported that *F. boothii* from maize was similarly aggressive to maize stalks as *F. graminearum* isolates obtained from maize or wheat (Zhang et al. 2016). In the present study, I found that severity was more than twice as high when *F. meridionale* was inoculated singly compared with *F. graminearum*. This supports my hypothesis that *F. meridionale* is more aggressive to maize, which may contribute to its dominance in naturally infected ears and stalks of maize in a region where both species co-exist and *F. graminearum* is dominant in wheat (Kuhnem et al. 2016).

The current work is the first field inoculation study to confirm the ability of *F. meridionale* to produce significant amounts of NIV in maize ears. NIV was recently found to be the most common mycotoxin in surveys of commercial maize grain in Brazil (Oliveira

et al. 2017), which is consistent with the dominance of this pathogen in causing GER epidemics. The higher toxin amount/severity ratio for *F. graminearum* than *F. meridionale* observed is consistent with reports of the generally high toxigenic potential of *F. graminearum* of the DON type versus the NIV-producing FGSC (Nicolli et al. 2015; Tóth et al. 2005). Levels of NIV were correlated with aggressiveness to ears, suggesting that NIV plays a role in the process of infection and colonization.

During sequential co-inoculations, GER severity and NIV production by *F. meridionale* were both reduced if *F. graminearum* was inoculated following *F. meridionale* at the same infection site. It seems that *F. meridionale* facilitated *F. graminearum* infection, but its own development was reduced as a result. Previous studies of maize ears inoculated with a mix of species suggested that *F. graminearum* predisposed ear tissues to infection by a weaker species, *F. verticillioides*, in mixed inoculation treatment (Reid et al. 1999). The damage caused by the first species to be inoculated may have an effect similar to wounding caused by lepidopteran larvae that facilitates colonization by subsequently applied inoculum (Picot et al. 2012). However, when *F. graminearum* was inoculated first in my experiments, its growth was apparently ultimately suppressed by the more aggressive *F. meridionale*, evidenced by absence of DON in these samples.

The collective results of prior extensive surveys of species composition frequency, together with the results of the field experiments reported here, suggest that relative aggressiveness and a competitive advantage is likely to be one of the drivers shaping FGSC composition in maize in Brazil. My findings are important to breeders because they can inform the selection of appropriate adapted species to be used as inoculum when screening for host resistance. Ideally, further studies should focus on screening of regionally-

dominant inoculum where the hybrids shall be deployed, as well as testing the significance of the species x hybrid interactions using larger number of host genotypes, similar to what has been done for wheat genotypes challenged with *F. graminearum* and *F. meridionale* (Mendes et al. 2018).

2.6 Tables

Table 2.1. Single and sequential inoculation treatments of *Fusarium graminearum* (Fgra) and *Fusarium meridionale* (Fmer) on maize ears.

Treatment[a]	**Silking + 4 days**	**Silking + 10 days**
Fgra[b]	*F. graminearum*	-
Fmer[c]	*F. meridionale*	-
Fgra → Fmer	*F. graminearum*	*F. meridionale*
Fmer → Fgra	*F. meridionale*	*F. graminearum*
Mock inoculations	Water	Water

[a] All inoculations were done by injecting 2 mL of the macroconidia suspension at 5×10^5 macroconidia/ml. All isolates were obtained from naturally infected maize kernels (Stumpf et al. 2013).

[b]Mix of two *F. graminearum* (15ADON) isolates.

[c]Mix of two *F. meridionale* (NIV) isolates.

Table 2.2. Estimated differences in mean Gibberella ear rot (GER) severity between pairs of inoculum treatments of *Fusarium graminearum* (Fgra) and *F. meridionale* (Fmer) isolates on three different hybrids (P30F53 HR®, STATUS TL® and RB9004 PRO2®) in three different field locations in Brazil during 2012, 2013 and 2017 growing seasons in Lages-SC, Eldorado do Sul-RS and Viçosa-MG, Brazil.

Contrasts	**Estimate**	**SE[a]**	**df[b]**	**t.ratio**	**P-value[c]**
Fgra - (Fgra→Fmer)	-14.50	4.87	247	-2.97	0.017
Fgra - Fmer	-28.30	4.66	242	-6.07	< 0.001
Fgra - (Fmer→Fgra)	-16.50	4.75	244	-3.47	0.004
(Fgra→Fmer) - Fmer	-13.80	4.54	246	-3.05	0.013
(Fgra→Fmer) - (Fmer→Fgra)	-2.00	4.55	240	-0.44	0.972
Fmer - (Fmer→Fgra)	11.80	4.41	239	2.69	0.038

[a]SE = Standard error.

[b]Degrees-of-freedom method: Kenward-Roger.

[c]*P*-value adjustment: Tukey method for comparing a family of 4 estimates.

2.7 Figures

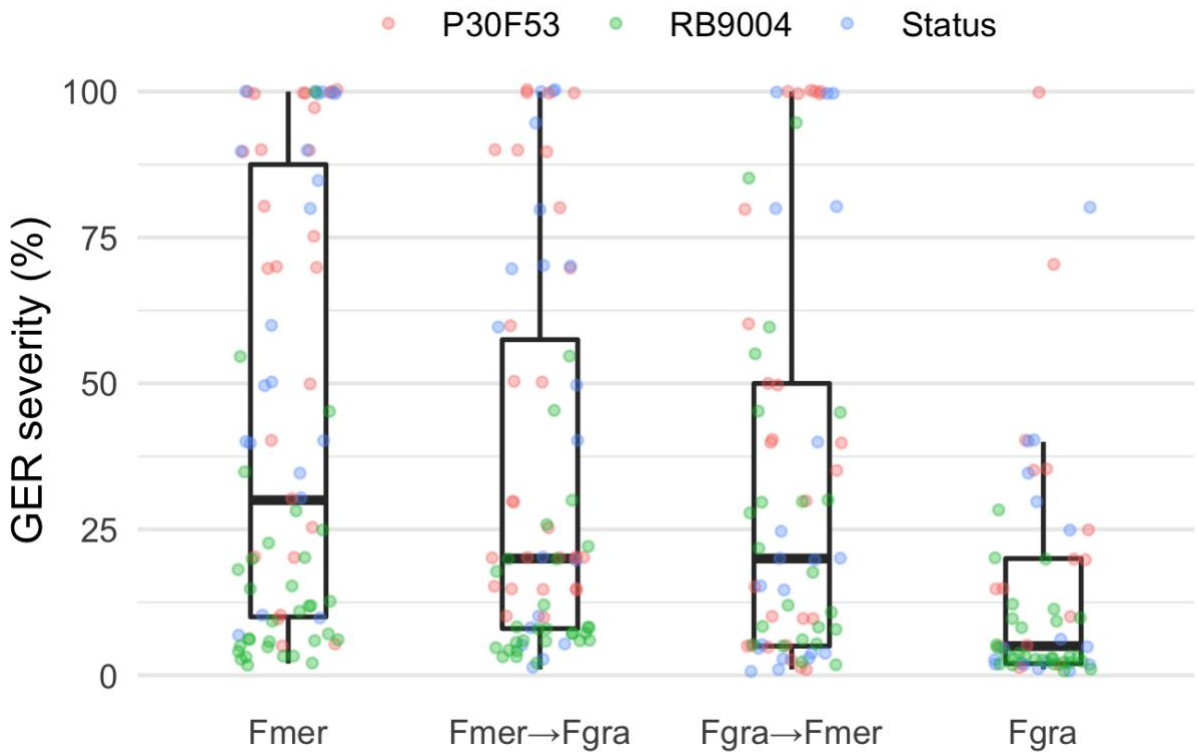

Figure 2.1. Distribution of Gibberella ear rot (GER) severity among the inoculation treatments of *Fusarium graminearum* (Fgra) and *F. meridionale* (Fgra) isolates on three different hybrids (P30F53 HR®, STATUS TL® and RB9004 PRO2®) in three different locations in Brazil. The first two maize hybrids were planted during the 2012 growing season in Lages Santa Catarina state (SC) and Eldorado do Sul, Rio Grande do Sul state (RS), and during the 2013 growing season in Eldorado do Sul, RS. The RB9004 PRO2® maize hybrid was cultivated during the 2017 growing season in Viçosa, Minas Gerais state (MG). The line within each box represents the median, the top and bottom lines of the boxes represent the 75th and 25th percentiles, respectively. The vertical bars extending beyond the boxes show the 10th and 90th percentiles, and the dots represent the GER severity of each inoculated ear.

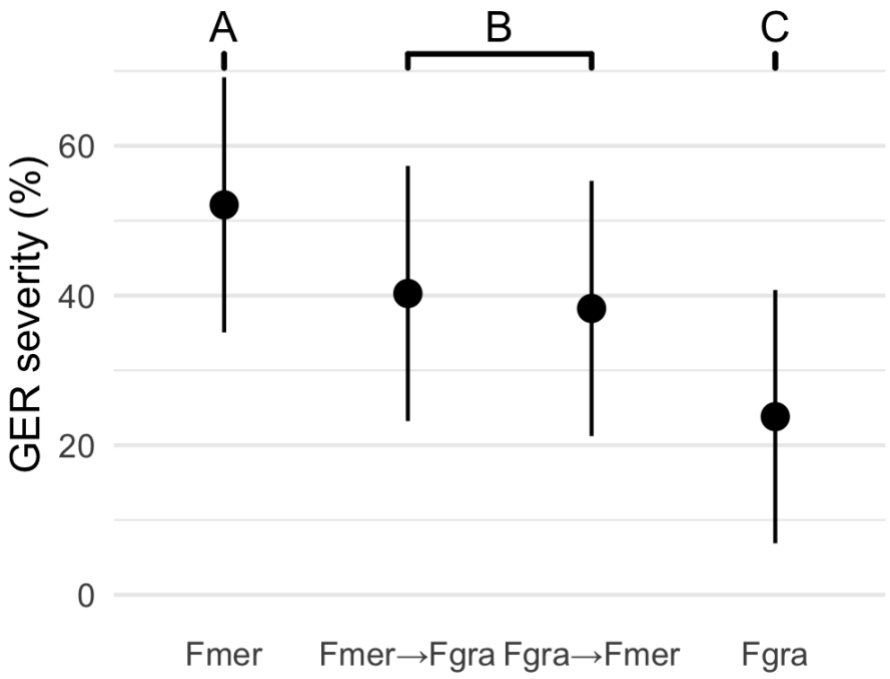

Figure 2.2. Least square means and confidence intervals from linear mixed analyses of the effect of single and sequential inoculation treatments of *Fusarium graminearum* (Fgra) and *F. meridionale* (Fgra) isolates on Gibberella ear rot (GER) severity of inoculated maize ears of three different hybrids (P30F53 HR®, STATUS TL® and RB9004 PRO2®) in three different locations in Brazil. Means with the same letters are not significantly different from each other based on Tukey test ($P = 0.05$).

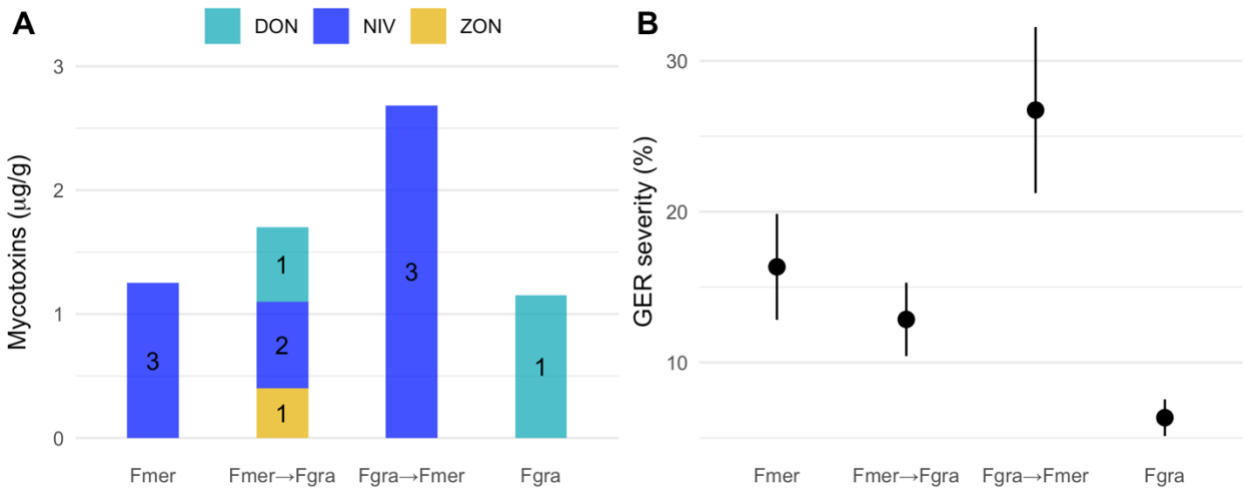

Figure 2.3. (A) Mean production of deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZON) and **(B)** mean Gibberella ear rot (GER) severity and standard error resulting from *Fusarium graminearum* and *F. meridionale* isolates inoculated onto maize ears of hybrid RB9004 PRO2® in single and sequential inoculation treatments in a field trial conducted in Viçosa 2017. Numbers represent how many of the four blocks (bulked sample of kernels from 10 inoculated ears) had detectable mycotoxins. Measurements of 15-acetyl-deoxynivalenol (15ADON) and 3ADON, and in the missing blocks, were under the limit of detection (< 0.25 µg/g).

2.8 SUPPLEMENTARY MATERIAL

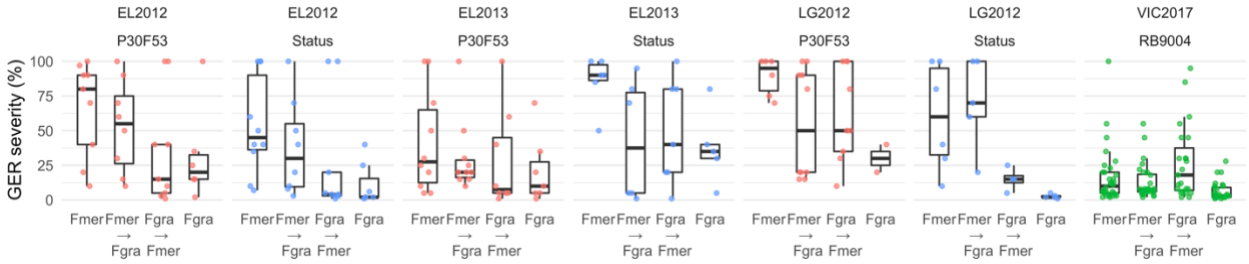

Figure S2.4. Distribution of Gibberella ear rot (GER) severity among the inoculation treatments of *Fusarium graminearum* (Fgra) and *F. meridionale* (Fgra) isolates on three different hybrids (P30F53 HR®, STATUS TL® and RB9004 PRO2®) in three different locations in Brazil by trial. The first two maize hybrids were planted during the 2012 growing season in Lages (LG), Santa Catarina state (SC) and Eldorado do Sul (EL), Rio Grande do Sul state (RS), and during the 2013 growing season in Eldorado do Sul, RS. The RB9004 PRO2® maize hybrid was cultivated during the 2017 growing season in Viçosa (VIC), Minas Gerais state (MG). Each data point represents the GER severity in a single ear.

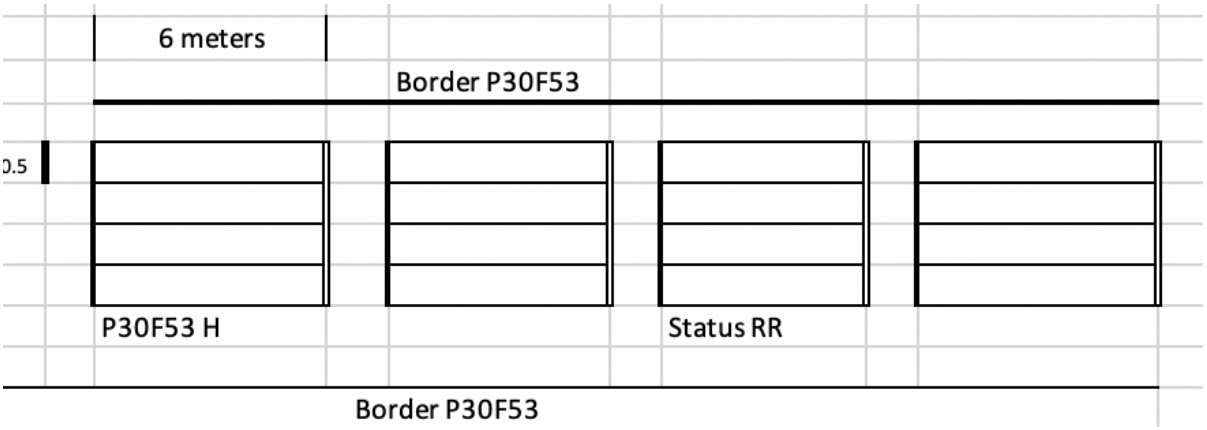

Figure S2.5. Schematic representation of the trials conducted in Southern Brazil under subtropical conditions.

Figure S2.6. Daily average of meteorological data recorded by an automatic meteorological station located within the field trial conducted in Viçosa, Minas Gerais state (MG) during the 2017 growing season. Minimum and maximum air temperature (°C) are represented in red and blue solid lines, respectively. Precipitation (rain plus irrigation) is represented by vertical bars. The RB9004 PRO2® maize hybrid was planted on April 25, inoculated on the 7th of August and harvested on the 25th of September.

CHAPTER 3. GIBBERELLA EAR AND STALK ROT CAUSED BY *FUSARIUM MERIDIONALE* AND *F. GRAMINEARUM*: AGGRESSIVENESS AND MYCOTOXIN PRODUCTION

Abstract

Gibberella ear (GER) and stalk rot (GSR) of maize in Brazil are caused mainly by *Fusarium meridionale*, while *F. graminearum*, the dominant pathogen causing Fusarium head blight (FHB) in wheat worldwide, is the main contributor to FHB epidemics in the same region. One hypothesis for this observed shift in dominance is that *F. meridionale* is more aggressive as a maize pathogen, while *F. graminearum* is more aggressive as a wheat pathogen. A collection consisting of 16 isolates of *F. graminearum* (12 from wheat and four from maize) and 24 isolates of *F. meridionale* (8 from wheat and 16 from maize) was tested for aggressiveness (on ears and stalks) and toxin production (in kernels) in field inoculation studies involving four maize hybrids. Field trials were conducted during the winter and the summer growing seasons. Ear and stalk inoculations were performed four days after silking. Inoculated maize ears and stalks were harvested at R5-R6 and disease severity was estimated. The amount of deoxynivalenol (DON) and its acetylated forms (15ADON and 3ADON), nivalenol (NIV) and zearalenone (ZON) were quantified in harvested grains from one season (summer). Average GER severity induced by *F. meridionale* (13.94%) was twice as high as that produced by *F. graminearum* (7.15%). Host of origin (wheat versus maize) had no significant effect on GER severity. However, when comparisons were limited to strains from either wheat or from maize, isolates of *F. meridionale* and *F. graminearum* from wheat were not significantly different in aggressiveness to maize ears, whereas isolates of *F. meridionale* from maize were twice as aggressive as isolates of *F. graminearum* from maize. There was a lot of variability in GER and GSR severity among the isolates of the same species. The GER and GSR severity varied from 0.33 to 100% and from 0.005 to 81.17%, respectively. On average, *F. graminearum* (18.40%) was slightly more aggressive than *F. meridionale* (16.10%) in maize stalks, regardless of the hybrid and host of origin. The primary mycotoxins produced by *F. graminearum* in maize ears were DON and 15ADON (7/16 strains), and NIV was the only toxin produced by *F. meridionale* (17/24 strains). Three isolates of each species produced ZON. The rest of the strains did not produce detectable mycotoxins. My results provide a basis for understanding the epidemiology of both species in Brazil, contributing new knowledge to explain the predominance of *F. meridionale* associated with maize.

KEYWORDS: *Zea mays*, nivalenol, deoxynivalenol, comparative epidemiology.

3.1 Introduction

Members of the *Fusarium graminearum* species complex (FGSC) are ascomycete fungi that cause Fusarium head blight (FHB) in small grains, and Gibberella ear rot (GER) and stalk rot (GSR) on maize. These diseases cause significant reductions in yield and grain quality worldwide (Kazan et al. 2012; McMullen et al. 2012). They also pose a threat to food safety because of the ability of the causal fungi to produce mycotoxins that accumulate in the kernels at unsafe levels for both livestock and human ingestion (Rocha et al. 2005; Pestka 2010). The primary toxins produced by FGSC are the B-trichothecenes, which include deoxynivalenol (DON) and nivalenol (NIV) (and their acetylated forms), as well as zearalenone (ZON) (Miller and Greenhalgh 1991). DON and ZON are proposed to act as aggressiveness factors in maize stalks (Quesada-Ocampo et al. 2016), and the importance of DON for aggressiveness has been well-established on wheat heads (Bai et al. 2002; Desjardins et al. 1996; Maier et al. 2006). DON also reportedly contributes to GER severity (Harris et al. 1999). The role of NIV during maize ear and stalk infection is less well understood (Maier et al. 2006).

In Brazil, GER and GSR are more prevalent in the southern subtropical climates where small grains are typically cultivated in a no-tillage system, and FHB is of common occurrence (Del Ponte et al. 2009). Ear and stalk rots caused by FGSC are increasing in importance as the application of improved technologies, and agronomic practices including irrigation and double-cropping have expanded (Costa et al. 2019). The recent promulgation of mycotoxin limits for maize-based food and feed produced in Brazil (ANVISA 2011), and the unpredictability of fungicide performance for disease control (Andriolli et al. 2016), means that breeding for host resistance is a priority (Mesterházy et al. 2012).

Resistance to either GSR or GER in maize is a complex trait that is influenced by genetic background and environmental factors, as well as by the pathogen population (Mesterházy et al. 2012; Yang et al. 2010). To date, there are no reports of complete resistance to GER or GSR, and the mechanisms of quantitative resistance are not completely understood (Mesterházy et al. 2012). As discussed in Chapter 1, breeders should include adapted strains of regionally dominant species when screening for host resistance.

The hypothesis that FGSC pathogen species differ in their ability to survive and compete in different crops is suggested by the results of surveys in regions where multiple FGSC species and cereal hosts occur (Carter et al. 2000; Boutigny et al. 2011; Del Ponte et al. 2015; Kuhnem et al. 2016; Umpiérrez-Failache et al. 2013). There are some controlled studies that also support this hypothesis: for example, the most prevalent species causing FHB, *F. graminearum* 15ADON, has been shown to be more fit as a pathogen of wheat compared with other species (Liu et al. 2017; Nicolli et al. 2018; Zhang et al. 2016). In Chapter 1 of my dissertation, I reported that *F. meridionale* was more aggressive and competitive than *F. graminearum* when it was used to inoculate or co-inoculate maize ears. However, the small sample size (two isolates of each species, from only a single host), the combining of the isolates, thus masking any intraspecific differences, and the focus on only one stage of the cycle (colonization) and one single organ (ears), limited my ability to conclude that these differences have a major role in shaping FGSC composition in the field. In the current study, the number of isolates was increased, isolates were collected from both maize and wheat, isolates were evaluated individually, and their ability to colonize stalks was also studied. The main objective of my work here was to compare *F.*

graminearum and *F. meridionale* aggressiveness on maize ears and stalks, and mycotoxin production in ears.

3.2 Materials and Methods

3.2.1 Collection of isolates

Forty-one isolates of *F. graminearum* and *F. meridionale* that were collected during previous surveys from symptomatic maize (Kuhnem et al. 2016) and wheat kernels (Del Ponte et al. 2015) were used in these trials. The isolates were previously identified simultaneously to species and trichothecene chemotype (Del Ponte et al. 2015, Kuhnem et al. 2016). The isolates were recovered from storage and their identities were confirmed by using the Fg16F/R primer set (Nicholson et al. 1998) that yields distinct fragment sizes for *F. meridionale* (~500 bp) or *F. graminearum* (~450 bp) (Astolfi et al. 2011; Castañares et al. 2016; Del Ponte et al. 2015; Nicholson et al. 1998). The 40 isolates were selected to be representative geographically and for time of sampling. The number of isolates from each host and each species was based on the reported frequencies in surveys. Thus a total of 25 *F. meridionale* (nine from wheat and 16 from maize) and 16 *F. graminearum* (12 from wheat and four from maize) were selected. The isolates are described in Table S3.1.

3.2.2 Inoculum preparation

Spore suspensions were prepared by growing the isolates on *Spezieller Nahrstoffarmer* agar (SNA) plates for 10 days at 23 °C with a 12-h dark/light cycle. The macroconidia were

filtered through two layers of cheesecloth to reduce the mycelial fragments present in the inoculum. The macroconidial suspension was then quantified by using a hemocytometer. Sterile water was added to the inoculum to achieve the desired macroconidial concentration ($2 \times 10_5$ macroconidia/ml) (Reid et al. 2002; Kuhnem et al. 2015; Anderson et al. 2016; Chungu et al. 1996).

3.2.3 Field experiments

Field experiments were conducted at the experimental station at the Universidade Federal de Viçosa (20º44'44" S, 42º50'59" W, 661 m above sea level) during two growing seasons consisting of a winter crop in 2017 and a summer crop in 2018. In each growing season, two field trials were sown (60 cm between rows) on different dates three weeks apart and with different commercial maize hybrids. During the 2017 growing season, SupremoViptera® (Syngenta) and RB9004 PRO2® (KWS sementes) hybrids were sown between April and May. The hybrids MG580PW® (Dow AgroSciences) and BM820® (Sementes Biomatrix) were planted between October and early-November during the 2018 growing season. Two trials were planted side-by-side, one for the GER assay and the other for the GSR assay. Plants were fertilized following chemical soil analyses, and sprinkler-irrigated as needed.

The experiment was laid out in a randomized complete block design with four replications. Each block was consisted of three rows. The isolates were randomly assigned to the plot (experimental units consisted of a 1-m-long row). The central three plants in

each plot were inoculated. All plots were bordered by non-inoculated rows of the same maize hybrid.

3.2.4 Inoculation procedures

GER trials. Plants were inoculated by injecting 2 ml of each spore suspension into the silk channel of the primary ear of each plant four days after silk emergence by using a syringe with a 5 cm long and 3 mm diameter obtuse needle (Reid et al. 2002) (Fig. 3.1A). Ears mock-inoculated with sterile distilled water served as negative controls. The plots were irrigated for two consecutive days after inoculation. When plants reached the R5-R6 stage (dent to physiological maturity), ears were hand-picked and husked (Fig. 3.1B). GER severity was scored visually as the percent symptomatic area of each ear (Reid and Zhu 2005).

GSR trials. Maize stalks were inoculated four days after silk emergence. One milliliter of each inoculum suspension (2×10^5 macroconidia/ml) was injected at a 45° angle downward into the middle of the first internode above the uppermost aerial root of each plant by using a syringe with an obtuse needle (Reid and Zhu 2005) (Fig. 3.1C). The obtuse needle was 5 cm long and 3 mm diameter with an opening on the underside near the tip (Reid and Zhu 2005). A mock inoculation (sterile distilled water) was included as a negative control. Plants were irrigated for two consecutive days after inoculation. At the R5-R6 stage (dent to physiological maturity), stalks were split longitudinally, and symptomatic internodes

were photographed (Fig. 1D). The percent diseased (discolored) area of the internode (Reid and Zhu 2005) was measured by using Assess software (Lamari 2002).

3.2.5 Meteorological data

Weather variables including precipitation (P, rain and irrigation), temperature (T, maximum and minimum), and relative humidity (RH), were recorded hourly at the experimental site by using an automatic meteorological station (Squitter, Squitter Soluções em Monitoramento Ambiental, São José dos Campos, São Paulo, Brazil). The station was located within the field, and data were collected from sowing to maize harvest. Daily values of the weather variables are presented in Figure S3.6.

3.2.6 Mycotoxin analysis

Mycotoxins were analyzed in maize hybrid BM820® kernels harvested from the experiment conducted in 2018. Harvested ears were dried on a greenhouse bench for five days, shelled, and grains were kept at -20 °C until analysis. Mycotoxin was determined by bulking grains of all individual ears inoculated with the same isolate. A 10-g sub-sample of each pooled replicate was ground by using a coffee grinder and then homogenized. The amount of DON and its acetylated forms (15ADON and 3ADON), NIV, and ZON were quantified using a gas chromatography–mass spectrometry method as described previously (Fuentes et al. 2005; Mirocha et al. 1998).

3.2.7 Data analysis

The effects of the main factors (species and host of origin), and the interaction between them, were evaluated in a multilevel nested mixed model framework. Maize hybrids, isolates and replicates were treated as random effects. GSR and GER severity data (on a percentage scale) were log-transformed (logSEV = log[SEV + 1]) to stabilize variances. The mixed model analysis was performed using the lmer function of package 'lmer4' (Bates et al. 2015) of R (R Core Team 2019). The effects were tested at a 5% significance level. The 'emmeans' package (Lenth 2019) was used to estimate the least square means and respective confidence intervals. The function 'cld' from R package 'multcomp' (Hothorn et al. 2008) was used for comparing the treatment means at 5% significance.

3.3 Results

3.3.1 Gibberella ear rot

All isolates were pathogenic to maize ears, but there was a large degree of intraspecies variation in symptom severity, and ranges overlapped between the two species (Fig. 3.2). Mean severity differed between the species, but was dependent on the host of origin, as suggested by the significant interaction term (P = 0.034). The severity of disease was similar for isolates from wheat and from maize for *F.graminearum* (P = 0.079) and for *F. meridionale* (P = 0.25). However, when comparing isolates obtained only from maize, the severity was twice as high in ears inoculated with *F. meridionale* versus *F. graminearum* (P = 0.002). On average, the GER severity on ears inoculated with *F. graminearum* and *F. meridionale* from maize was 7.2% (± 5.3 standard error [SE]) and 13.9% (± 9.4 SE),

respectively (Fig. 3.4A). In contrast, there was no difference in severity between the two species when isolates were obtained only from wheat ($P = 0.464$). In this case, the average GER severity was 10.6% (± 7.3 SE) and 11.8% (± 8.1 SE) for *F. graminearum* and *F. meridionale*, respectively (Fig. 3.4A).

3.3.2 Gibberella stalk rot

Similar to GER, all isolates were pathogenic to the stalks. Mean GSR severity varied among the isolates ($P < 0.001$), ranging from trace levels to 81.2% (median = 18.5%) (Fig. 3.3). The interaction tested in the mixed model (species *vs* host) did not affect the GSR severity ($P = 0.168$). There was no significant difference between isolates from maize versus wheat ($P = 0.659$). However, there was a significant effect of the species, regardless of the hybrid or the host from which the isolates had been obtained ($P = 0.021$). On average, *F. graminearum* was slightly more aggressive on maize stalks (18.4% ± 7.1 SE) than *F. meridionale* (16.1% ± 6.2 SE) (Fig. 3.4B). There was no significant correlation between stalk and ear disease severity ($\rho = -0.10$; $P = 0.218$).

3.3.3 Mycotoxin analysis

Not all isolates produced trichothecenes at detectable levels in the composite samples of kernels from all replicates. A larger proportion of *F. meridionale* isolates (17/24) produced detectable trichothecenes compared to *F. graminearum* (7/16). ZON was not detected in kernels from any of the ears inoculated with *F. meridionale*. This mycotoxin was found

only in samples of kernels inoculated with three *F. graminearum* strains from maize, and three from wheat (Fig. 3.5).

NIV was detected only in kernels from ears inoculated with *F. meridionale*, while DON/15ADON were only detected in ears inoculated with *F. graminearum*. One sample of kernels from ears inoculated with a *F. graminearum* strain from wheat had levels of 3ADON that were equivalent to 15ADON. This strain produced the highest amount of DON overall (DON = 52.15 μg/g) and produced ZON (Fig. 3.5). None of the other *F. graminearum* strains produced detectable 3ADON. On average, *F. graminearum* strains from wheat produced approximately twice as much DON (13.36 μg/g) as those from maize (6.45 μg/g). In kernels from ears inoculated with *F. meridionale*, NIV levels ranged from 0.25 to 7.00 μg/g across the strains. Isolates from maize produced slightly more NIV (1.80 μg/g) than isolates from wheat (1.35 μg/g).

There was a significant correlation between GER severity and DON production by *F. graminearum* isolates (ρ = 0.81; P = 0.027). For *F. meridionale* isolates, a significant positive correlation was also observed between GER severity and NIV production (ρ = 0.79; $P < 0.01$) (Fig. 3.5).

3.4 Discussion

The presence of multiple *Fusarium* spp. in association with maize ear or stalk symptoms is well-known, and the diversity of species varies across regions (Kelly et al. 2017; Logrieco et al. 2002; Oldenburg et al. 2017; Picot et al. 2012). In the past, variation in species composition in different regions was most often attributed to prevailing weather

conditions. Changes or variability in climatic patterns (Vaughan et al. 2016) or introduction of highly aggressive species (Ward et al. 2008; Valverde-Bogantes et al., 2019) have also been implicated as important drivers of rapid changes in relative dominance of species among pathogenic populations. The importance of the host on FGSC composition has emerged more recently from work done to identify FGSC in larger collections of strains from multiple hosts in the same region (Del Ponte et al. 2015; Gomes et al. 2015; Kuhnem et al. 2016). Survey and experimental data (controlled environment and field studies) from Brazil have shown that *F. meridionale* is dominant as a maize pathogen, while *F. graminearum* is dominant as a wheat pathogen in regions where these species and crops co-occur. Results of the study reported in this third chapter of my dissertation support the hypothesis that increased aggressiveness of *F. meridionale* to maize ears plays a role in host dominance, given that *F. meridionale* strains caused twice as much GER, on average, as *F. graminearum.* Closer scrutiny of the data revealed that this difference occurred only when those strains originated from maize, and not when they came from wheat. This result was consistent with my data in Chapter 1, in which a more limited sample of *F. meridionale* strains only from maize were also twice as aggressive as the *F. graminearum* strains that also came from maize. This host effect could be due to differential selection, either selection for more aggressive *F. meridionale* strains by maize, or of more aggressive *F. graminearum* strains by wheat, or a combination of the two.

The NIV mycotoxin was recently reported for the first time in Brazil and was more frequently found than DON in surveys of commercial maize grain (Oliveira et al. 2017). Alarmingly, NIV is about 10 times more toxic to animals than DON (Desjardins 2008). The results in this chapter confirm the results from Chapter 2, demonstrating that *F.*

meridionale isolates causing GER produce NIV in maize ears. My results support the need to expand surveys of NIV in Brazil, and further revise the legislation which currently considers only DON and ZON (ANVISA 2011). *Fusarium graminearum*-inoculated ears contained DON and no NIV, but levels of DON were seven times higher than NIV levels. In this chapter, evaluation of more isolates, and inoculating separately instead of pooling them, showed that both NIV and DON were positively correlated with GER severity. There was also a relationship between mycotoxin production and host of origin, with isolates of *F. graminearum* from wheat producing more DON than those from maize, and isolates of *F. meridionale* from maize producing more NIV than the strains from wheat. This implies that there is selection for increased mycotoxin levels by these hosts and suggests that NIV plays a more important role in colonization of maize versus wheat, while higher levels of DON may be more important for wheat colonization. A role for DON in colonization of maize is known (Bai et al. 2002; Desjardins et al. 1996; Harris et al. 1999; Maier et al. 2006). However, the role of NIV mycotoxin in pathogenesis on maize is less understood and needs to be further explored. Not all of the strains I tested produced detectable levels of mycotoxins in the composite sample of kernels from field inoculations. Low levels of mycotoxin were likely undetected in those cases due to technical limitations of the sampling and detection methods. The ability of the genotyping to predict the chemotype was confirmed in my study (Desjardins 2008; Ward et al. 2008). Thus, DON and 15ADON were produced by *F. graminearum* isolates previously identified as the 15ADON genotype, while *F. meridionale* strains previously identified as the NIV genotype produced only NIV mycotoxins.

Previous surveys in southern Brazil showed that *F. meridionale* was much more abundant among isolates from perithecia formed on corn stubble in wheat fields, as well as from maize plants with stalk rot symptoms (Kuhnem et al. 2016). Its relatively high frequency on maize tissues in comparison with *F. graminearum* suggested a higher ability to colonize maize stalks, but this was not confirmed by my data when stalks were inoculated with either species under controlled conditions. Intriguingly, it was found that *F. graminearum* was actually more aggressive on stalks than *F. meridionale*, although the difference was very small. In a previous study, differences in aggressiveness were not found in a sample of four *F. meridionale* and two *F. graminearum* strains inoculated on stalks of seedlings (25-day-old plants) in the greenhouse (Kuhnem Júnior et al. 2013). The difference in my field study may be related to the influence of environmental factors or host age, both of which could increase susceptibility to *F. graminearum* relative to *F. meridionale*. There may be other reasons for the dominance of *F. meridionale* on maize stalks in the field, including inoculum availability and differential responses to environmental factors during perithecial formation or overwintering survival. In the first chapter I showed that *F. meridionale* was more competitive in maize ears during mixed infections, and so another explanation for dominance of *F. meridionale* perithecia on maize stalks could be that it outcompetes *F. graminearum* during stalk colonization when both species are present.

Resistance to GSR and GER in maize is complex, and is influenced by genetic background as well as environmental factors (Mesterházy et al. 2012; Yang et al. 2010). Previous work has suggested that GSR has a direct relationship with GER, and that breeding for resistance should take both diseases into account (Mesterházy et al. 2012).

Thus, GSR was shown to enhance development and drying of the ear by interrupting the water supply, and this in turn reduced levels of ear rot by more than half (Mesterházy et al. 2012). My study found no correlation in the degree of aggressiveness of individual isolates to ears versus stalks. A previous study also found no correlation between GER and seedling blight (Kuhnem et al. 2015). It would be instructive to further explore pathogenicity by these two species by using a larger set of maize cultivars, and co-inoculation with both diseases at once to study potential interactions.

3.5 Figures

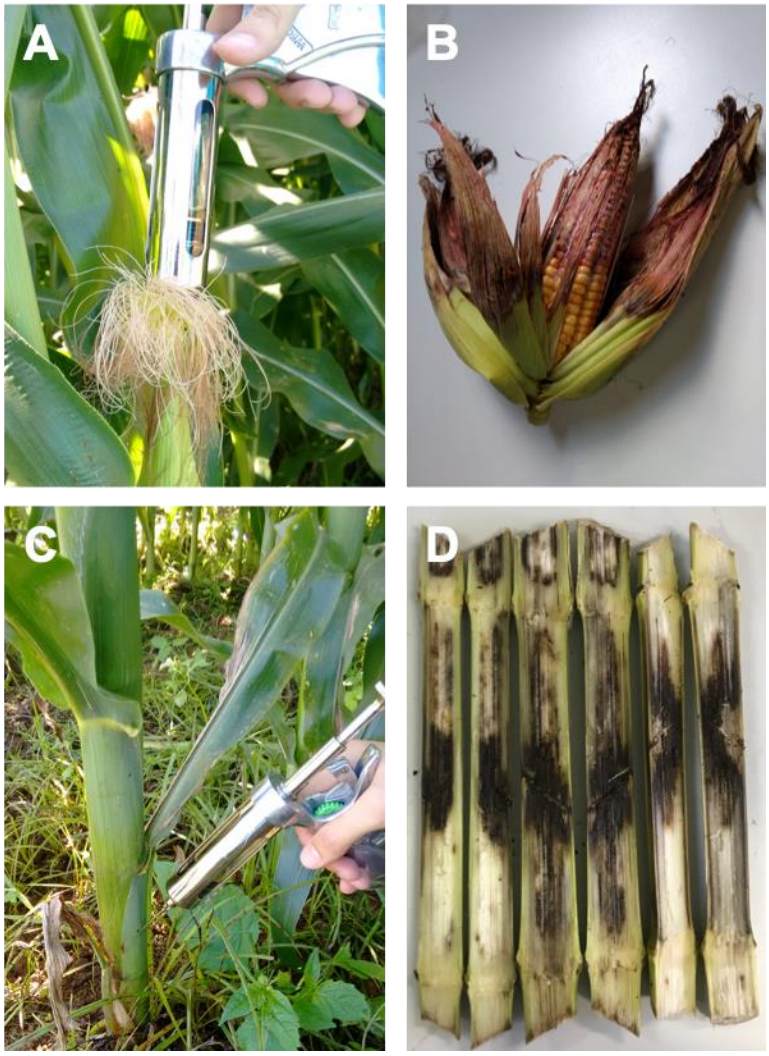

Figure 3.1. Procedure used to inoculate maize ears (A) and stalks (C) with *Fusarium graminearum* and *F. meridionale* isolates, and symptoms of Gibberella ear (B) and stalk (D) rot at dent to physiological maturity stage.

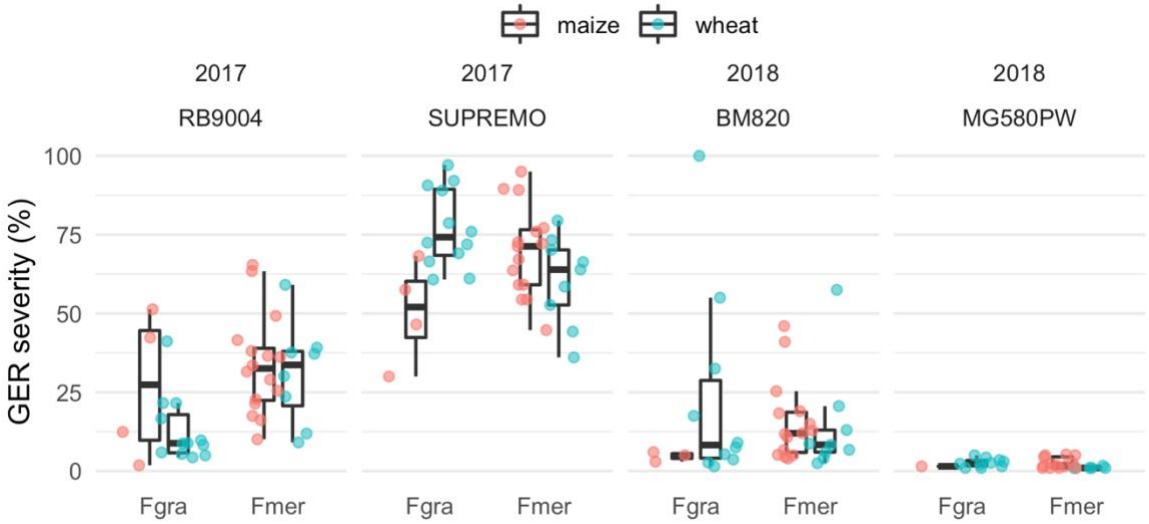

Figure 3.2. Severity of Gibberella ear rot on four different commercial maize hybrids (Supremo Viptera®, RB9004 PRO2®, MG580PW®, BM820®) inoculated with 16 isolates of *F. graminearum* (Fgra) or 24 isolates of *F. meridionale* (Fmer). Data points for the isolates obtained from infected maize kernels are shown as red dots, and from symptomatic wheat heads as blue dots. The first two maize hybrids were planted during the 2017 growing season, and the last two were cultivated during the 2018 growing season. The line within the box represents the median, whereas the top and bottom lines of the boxes represent the 75th and 25th percentiles of the data, respectively. The vertical bars extending beyond the boxes represent 10th and 90th percentiles, and the dots represent the mean GER severity for each isolate across all inoculated ears.

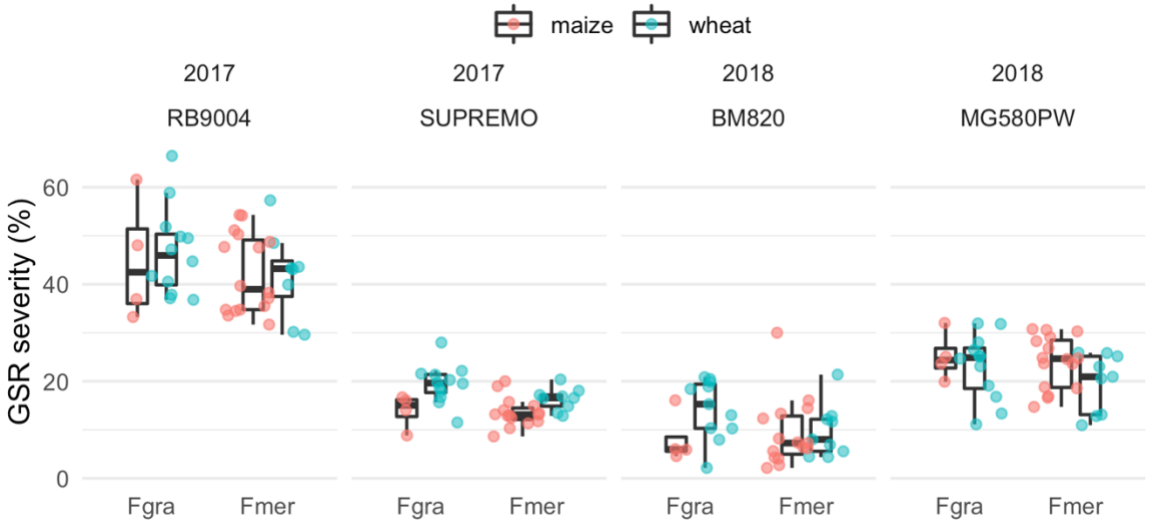

Figure 3.3. Severity of Gibberella stalk rot on four different commercial maize hybrids (Supremo Viptera®, RB9004 PRO2®, MG580PW®, BM820®) inoculated with 16 isolates of *F. graminearum* (Fgra) or 24 isolates of *F. meridionale* (Fmer). Data points for the isolates obtained from infected maize kernels are shown as red dots, and from symptomatic wheat heads as blue dots. The first two maize hybrids were planted during the 2017 growing season and the last two were cultivated during the 2018 growing season. The line within the box represents the median, whereas the top and bottom lines of the boxes represent the 75th and 25th percentiles of the data, respectively. The vertical bars extending beyond the boxes represent 10th and 90th percentiles, and the dots represent the mean GSR severity for each isolate across all inoculated stalks.

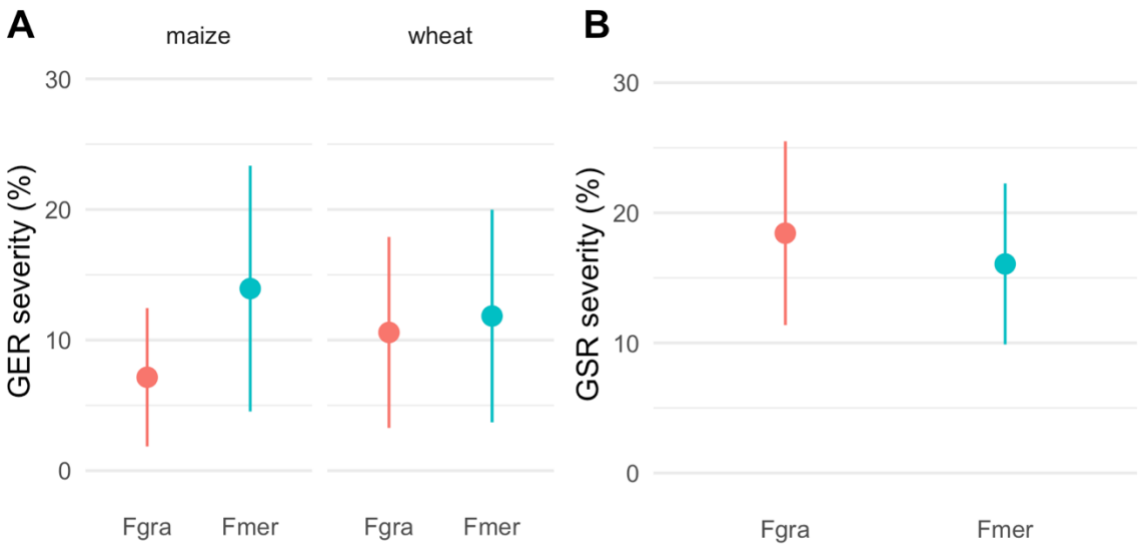

Figure 3.4. Least square means and standard error from linear mixed analyses of the effect of 16 isolates of *F. graminearum* (Fgra) or 24 isolates of *F. meridionale* (Fmer) obtained from either wheat or maize on (**A**) Gibberella ear rot (GER) and (**B**) Gibberella stalk rot (GSR) severity of inoculated maize ears and stalks across four different commercial maize hybrids (RB9004 PRO2®, SUPREMO®, BM820® and MG580PW®) in two growing seasons in Brazil. The first two maize hybrids were planted during the 2017 growing season and the last two were cultivated during the 2018 growing season.

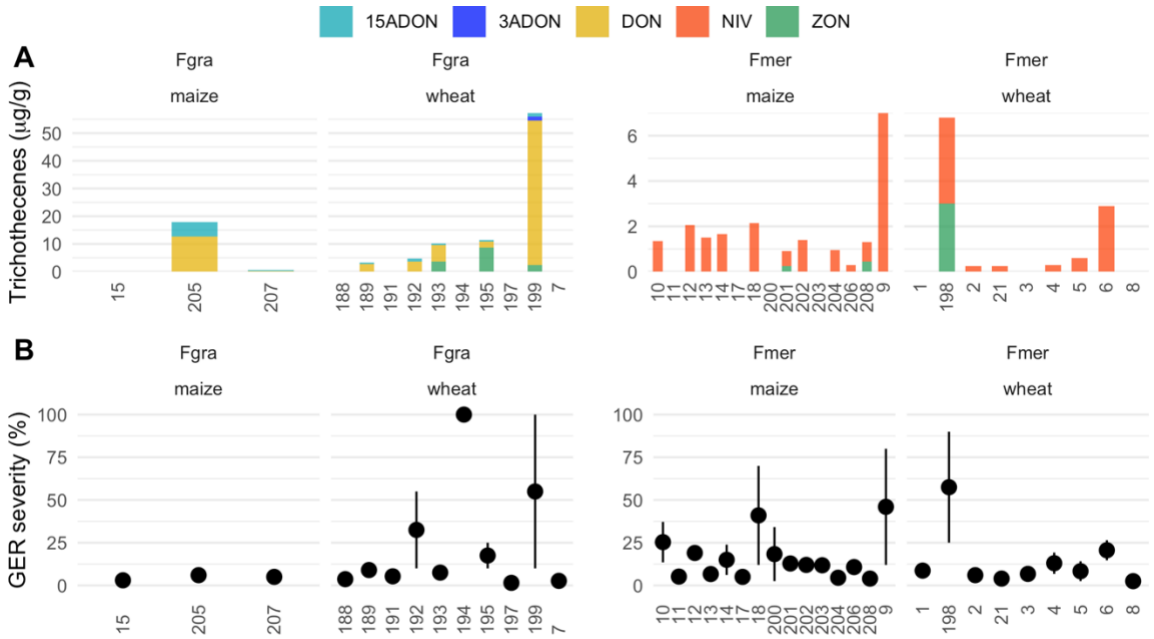

Figure 3.5. (A) Mean production of deoxynivalenol (DON), nivalenol (NIV), and zearalenone (ZON) and **(B)** mean Gibberella ear rot (GER) severity (%) and standard error of *Fusarium graminearum* and *F. meridionale* isolates inoculated onto maize ears of hybrid BM820® in a field trial conducted in Viçosa during the 2018 summer season. Values represent a bulked sample of kernels from 5-6 inoculated ears. Limit of detection (< 0.25 µg/g).

3.6 SUPPLEMENTARY MATERIAL

Table S3.1. Summary information for 41 arbitrarily selected isolates representing *Fusarium graminearum* (*n* = 16) and *F. meridionale* (*n* = 25) obtained from naturally infected maize kernels and symptomatic wheat heads in surveys of commercial fields in southern Brazil from 2009 to 2011.

Species[a]	Id.[b]	Host	Year	Municipality	State[c]
Fgra	188	wheat	2009	Panambi	RS
Fgra	189	wheat	2009	Ijuí	RS
Fgra	190	wheat	2010	Coxilha	RS
Fgra	191	wheat	2007	Cruz Alta	RS
Fgra	192	wheat	2007	Ernestina	RS
Fgra	193	wheat	2010	Tapejara	RS
Fgra	194	wheat	2011	Não-me-Toque	RS
Fgra	195	wheat	2011	Palmeira das Missões	RS
Fgra	196	wheat	2010	Caseiros	RS
Fgra	197	wheat	2010	Coxilha	RS
Fgra	07	wheat	2010	Estação	RS
Fgra	199	wheat	2011	Ijuí	RS
Fgra	15	maize	2011	Bom Jesus	RS
Fgra	205	maize	2011	Vacaria	RS
Fgra	207	maize	2011	Bom Jesus	RS
Fgra	209	maize	2011	Bom Jesus	RS
Fmer	01	wheat	2009	Ernestina	RS
Fmer	02	wheat	2009	Coxilha	RS
Fmer	03	wheat	2007	Nonoai	RS
Fmer	04	wheat	2011	Marau	RS
Fmer	05	wheat	2011	Sertão	RS
Fmer	198	wheat	2010	Água Santa	RS

Table S3.1. (continued)

Species[a]	Id.[b]	Host	Year	Municipality	State[c]
Fmer	06	wheat	2010	Tapejara	RS
Fmer	08	wheat	2009	Santa Bárbara do Sul	RS
Fmer	21	wheat	2007	Nonoai	RS
Fmer	09	maize	2011	Marechal Cândido Rondon	PR
Fmer	10	maize	2011	Ponta Grossa	PR
Fmer	11	maize	2011	Irati	PR
Fmer	12	maize	2011	Casca	RS
Fmer	13	maize	2011	Eldorado do Sul	RS
Fmer	14	maize	2011	Bom Jesus	RS
Fmer	16	maize	2011	Taguaí	SP
Fmer	17	maize	2011	Paranapanema	SP
Fmer	18	maize	2011	Alambari	SP
Fmer	200	maize	2011	Ponta Grossa	PR
Fmer	201	maize	2011	Ponta Grossa	PR
Fmer	202	maize	2011	Ponta Grossa	PR
Fmer	203	maize	2011	Palmeira	PR
Fmer	204	maize	2011	Palmeira	PR
Fmer	206	maize	2011	Vacaria	RS
Fmer	208	maize	2011	Bom Jesus	RS

[a] Species and trichothecene genotype identified using the multilocus genotype method (Ward et al. 2008). Fgra = *F. graminearum* with 15-acetyl-deoxynivalenol genotype, Fmer = *F. meridionale* with nivalenol genotype.

[b] Isolates were obtained either from maize kernels (Kuhnem et al. 2016) or symptomatic wheat heads (Del Ponte et al. 2015) in previous surveys.

[c] Brazilian states: RS = Rio Grande do Sul, PR = Paraná, SP = São Paulo.

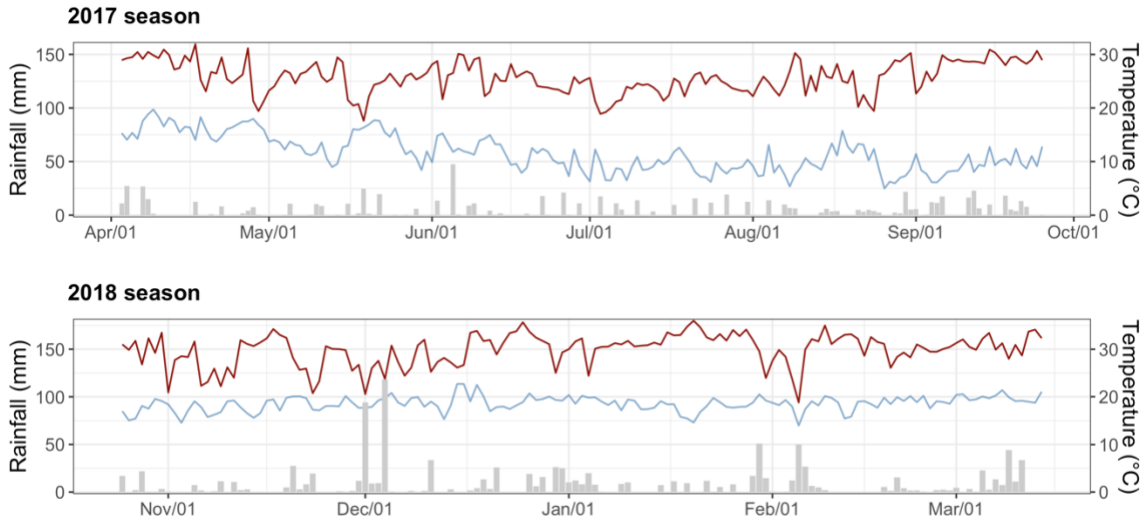

Figure S3.6. Daily values of meteorological data recorded by an automatic meteorological station located within the field trial. Minimum and maximum air temperature (°C) are represented in red and blue solid lines, respectively. Precipitation (rain plus irrigation) is represented by vertical bars. During the 2017 growing season, SupremoViptera® (Syngenta) and RB9004 PRO2® (KWS sementes) maize hybrids were planted on 4th and 25th of April, inoculated on 15th of June and 7th of August and harvested on 18th of August and 25th of September, respectively. The maize hybrids MG580PW® (Dow AgroSciences) and BM820® (Sementes Biomatrix) were planted planted on 25th of October and 20th of November 2017, inoculated on 5th of January and 9th of February and harvested on 11th of February and 15th of March during the 2018 growing season, respectively.

CHAPTER 4. UNRAVELLING SHIFTS IN DOMINANCE OF *FUSARIUM MERIDIONALE* AND *F. GRAMINEARUM* ON MAIZE VERSUS WHEAT IN BRAZIL: A MULTIVARIATE PHENOTYPIC ANALYSIS

Abstract

Fusarium head blight (FHB) and Gibberella ear and stalk rot (GER and GSR) are diseases of worldwide importance affecting wheat and maize, respectively. In Brazil, a handful of *Fusarium graminearum* species complex (FGSC) members cause these diseases, but the dominant species varies according to the host. A comparison of various pathogenic and fitness-related traits was undertaken for a large number of strains representative of the two species and hosts of origin in order to enhance understanding of host association. A collection of 45 strains, including 18 *F. graminearum* (12 from wheat and six from maize) and 27 *F. meridionale* (nine from wheat and 18 from maize), was compared for 17 phenotypic traits. Although there was significant intraspecies variation for most traits, strains were strongly structured by species regardless of the host of origin, based on a multivariate analysis. *Fusarium graminearum* was a more aggressive pathogen of wheat, and produced more abundant macroconidia, perithecia, and ascospores in culture. All *F. graminearum* strains produced primarily deoxynivalenol (DON), and more of its acetylated form 15ADON versus 3ADON, in rice cultures or on wheat heads. In contrast, *F. meridionale* grew faster in culture, and all *F. meridionale* strains produced mainly nivalenol (NIV) both *in vitro* and in wheat heads, with the exception of two strains from maize that produced more DON than NIV *in planta*. Overall, traits related to increased sexual or asexual reproduction and aggressiveness to wheat heads contributed the most to distinguish isolates of *F. graminearum*. On the other hand, faster mycelial growth in culture and reduced colonization of maize silks was highly associated with *F. meridionale* strains. This study provides a baseline for improving our knowledge of the biology and ecology of FGSC species infecting wheat and maize.

KEYWORDS: Fusarium head blight, Gibberella ear rot, Gibberella stalk rot.

4.1 Introduction

A handful of species of the *Fusarium graminearum* species complex (FGSC) are pathogens of major cereal crops. In wheat and maize, they cause the flowering diseases known as Fusarium head blight (FHB) (a.k.a. wheat scab) and Gibberella ear rot (GER), respectively (Goswami and Kistler 2004; Kuhnem et al. 2015; Munkvold 2003a). These diseases, together with Gibberella stalk rot (GSR) of maize also caused by the same pathogens, cause significant economic losses due to grain yield reduction. In addition, the flowering diseases degrade crop value via contamination of grain with mycotoxins, including deoxynivalenol (DON) which is regulated for maximum tolerance limits (McMullen et al. 2012; Sutton 1982). *Fusarium graminearum* is the most common species causing FHB in wheat and barley worldwide. This species produces mainly DON, although there are some geographically-defined subpopulations that produce more nivalenol (NIV) (Del Ponte et al. 2015; Kelly and Ward 2018). *Fusarium meridionale* strains are typically NIV-producers, and are regionally important as causal agents of GER and GSR in South America (Kuhnem et al. 2016; Sampietro et al. 2011) and Nepal (Desjardins and Proctor 2011). *Fusarium meridionale* is also the second most common species causing FHB in wheat in Brazil, after *F. graminearum* (Del Ponte et al. 2015). Several other *Fusarium* species, both NIV-producing (*F. graminearum*, *F. asiaticum*, *F. cortaderiae*, and *F. austroamericanum*) and non-producing (*F. culmorum*, *F. cerealis* and *F. poae*), also cause GER and GSR in maize in other regions of the world (Basler 2016; Kuhnem et al. 2016; Lee et al. 2012; Ndoye et al. 2012). FGSC members produce zearalenone (ZON) in addition to the type-B trichothecenes, and all these compounds are extremely harmful for human and animal health (Chen et al. 2019). Maximum limits of DON on grains, food and feed are well established for several countries (van Egmond et al. 2007). Although NIV-

producing species are also found associated with maize and wheat, NIV has not been regulated by any country so far (Ferrigo et al. 2016; Park et al. 2018).

In the first chapter of this dissertation, I demonstrated that a mixture of two *F. meridionale* isolates was twice as aggressive (resulted in higher disease severity) in maize ears compared with a mixture of two *F. graminearum* strains. In the second chapter, the sample size was increased ($n \geq 18$ strains) for both species, and included isolates from both crops. The isolates were also evaluated separately rather than in mixtures. The same pattern was confirmed, showing that *F. meridionale* was, on average, twice as aggressive on ears compared with *F. graminearum*. This could explain the prevalence of this species among strains recovered from maize kernels (67%), compared with *F. graminearum* isolates (18%) (Kuhnem et al. 2016). However, *F. graminearum* was 14% more aggressive to maize stalks than *F. meridionale*, so aggressiveness alone cannot explain the relative over-representation of *F. meridionale* (53%) among strains recovered from corn stubble (Kuhnem et al. 2016).

There was substantial intraspecies variation in aggressiveness to maize ears and stalks, and the ranges overlapped between the two species. It is important to consider that aggressiveness-related traits alone may be insufficient to explain the dominance of one species over the other in the field, and that other stages of the disease cycle (e.g. sexual reproduction, dispersal, etc.) may also play important roles in shaping species composition. Pathogen aggressiveness is a complex trait, not only because of its quantitative inheritance, governed by multiple genes with additive effects, but also because it is greatly influenced by interaction with environmental factors (Cumagun and Miedaner 2004). The visual severity of disease on ears is a consequence of the ability of the pathogen to colonize the

ears after inoculation, which can be significantly influenced by environmental conditions. For example, previous studies have suggested higher sensitivity to temperature of *F. meridionale* compared with *F. graminearum* (Kuhnem et al. 2016). This may provide some advantage to the latter during mixed infections in warmer environments (Kuhnem et al. 2016). *In vitro* studies showed that *F. graminearum* was able to produce perithecia and ascospores faster than other FGSC species *in vitro*, under optimum and constant temperatures (Liu et al. 2017; Nicolli et al. 2018). This may lead to differences in timing of inoculum production, which may be important given that these pathogens infect specifically during flowering. It is possible that the amount of *F. meridionale* airborne inoculum is higher than *F. graminearum* during maize flowering. This could be tested by collecting airborne spores over the maize canopy. A similar experiment done with wheat demonstrated that spores of both species could be collected in the air above the canopy during wheat flowering, and that about two-thirds of the airborne inoculum was *F. graminearum* (Del Ponte et al. 2015).

My goal in this chapter of my dissertation was to more fully characterize the two species, and to better understand the biological significance of the phylogenetic species designations by conducting a multi-phenotype study of a representative sample of strains from both hosts. These comparisons included inoculations of both hosts with the same strains, together with a more complete evaluation of other traits that have been related to fitness and to the saprophytic stage of the disease cycle (Del Ponte et al. 2015; Gomes et al. 2015; Kuhnem et al. 2016; Yang et al. 2018; Zhang et al. 2016). In this chapter, I used the same isolates from previous chapters, plus a few additional representatives, to inoculate wheat plants. I measured aggressiveness and toxin production in wheat heads, and I also

compared *in vitro* growth, fungicide sensitivity, toxin production, and the production of sexual and asexual spores.

4.2 Materials and Methods

Forty-five isolates were selected from a larger collection obtained from maize kernels (Kuhnem et al. 2016) and symptomatic wheat heads (Del Ponte et al. 2015) during surveys of commercial fields in southern Brazil from 2009 to 2011 (Fig. S4.3). Eighteen isolates of *F. graminearum* (12 from wheat and six from maize) and 27 isolates of *F. meridionale* (nine from wheat and 18 from maize) were selected to represent the geographic and temporal diversity of the collection (Table S4.31). The isolates were previously assigned to species and trichothecene chemotype by a MLGT assay using a Luminex flux cytometer (Ward et al. 2008) and had been preserved as a permanent collection for future studies. After recovery from storage, the identities of the isolates were confirmed using the Fg16F/R primer set (Nicholson et al. 1998). This primer set generates different fragment sizes that identify isolates as either *F. meridionale* (~500 bp) or *F. graminearum* (~450 bp) (Astolfi et al. 2012; Castañares et al. 2016; Del Ponte et al. 2015; Nicholson et al. 1998).

4.2.1 Saprophytic and fitness traits

Mycelial growth. Three equidistantly positioned colonies were produced by placing 5-µl drops of a spore suspension (10,000 macroconidia/ml) onto a 9-cm PDA plate as described previously (Zhan and McDonald 2011) with some modifications (Spolti et al. 2014a). The media were prepared as a single batch and poured into 9-cm plastic Petri dishes (15 ml). Inoculated plates were allowed to dry before being randomly placed in the growth chamber.

The cultures were incubated at 23 °C in darkness for 5 days. Radial mycelial growth was obtained by averaging two perpendicular measurements. The entire assay was repeated three times.

Asexual spore production. Mycelial plugs from a 5-day-old PDA plate were used to inoculate Mung Bean Agar plates (MBA: 40 g of mung bean/liter, placed in boiling distilled water for 23 min [~50% of seed pericarps split while cooking] filtered through two layers of cheesecloth, adjusted to 1 liter, 15 g of agar) (Evans et al. 2000). Each isolate was cultured for seven days at 23 °C under constant lights. Three agar discs (6-mm in diameter) were taken from the edges of each colony and added to a microcentrifuge tube containing 1 ml of sterile water. Spores were harvested by vortexing each tube for 20 s (Nicolli et al. 2018). The macroconidial concentration was quantified by using a hemocytometer and expressed as the number of macroconidia per ml. Each plate was considered as one replicate (three plates per isolate) and the entire assay was repeated once.

Sexual fertility. The sexual fertility was assessed based on the ability of each isolate to produce perithecia and ascospores on carrot agar following a standard protocol with some adaptations (Cavinder et al. 2012). Carrot agar plates (6.0-cm-diameter) were inoculated by placing a 5-µl drop of a spore suspension (1×10^4 macroconidia/ml) of each isolate in the center. Plates were incubated in a growth room at 23°C under constant luminosity until the mycelium reached the edge of the plate (~ 4 days). Aerial mycelia were gently removed using a toothpick and then 1 ml of 2.5% Tween 80 in water was distributed with a sterile plastic micro-pestle. Plates were returned to the light. At 21 days after induction, perithecia were quantified under a stereomicroscope within a 1-cm^2 area at two different positions on the plate and expressed as mean perithecia per cm^2. Ascospore production was measured

21 days after induction by applying 5 ml of sterile deionized water and gently rubbing the plate surface with a sterile plastic micro-pestle. Ascospore suspensions were filtered through two layers of cheesecloth. Ascospore concentration was estimated by using a hemocytometer, and expressed as the number of ascospores per ml. Each plate was considered as one replicate, and placed on different benches in the growth room (blocks, three plates per isolate). The entire assay was performed once.

4.2.2 Pathogenic and toxigenic traits

Maize silk infection assay. Maize silk infection was assessed by using a standard protocol with some modifications (Seong et al. 2005). Susceptible sweet corn hybrid Golden Jubilee was sown in the greenhouse to produce fresh ears every week. Briefly, three seeds were planted per 25-cm pot filled with a mixture of three parts ProMix BX (Premiere Horticulture Ltd., Riviere du Loup, PQ, Canada) and one-part topsoil. Maize plants were grown in a greenhouse with a 14 h photoperiod and temperatures ranging from 25 to 28 °C. Seedlings were thinned to two plants per pot and fertilized weekly with a solution of Peters 20-20-20 fertilizer (Scotts-Sierra Horticultural Product Co. Marysville, OH). At the silking stage, primary maize ears were harvested, and the exposed portions of silks were trimmed off. In the laboratory, 4-5 maize silks were sliced into 5-cm sections and the sections were bundled and aligned on a water-soaked Whatman No 1 filter paper. Three silk bundles were positioned alongside each other inside a 90-mm petri dish. A mycelial agar plug taken from the edge of a 7-day-old culture of each isolate growing on MBA (6-mm in diameter) was placed upside down covering the lower ends of the silks.

Uninoculated MBA plugs were used as negative controls. Plates were placed inside a closed plastic box to maintain high humidity. Boxes were incubated in a growth room at 23 °C under constant light for 4 days. Infection was assessed by measuring the extent of tissue discoloration using digital calipers. The entire assay was repeated twice.

Aggressiveness on wheat heads. Seeds of spring wheat variety Wheaton, which is highly susceptible to FHB, were sown in cone containers filled with a mixture of ProMix BX and topsoil (3:1). Wheat plants were grown in a greenhouse with a 14 h photoperiod and temperatures ranging from 25 to 28 °C. Seedlings were fertilized weekly with a solution of Peters 20-20-20 fertilizer (Scotts-Sierra Horticultural Product Co. Marysville, OH) and maintained until flowering. A spore suspension was prepared by growing each isolate on MBA for 7-14 days under constant lights. The macroconidial suspensions were filtered through two layers of cheesecloth. The concentration of each suspension was then quantified by using a hemocytometer and adjusted to 1×10^4 macroconidia/ml. The wheat heads were inoculated with a 10-µl drop of the spore suspension (1×10^4 macroconidia/ml) placed inside each lateral floret of the central spikelet at early- to mid-anthesis. Each head was individually covered with a plastic bag for 24 h and maintained in a containment growth chamber set for 25 °C and a 14 h photoperiod until harvest (plant maturity). FHB severity was assessed at four, seven- and 10-days post-inoculation, and expressed as the number of FHB-symptomatic (bleached) spikelets per inoculated spike. Three replicates (individual heads) per isolate were included. The experiment was repeated three times.

Trichothecene production in planta. Harvested wheat heads from the aggressiveness assay were dried at room temperature and kept in a cold room (4°C) until analysis. Mycotoxin production by each of the 45 isolates was determined by bulking the samples

from each experiment and considered each bulked sample as a replicate. The wheat heads were ground in a coffee grinder to obtain at least a 5-g sample of each replicate. The ground samples were sent to the Virginia Tech Deoxynivalenol (DON) Testing Laboratory, Blacksburg (Virginia) where the amount of DON and its acetylated forms (15ADON and 3ADON), NIV, and ZON were quantified by using a gas chromatography–mass spectrometry method as described previously (Fuentes et al. 2005; Mirocha et al. 1998).

Trichothecene production in rice. Mycotoxin production in rice culture was determined by using a standard protocol with some modifications (Spolti et al. 2014b; Puri and Zhong 2010; Burlakoti et al. 2008; Walker et al. 2001). Rice grains (30 g) were soaked in 13 ml of sterile deionized water overnight (~10 h) in a 250-ml Erlenmeyer flask. All flasks were autoclaved for 30 minutes and inoculated with 500 µL of a spore suspension ($1 \times 10_4$ macroconidia/ml) of each isolate. Three flasks (replicates) were prepared for each isolate, and all isolates were cultured at the same time in the same location. The flasks were shaken manually every two days so that the fungus evenly colonized the substrate. The cultures were incubated for 28 days at 23 °C in the dark. The entire assay was performed once. The colonized rice cultures were transferred into a 50-ml centrifuge tube, frozen overnight at -80 °C, and then lyophilized for 72 h at -40 °C. The rice cultures were ground in a coffee grinder and a 5-g sample was sent to the Virginia Tech Deoxynivalenol (DON) Testing Laboratory, for mycotoxin analysis. The amounts of DON and its acetylated forms (15ADON and 3ADON), NIV, and ZON were quantified by using a gas chromatography–mass spectrometry method as described previously (Fuentes et al. 2005; Mirocha et al. 1998).

4.2.3 Tebuconazole sensitivity

Sensitivity to tebuconazole (Folicur 3.6F; 38.7% active ingredient; Bayer CropScience, Research Triangle Park, NC), was estimated by measuring radial growth of the isolates on PDA adjusted to different fungicide concentrations in three replicates each as described previously (Spolti et al. 2012b; Spolti et al. 2014b; Chen et al. 2007). The tested concentrations were: 0 (non-amended agar - PDA), 0.5, 1.0, 2.0, 4.0 and 8.0 µg a.i./ml. Plates were incubated at 23°C in darkness for 5 days. Colony diameters were measured in perpendicular directions using digital calipers, and the original plug diameter was subtracted. Effective concentration leading to a 50% reduction of mycelial growth (EC_{50}) was calculated based on the linear regression analysis between the relative mycelial growth inhibition (percent) and the log-transformed fungicide concentrations. The entire experiment was repeated twice.

4.2.4 Data analysis

All experiments were conducted as a completely randomized design with three replicates. Data from assays conducted two or three times were combined for analysis. The overall mean and the standard deviation and Cohen's *d* were estimated using the 'effsize' R package (Torchiano 2019). A multivariate analysis of variance (MANOVA) was performed. Mycotoxin data were not included in the multivariate analysis because not all isolates produced the same mycotoxin. Prior to the analysis, all variables were transformed [log(X + 1)] for normality and homoscedasticity. A principal components analysis (PCA) was performed using the mean values of the variables for each isolate, averaged over

replicates and experiments. The contribution of each variable to each principal component was also estimated. A correlogram was made using the overall means for each pair of variables using the package 'corrplot' (Wei and Simko 2017). The packages 'FactoMineR' (Lê et al. 2008) and 'factoextra' (Kassambara and Mundt 2017) were used for the PCA. All analyses were run in R (R Core Team 2019).

4.3 Results

4.3.1 Saprophytic and fitness traits

Fusarium meridionale grew significantly more quickly *in vitro* than *F. graminearum*, but *F. graminearum* produced more macroconidia (Table 4.1). All of the *F. graminearum* strains and all but one of the *F. meridionale* strains, produced perithecia *in vitro*. In both cases, there was substantial intraspecies variation in both perithecial and ascospore production (Fig. 4.3A-B). Two *F. meridionale* isolates produced protoperithecia on carrot agar but did not produce ascospores (Fig. 4.3B). Overall, *F. graminearum* isolates produced 2.5 times more perithecia, and 17.5 times more ascospores than *F. meridionale* isolates (Table 4.1). In general, host origin had no significant effect on fertility of either species, however, perithecia of *F. graminearum* from wheat produced significantly more ascospores than those from maize; $398.61 \pm 378.28 \times 10_4$ ascospores/ml ($n = 36$) versus $91.39 \pm 74.61 \times 10_4$ ascospores/ml ($n = 18$), respectively.

4.3.2 Pathogenic traits

Aggressiveness on maize silks. Mean lesion lengths were 21.97 ± 9.56 mm (*n* = 36) and 26.87 ± 9.72 mm (*n* = 72) for *F. graminearum* isolates from maize and wheat, respectively. Mean lesion lengths for *F. meridionale* isolates from maize and wheat were 18.81 ± 11.38 mm (*n* = 108) and 19.29 ± 11.69 (*n* = 54) respectively (Fig. 4.4B). On average, the effect size when comparing *F. meridionale* to *F. graminearum* was considered medium (Cohen's *d* = 0.58), and the former was 33% less aggressive than the latter (Table 4.2).

Aggressiveness on susceptible wheat heads. All strains were able to produce typical FHB symptoms, but the disease severity at 10 days post inoculation varied greatly among strains (Fig. 4.4A), ranging from 9.16 to 40.99%, and from 5.26 to 10.66% for *F. graminearum* and *F. meridionale*, respectively (Fig. 4.4A). The mean FHB severity for maize isolates of *F. graminearum* was 20.07 ± 18.76% (*n* = 48) and for wheat isolates it was 26.63 ± 21.20% (*n* = 94). This difference was not statistically different. Within *F. meridionale*, the mean severities for maize and wheat strains were also not statistically different, at 7.20 ± 3.17% (*n* = 142) and 6.67% 1.81 (*n* = 70), respectively (Fig. 4.4A). On average, *F. graminearum* isolates were significantly more aggressive on wheat heads than *F. meridionale* isolates, causing at least three times more disease (Table 4.2).

4.3.3 Toxigenic traits

Mycotoxin production in wheat heads. All *F. graminearum* strains produced primarily DON and small amounts of the two acetylated forms (Table 2). DON ranged from 53.64 to 209.68 μg/g (Fig. 4.5). The chemotype confirmed the previous PCR-genotype analysis:

15ADON was 6.5 times higher than 3ADON (Table 4.2). There were seven 15ADON strains from maize that produced trace levels of NIV (0.31 μg/g) (Fig. 4.5). Consistent with the PCR-genotyping, all *F. meridionale* isolates produced NIV at levels 17-fold higher than *F. graminearum* isolates. Four *F. meridionale* strains were also producers of 15ADON and DON (Fig. 4.5). Exceptionally, two *F. meridionale* strains from maize produced even more DON than NIV in wheat heads (Fig. 4.5). Neither *F. graminearum* nor *F. meridionale* isolates produced detectable levels of ZON (Table 4.2).

Mycotoxin production in rice cultures. All *F. graminearum* isolates were able to produce DON and 15ADON. Most (15 out of 18) isolates also produced 3ADON (Fig. 4.6), and 11 produced NIV. Three of this last group of 11 were obtained from maize. All isolates produced ZON in rice cultures (Table 4.2, Fig. 4.6). All *F. meridionale* isolates, regardless of their host of origin, produced NIV (Fig. 4.6). In addition, one *F. meridionale* isolate from wheat produced DON, one from each host produced 15ADON, and seven from maize and five from wheat produced 3ADON (Fig. 4.6). ZON was produced by eight isolates from maize and three from wheat (Table 4.2, Fig. 4.6). One *F. meridionale* isolate from wheat produced all four mycotoxins on rice (strain 198, Fig. 4.6).

4.3.4 Tebuconazole sensitivity

The mean EC_{50} values ± S.D. (standard deviation) for *F. graminearum* isolates from maize and wheat were 0.84 ± 0.55 μg/ml (n = 12) and 0.50 ± 0.36 μg/ml (n = 24) respectively. Only three *F. graminearum* isolates, all from maize, showed an EC_{50} higher than 1.0 μg/ml (strain '211' = 1.17, '15' = 1.19 and '210' = 1.46 μg/ml) (Fig. 4.7). For *F. meridionale*,

mean EC_{50} ratings were 0.21 ± 0.16 μg/ml (n = 36) and 0.20 ± 0.13 μg/ml (n = 18) for maize and wheat isolates, respectively (Fig. 4.7). All *F. meridionale* isolates showed an EC_{50} lower than 0.5 μg/ml, with the exception of two maize strains (strain '20' = 0.51 and '09' = 0.52 μg/ml) (Fig. 4.7). Overall, *F. meridionale* isolates were three times more sensitive to tebuconazole than *F. graminearum* isolates (Table 4.1).

4.3.5 Overall species related fitness

Based on MANOVA results, there was weak evidence against the null hypothesis of interaction between species and host of origin (P = 0.078), contrasting with a strong effect of species (P < 0.001). The correlation analysis showed 75 significant associations (P < 0.05) over each of the 117 pairwise comparisons for all the 17 variables (Fig. S4.9). Overall, FHB severity at 10 dpi was highly associated with DON production in wheat heads (Fig. S4.9). NIV production was negatively associated with DON production, either in wheat heads or in rice cultures (Fig. S4.9). Mycelial growth was negatively associated with all other variables with the exception of NIV production, which was also negatively associated with all the other traits.

The PCA suggested that macroconidial production, aggressiveness to wheat heads at 10 dpi, and ascospore production contributed the most for the PC1: 24%, 23% and 16% respectively of the total, with 49.4% of all variation explained by this first PC (Fig. 4.8). The second PC explained 17.2% of the variation, with aggressiveness on maize silks and mycelial growth contributing 51% and 40%, respectively (Fig. 4.8). The third PC explained 13.6% of the variation among isolates, with a contribution of 44% from the variable

tebuconazole sensitivity (EC_{50}) and 34% from perithecial production. Those last two parameters were also negatively associated with PC2 (ρ = -0.20 and ρ = -0.13, respectively). Interestingly, mycelial growth was the only variable negatively associated with the first PC (ρ = -0.57). Cluster analysis showed that strains were generally structured by species (Fig S2). Exceptions were four isolates, including one *F. graminearum* from wheat, two *F. graminearum* from maize, and one *F. meridionale* from maize, that did not group together with the other strains (Fig. S4.10). Clustering was not related to host (wheat versus maize) or to geographic origin.

4.4 Discussion

The work in this chapter confirms that *F. graminearum* and *F. meridionale* have distinct phenotypes for multiple pathogenicity, fitness, and saprophytic-related traits, thus confirming the biological significance of the phylogenetic species identity. My results also show that the host of origin has an impact on some, but not all, of the traits investigated, suggesting the presence of different selective pressures that are imposed by wheat versus maize on populations of the two species.

My results are consistent with previous studies that have proposed that *F. graminearum* is the most prevalent FHB causal agent in Brazil because it is more aggressive on wheat than *F. meridionale* (Astolfi et al. 2012; Del Ponte et al. 2015; Scoz et al. 2009; Nicolli et al. 2018). *Fusarium graminearum* isolates produced more perithecia, ascospores, and macroconidia in culture than *F. meridionale* isolates, which is also in agreement with previous reports (Bowden and Leslie 1999; Liu et al. 2017; Nicolli et al.

2018). Wheat isolates of *F. graminearum* produced more than three times as many ascospores per perithecium than maize isolates did, suggesting that there is selection for greater ascospore production by the wheat crop for this species. This might relate to the known importance of ascospores in the disease cycle of FHB (Leplat et al. 2013; McMullen et al. 2012; Pereyra et al. 2004). The results also imply that such high numbers of ascospores or conidia are less important for infection of maize ears. Infection of maize may be more efficient, perhaps due to a longer infectious period while the silks are exposed, or to a larger target area for infection. This would be an interesting topic for further study.

My study showed that *F. meridionale*, though it produced fewer sexual and asexual propagules, grew faster on PDA. This faster growth rate could be related to the competitiveness of *F. meridionale* versus *F. graminearum* in maize ears when both species are present. Interestingly, a significant correlation has been previously reported between mycelial growth on PDA and the production of ascospores on corn stalks (Spolti et al. 2014a), and this could be related to the prevalence of this species in maize stubble. Surprisingly, *F. meridionale* isolates were less aggressive on maize silks than isolates of *F. graminearum*, regardless of their host of origin. This suggests that the dominance of *F. meridionale* on ears is not due to relative aggressiveness on silks. However, it would be interesting to do co-inoculations of silks, given that in the first chapter I observed that *F. meridionale* out-competed *F. graminearum* on ears, when both species were present.

The tebuconazole sensitivity for isolates of both species obtained from maize was reported for the first time here. Tebuconazole has long been used to manage FHB in wheat, but its application to the control of maize diseases is more recent. Although no substantial differences were detected among isolates from different hosts, my results showed that *F.*

graminearum isolates overall were less sensitive to tebuconazole than *F. meridionale* isolates, which is in agreement with previous studies of isolates from wheat (Machado et al. *unpublished*, Spolti et al. 2012b; Nicolli et al. 2018; Machado et al. 2017). My findings are also consistent with a previous study done under greenhouse conditions, which demonstrated a higher fungistatic effect of tebuconazole against *F. meridionale* compared to *F. graminearum* when inoculated alone or in co-inoculations in wheat heads (Spolti and Ponte 2013). It has been suggested that the extended use of this fungicide against *F. graminearum* causing FHB in wheat has imposed selection pressure that has resulted in the population becoming less sensitive (Spolti et al. 2012b; Spolti et al. 2014b; Becher et al., 2010). It is possible that the population of *F. meridionale* in maize may also become more tolerant to fungicides, as use of this and other chemicals for management of maize diseases, including GER, becomes more common.

Results of the PCR-based chemotype assay among more than six hundred isolates from Brazil showed that all *F. graminearum* had the 15ADON genotype and all *F. meridionale* had the NIV genotype (Del Ponte et al. 2015; Kuhnem et al. 2016; Scoz et al. 2009; Astolfi et al. 2012). As expected, all *F. graminearum* isolates in my study produced large amounts of DON and more of their respective acetylate trichothecene, 15ADON. However, detectable trace amounts of 3ADON were also produced either in infected wheat heads or in rice cultures. Similarly, *F. meridionale* isolates produced more NIV. Surprisingly, two isolates from maize produced more DON than NIV in wheat heads, although these same isolates produced only NIV in maize ears, as reported in Chapter 3 of this dissertation. Interestingly these isolates, though they produced as much or more DON than some of the *F. graminearum* isolates, did not cause substantial disease in wheat heads.

This indicates that DON production is not sufficient to be an aggressive pathogen of wheat, and that other factors associated with *F. graminearum* are also necessary. It was also very interesting to see that the amount and type of mycotoxin produced by each strain varied depending on the substrate, implying that there is a regulatory aspect of the environment that interacts with the metabolic pathway. NIV strains differ from DON strains in possessing functional Tri13 and Tri7 proteins that hydroxylate and acetylate the C4 position of the trichothecene ring. If these proteins are absent, the pathway is diverted to production of DON (Lee et al., 2002; Kimura et al., 2003). The two *F. meridionale* strains that produced more DON than NIV on wheat heads had NIV genotypes, demonstrating that NIV chemotyping by genotype may not be accurate for all substrates. Other studies have reported a lack of correlation, at times, between mycotoxin genotypes and chemotypes (Mugrabi de Kuppler et al. 2011; Spolti et al. 2014a; Sampietro et al. 2012).

Although there is a large degree of intraspecific variation and overlap between species for individual traits, *F. meridionale* and *F. graminearum* were distinct from one another when all the traits were considered together. Traits related to sexual or asexual fertility and aggressiveness to wheat heads contributed the most to distinguish isolates of *F. graminearum*. In contrast, reduced aggressiveness on maize silks and mycelial growth were highly related to *F. meridionale* strains. Previous studies have failed to demonstrate these distinctions, leaving the biological significance of the phylogenetic species in some doubt (Spolti et al. 2012a; Kuhnem Júnior et al. 2013; Nicolli et al. 2015, 2018). In my study, I included a very large number of strains: strain-specific variation in individual traits can mask overall variation between species. Though not statistically significant, there is

weak evidence supporting a hypothesis of interaction between species and the host of origin in overall fitness, which should be more deeply explored in future studies.

Several traits were significantly correlated with one another. The highest correlation was observed between FHB severity and DON production in wheat heads. This is not unexpected, because DON is well known to be an aggressiveness factor for *F. graminearum* in wheat (Bai et al. 2002; Desjardins et al. 1996; Harris et al. 1999; Maier et al. 2006). DON production in rice culture, on the other hand, did not correlate with DON production *in planta*, suggesting that this trait represents the broader toxigenic potential of each isolate (Goswami and Kistler 2005; Walker et al. 2001). NIV production, either *in planta* or *in vitro*, was negatively associated with all the other traits except for mycelial growth. In earlier chapters of my dissertation, I observed that NIV and DON were both positively correlated with aggressiveness to maize ears, suggesting that toxin production is generally important in pathogenicity to cereals, but that wheat may provide a stronger selection than maize for higher levels of mycotoxins. There appeared to be no clustering of phenotypes related to geographic origin, thus no evidence for locally specialized populations.

Detailed surveys of the mycotoxins found in maize in Brazil are desperately needed. It would also be advisable for breeding programs to use the most aggressive isolates representing the fungal population in the region of interest to evaluate resistance in breeding lines. Increasing surveillance is needed to avoid the introduction of species into other countries, especially in locations where *F. meridionale* is absent, or not found yet, such as the United States and Canada. Likewise, it is critical to adjust the mycotoxin

regulations to include NIV as well as DON levels, considering its importance to public health.

4.5 Tables

Table 4.1. Summary information of saprophytic traits of 45 *Fusarium graminearum* and *F. meridionale* isolates obtained from either maize kernels or symptomatic wheat heads in Southern Brazil from 2009 to 2011.

	Species[a]				
Trait	*n*	F. gra.	*n*	F. mer.	Cohen's *d*[b]
Mycelial growth[c]	108	27.49 ± 2.41	162	30.93 ± 3.10	-1.21
Spore production[d]	108	99.89 ± 49.41	162	15.58 ± 28.32	2.21
Perithecial production[e]	54	305.31 ± 185.72	81	119.61 ± 103.00	1.31
Ascospore production[f]	54	296.20 ± 343.01	81	16.85 ± 27.49	1.28
EC_{50} [g]	36	0.61 ± 0.45	54	0.21 ± 0.15	1.30

[a] Isolates were simultaneously assigned to species and trichothecene genotype using MGLT assay (Ward et al. 2008). In total, 18 *F. graminearum* isolates (*n* = 6 from maize, *n* = 12 from maize) and 27 *F. meridionale* isolates (*n* = 18 from maize, *n* = 9 from maize) isolates were used in this study. Data are shown as means ± standard deviation.

[b] Cohen's *d.* Small (*d* > 0.2), medium (*d* > 0.5), and large (*d* > 0.8) effect sizes (Gent et al. 2018).

[c] Mycelial growth (mm) were determined on potato dextrose agar (PDA) at 23 °C incubated for five days under constant lights.

[d] Macroconidial production (×10^4 macroconidia/ml) on Mung Bean Agar (MBA) at 23 °C after seven days of incubation.

[e] Perithecial production (perithecia/cm^2) on carrot agar (CA) incubated at 23 °C for 21 days under constant lights.

[f] Ascospores (x10^4 ascospores/ml) harvested and counted from CA media at the 21st day.

[g] Effective concentration of tebuconazole (μg/ml) that reduces 50% of the mycelial growth of each of the isolates.

Table 4.2. Summary information of pathogenic and toxigenic traits of 45 *Fusarium graminearum* and *F. meridionale* isolates obtained from either maize kernels or symptomatic wheat heads in Southern Brazil from 2009 to 2011.

	Species[a]				
Trait	*n*	F. gra.	*n*	F. mer.	Cohen's *d*[b]
FHB severity (%)[c]	142	24.51 ± 20.58	212	7.02 ± 2.80	1.32
Silk infection (mm)[d]	108	25.24 ± 9.90	162	18.97 ± 11.45	0.58
Mycotoxins *in planta*[e]					
DON (μg/g)	54	125.51 ± 63.22	81	3.49 ± 6.60	2.34
15ADON (μg/g)	54	9.28 ± 4.28	81	1.34 ± 2.17	1.90
3ADON (μg/g)	54	1.42 ± 0.87	81	-	--
NIV (μg/g)	54	0.31 ± 0.21	81	5.34 ± 3.37	-1.55
ZON (μg/g)	54	-	81	-	-
Mycotoxins *in vitro*[f]					
DON (μg/g)	57	339.17 ± 340.96	78	-	--
15ADON (μg/g)	57	32.27 ± 27.31	78	-	--
3ADON (μg/g)	57	3.68 ± 3.79	78	3.02 ± 3.41	-0.18
NIV (μg/g)	57	7.49 ± 17.49	78	19.72 ± 22.41	-0.57
ZON (μg/g)	57	233.16 ± 527.75	78	26.99 ± 59.49	0.46

[a] Isolates were simultaneously assigned to species and trichothecene genotype using MGLT assay (Ward et al. 2008). In total, 18 *F. graminearum* isolates (n = 6 from maize, n = 12 from wheat) and 27 *F. meridionale* isolates (n = 18 from maize, n = 9 from wheat) isolates were used in this study. Data are shown as means ± standard deviation.

[b] Cohen's *d.* Small (d > 0.2), medium (d > 0.5), and large (d > 0.8) effect sizes (Gent et al. 2018).

[c] FHB severity (%) on 'Wheaton' plants inoculated by the single-floret method. Percentage of diseased spikelets in inoculated spikes at 10 days post inoculation.

[d] Lesion length (mm) on sweet corn hybrid Golden Jubilee silks.

[e] Trichothecene amount in grains from entire wheat heads (cv. Wheaton), inoculated in the greenhouse and averaged over three heads for each independent experiment; ppm = part per million (μg/g of samples). DON = deoxynivalenol; 3ADON = 3-acetyl-DON; 15ADON = 15-acetyl-DON; NIV = nivalenol.

[f] Mycotoxin amount (DON, 15ADON, 3ADON, NIV, and ZON = zearalenone) in rice cultures incubated at 23 °C for 28 days.

4.6 Figures

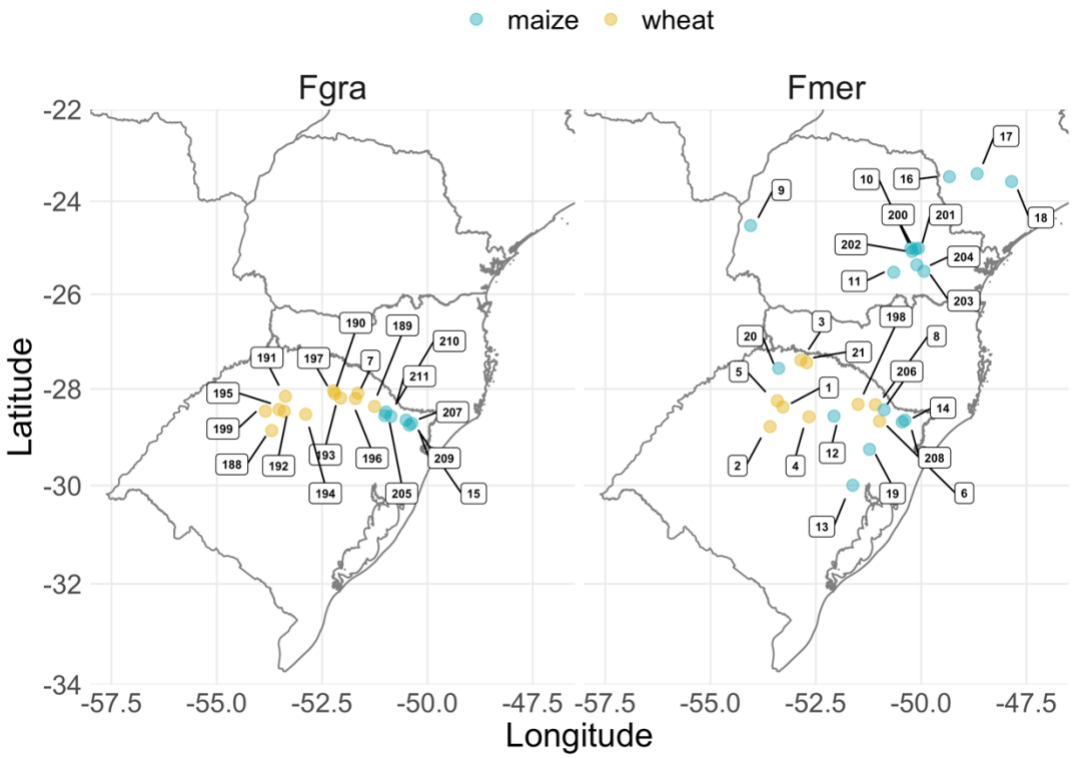

Figure 4.1. Geographic origin of 18 *Fusarium graminearum* isolates (n_{maize} = 6; n_{wheat} = 12) and 27 *F. meridionale* isolates (n_{maize} = 18; n_{wheat} = 9) obtained from symptomatic wheat heads and maize kernels in Southern Brazil from 2009 to 2011. Number associated with each datapoint represents the isolate identification in my collection.

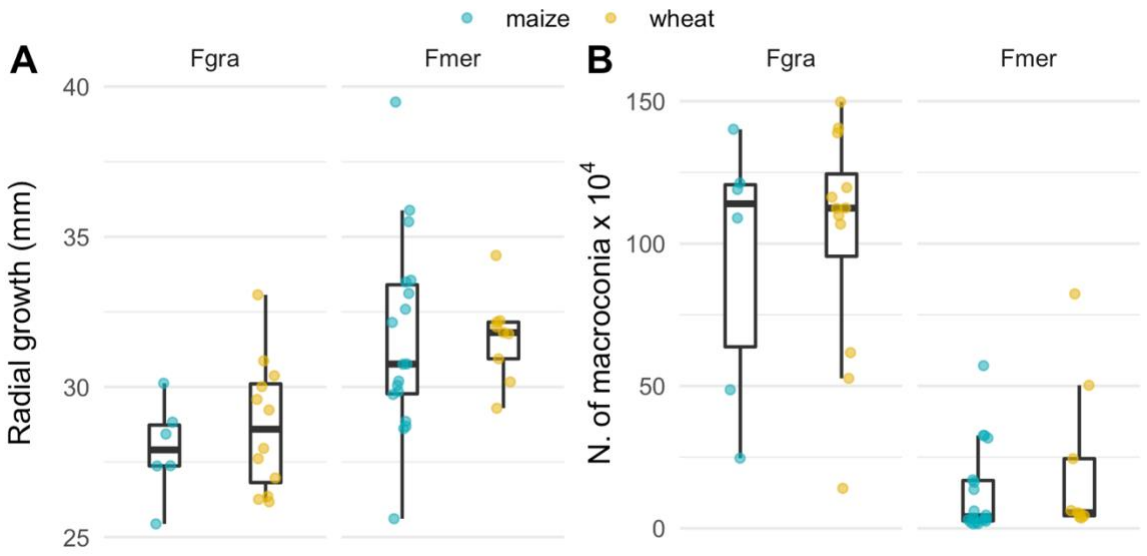

Figure 4.2. (A) Average radial growth on PDA, and **(B)** macroconidial production on MBA at 23°C of a sample of 45 isolates obtained from wheat heads or maize kernels. In total, 18 *F. graminearum* isolates (n_{maize} = 6; n_{wheat} = 12) and 27 *F. meridionale* isolates (n_{maize} = 18; n_{wheat} = 9) were utilized in this work. Datapoints for each isolate, averaged over three replicates of two independent experiments combined.

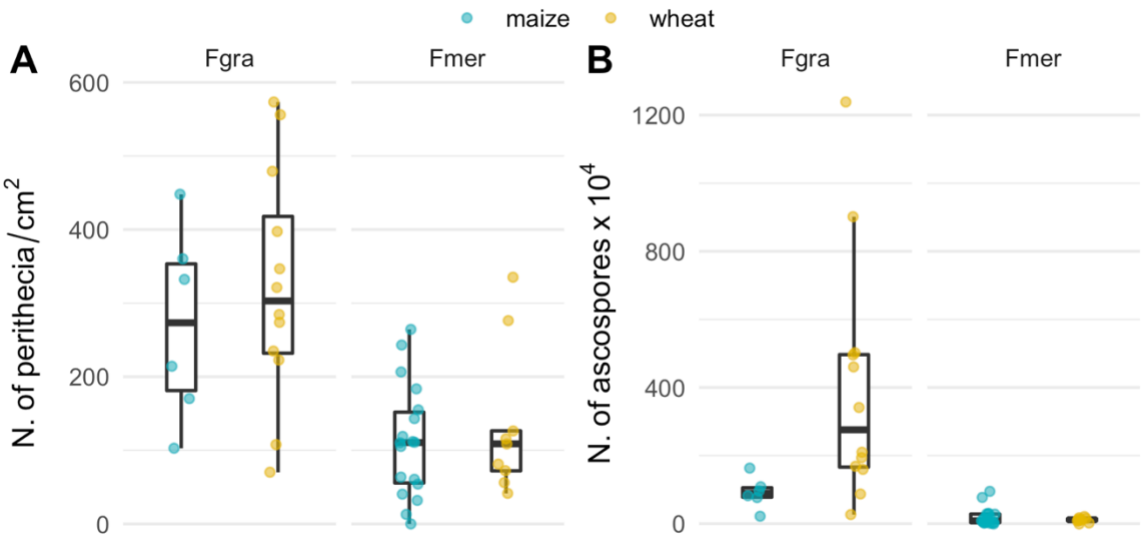

Figure 4.3. (A) Perithecial and **(B)** ascospore production on carrot agar incubated for among a sample of 45 isolates obtained from wheat heads or maize kernels. In total, 18 *F. graminearum* isolates (n_{maize} = 6; n_{wheat} = 12) and 27 *F. meridionale* isolates (n_{maize} = 18; n_{wheat} = 9) were utilized in this work. Datapoints for each isolate, averaged over three replicates of a single experiment.

Figure 4.4. Distribution of **(A)** Fusarium head blight severity (%) on susceptible spring wheat variety 'Wheaton' plants at 10 days post inoculation, and (**B)** lesion length on sweet corn hybrid Golden Jubilee silks among 18 *F. graminearum* isolates (n_{maize} = 6; n_{wheat} = 12) and 27 *F. meridionale* isolates (n_{maize} = 18; n_{wheat} = 9). Datapoints for each isolate, averaged over three replicates of three and two independent experiments combined, respectively.

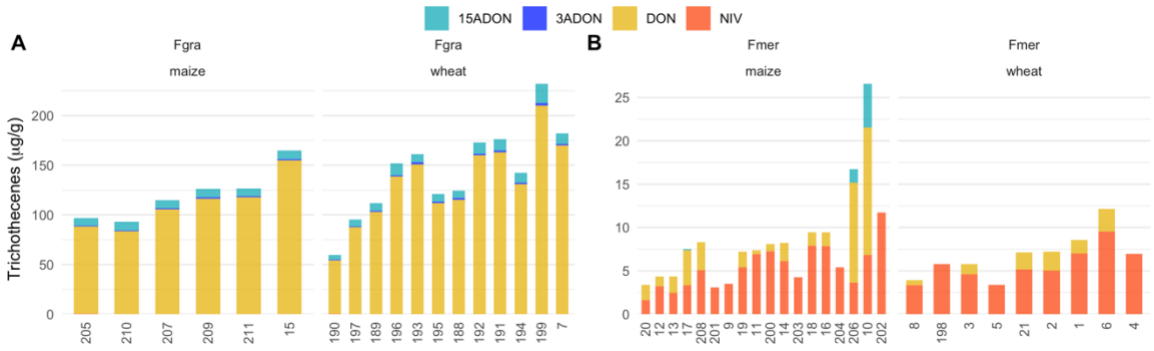

Figure 4.5. Mean production of trichothecenes: deoxynivalenol (DON), 15-acetyl-deoxynivalenol (15ADON) and 3ADON and nivalenol (NIV) by (**A**) the 18 *Fusarium graminearum* and (**A**) 27 *F. meridionale* isolates inoculated into wheat heads (cv. Wheaton).

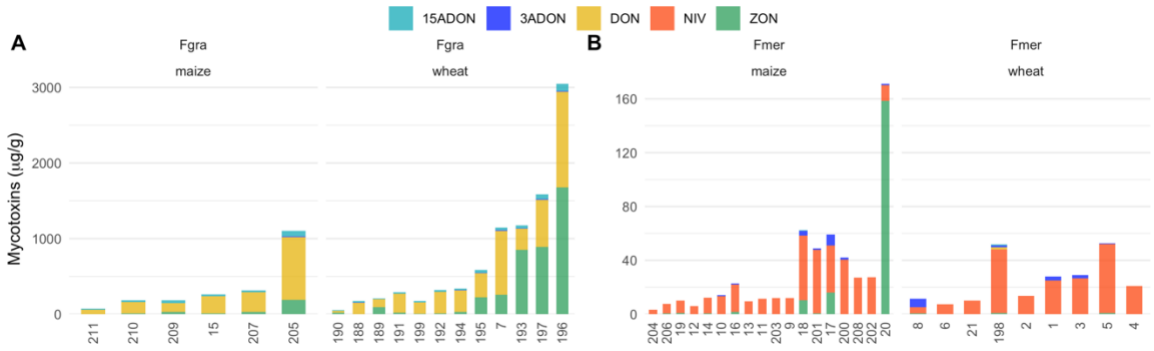

Figure 4.6. Mean production of deoxynivalenol (DON), 15-acetyl-deoxynivalenol (15ADON) and 3ADON, nivalenol (NIV) and zearalenone (ZON) by each of the (A) 18 Fusarium graminearum and (B) 27 F. meridionale isolates in rice cultures.

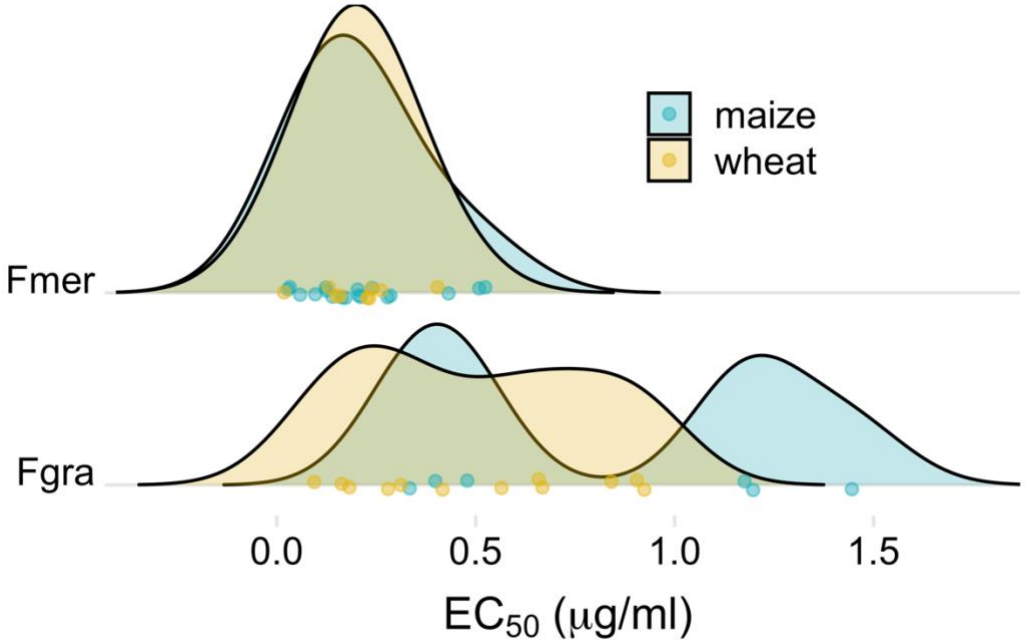

Figure 4.7. Density plots of the effective concentration of tebuconazole that reduces 50% of the mycelial growth (EC_{50}) of a sample of 18 *F. graminearum* isolates (n_{maize} = 6; n_{wheat} = 12) and 27 *F. meridionale* isolates (n_{maize} = 18; n_{wheat} = 9). Datapoints for each isolate, averaged over the two independent experiments combined.

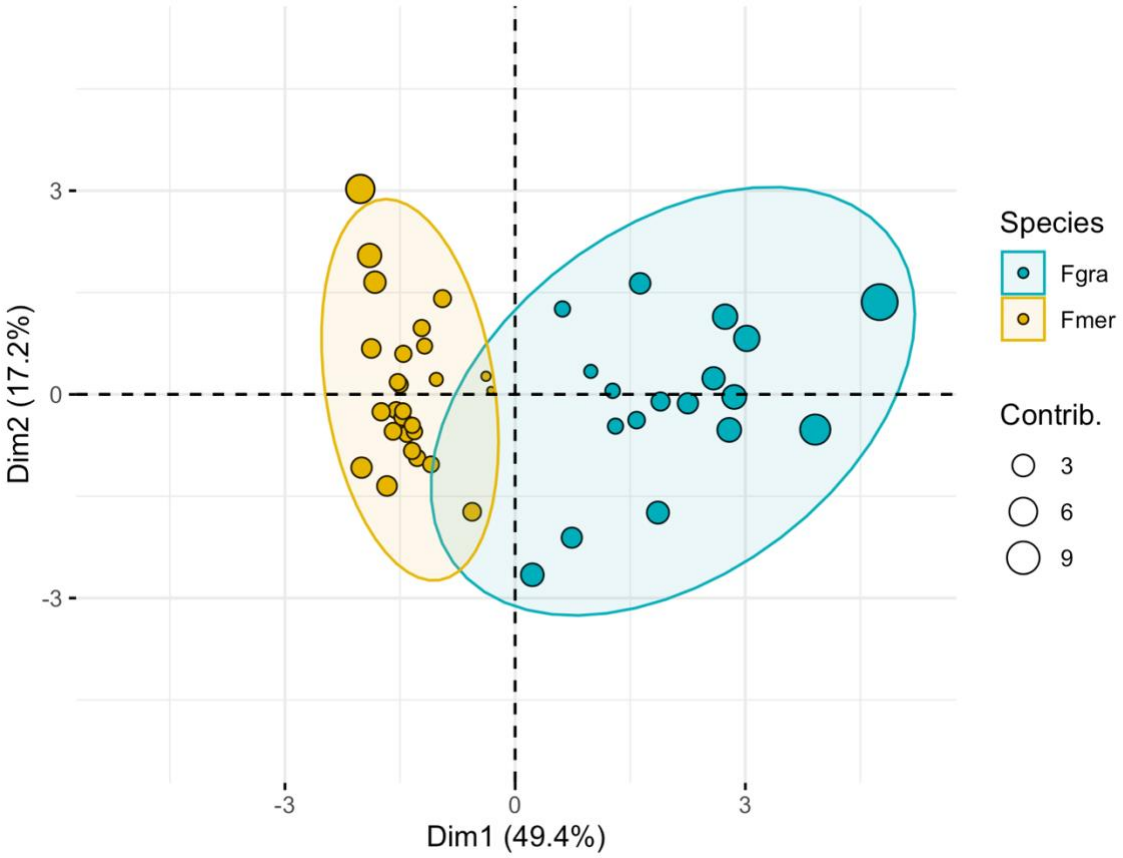

Figure 4.8. Scatterplot from the Principal Components Analysis (PCA) of a sample of 18 *F. graminearum* isolates (n_{maize} = 6; n_{wheat} = 12) and 27 *F. meridionale* isolates (n_{maize} = 18; n_{wheat} = 9) obtained from naturally infected maize kernels and symptomatic wheat heads in surveys of commercial fields in southern Brazil from 2009 to 2011. PCA was performed using data from a correlation matrix, and the estimated eigenvectors and eigenvalues were obtained for each principal component (PC). The first and second PC explained 49.4% and 17.2% of all the variation among the isolates. Contrib. = contribution (%) of each individual isolate in explaining the all variation among them.

4.7 SUPPLEMENTARY MATERIAL

Table S4.3. Summary information of the working collection of 45 arbitrarily selected isolates representing *Fusarium graminearum* (*n* = 18) and *F. meridionale* (*n* = 27) obtained from naturally infected maize kernels and symptomatic wheat heads in surveys of commercial fields in southern Brazil from 2009 to 2011.

Species[a]	Id.[b]	Host	Year	Municipality	State[c]
Fgra	188	wheat	2009	Panambi	RS
Fgra	189	wheat	2009	Ijuí	RS
Fgra	190	wheat	2010	Coxilha	RS
Fgra	191	wheat	2007	Cruz Alta	RS
Fgra	192	wheat	2007	Ernestina	RS
Fgra	193	wheat	2010	Tapejara	RS
Fgra	194	wheat	2011	Não-me-Toque	RS
Fgra	195	wheat	2011	Palmeira das Missões	RS
Fgra	196	wheat	2010	Caseiros	RS
Fgra	197	wheat	2010	Coxilha	RS
Fgra	07	wheat	2010	Estação	RS
Fgra	199	wheat	2011	Ijuí	RS
Fgra	15	maize	2011	Bom Jesus	RS
Fgra	205	maize	2011	Vacaria	RS
Fgra	207	maize	2011	Bom Jesus	RS
Fgra	209	maize	2011	Bom Jesus	RS
Fgra	210	maize	2009	Vacaria	RS
Fgra	211	maize	2009	Vacaria	RS
Fmer	01	wheat	2009	Ernestina	RS
Fmer	02	wheat	2009	Coxilha	RS
Fmer	03	wheat	2007	Nonoai	RS
Fmer	04	wheat	2011	Marau	RS

Table S4.3. (continued)

Species[a]	Id.[b]	Host	Year	Municipality	State[c]
Fmer	05	wheat	2011	Sertão	RS
Fmer	198	wheat	2010	Água Santa	RS
Fmer	06	wheat	2010	Tapejara	RS
Fmer	08	wheat	2009	Santa Bárbara do Sul	RS
Fmer	21	wheat	2007	Nonoai	RS
Fmer	09	maize	2011	Marechal Cândido Rondon	PR
Fmer	10	maize	2011	Ponta Grossa	PR
Fmer	11	maize	2011	Irati	PR
Fmer	12	maize	2011	Casca	RS
Fmer	13	maize	2011	Eldorado do Sul	RS
Fmer	14	maize	2011	Bom Jesus	RS
Fmer	16	maize	2011	Taguaí	SP
Fmer	17	maize	2011	Paranapanema	SP
Fmer	18	maize	2011	Alambari	SP
Fmer	200	maize	2011	Ponta Grossa	PR
Fmer	201	maize	2011	Ponta Grossa	PR
Fmer	202	maize	2011	Ponta Grossa	PR
Fmer	203	maize	2011	Palmeira	PR
Fmer	204	maize	2011	Palmeira	PR
Fmer	206	maize	2011	Vacaria	RS
Fmer	208	maize	2011	Bom Jesus	RS
Fmer	19	maize	2009	Caxias do sul	RS
Fmer	20	maize	2009	Boa Vista das Missões	RS

[a] Species and trichothecene genotype identified using the multilocus genotype method (Ward et al. 2008). Fgra = *F. graminearum* with 15-acetyl-deoxynivalenol genotype, Fmer = *F. meridionale* with nivalenol genotype.

[b] Isolates were obtained either from maize kernels (Kuhnem et al. 2016) or symptomatic wheat heads (Del Ponte et al. 2015) in previous surveys.
[c] Brazilian states: RS = Rio Grande do Sul, PR = Paraná, SP = São Paulo.

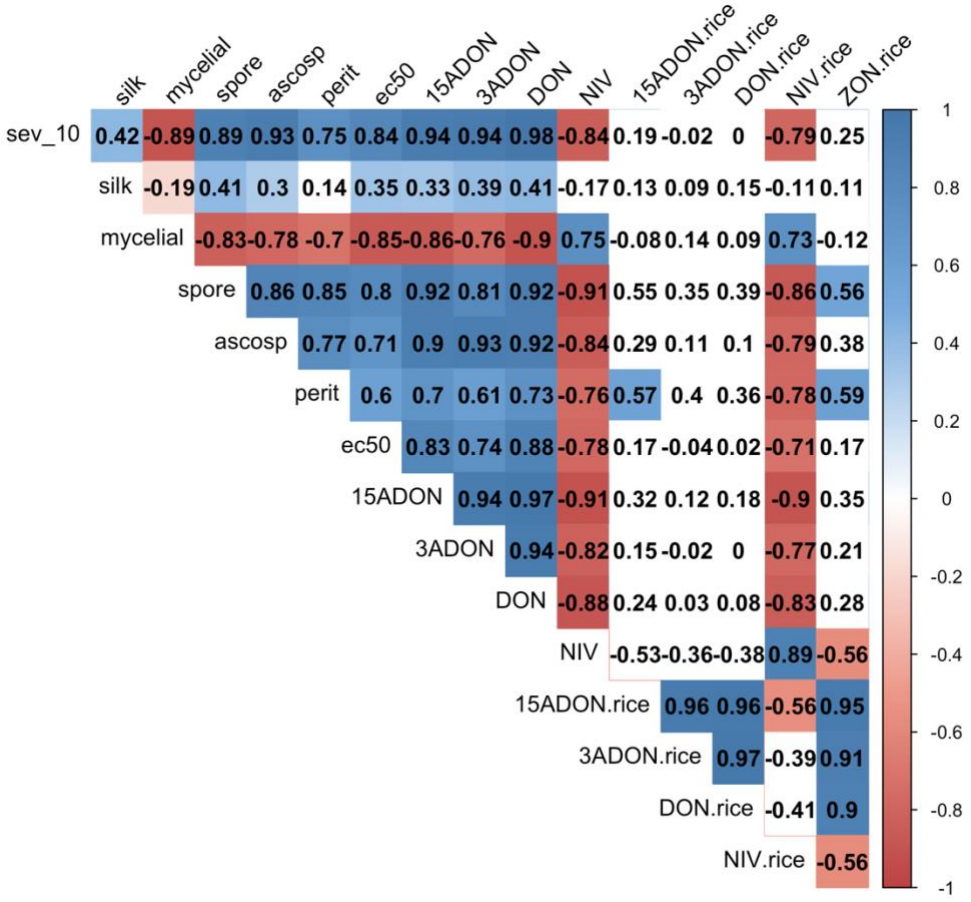

Figure S4.9. Pearson's correlation coefficients for all pairwise comparisons among saprophytic and pathogenic phenotypic traits disease-variables. Correlation coefficient was calculated using the average each trait per isolate. Negative correlation values are shown in red and positive values in blue. Lighter the color closer the correlation values to zero. Blanks values are not significant at 95% of confidence.

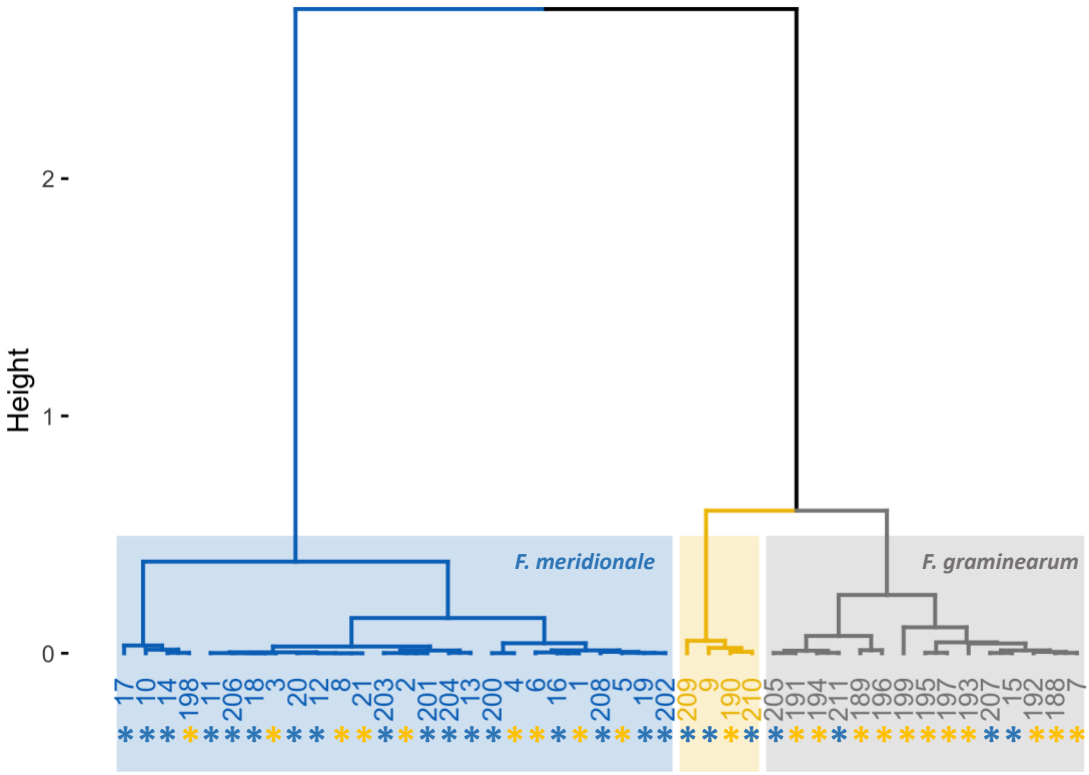

Figure S4.10. Dendrogram for a sample of 18 *F. graminearum* isolates (n_{maize} = 6; n_{wheat} = 12) and 27 *F. meridionale* isolates (n_{maize} = 18; n_{wheat} = 9) obtained from naturally infected maize kernels and symptomatic wheat heads in surveys of commercial fields in southern Brazil from 2009 to 2011 clustered according to their saprophytic and pathogenic traits based on the standardized Euclidean distance and the complete linkage method. Isolates in the yellow box include members of both species (*F. meridionale* strain 9 and *F. graminearum* strains 190, 209 and 210). Host origin is indicated by the asterisks, blue for maize and yellow for wheat. There is no obvious association with host origin in the clustering patterns.

CHAPTER 5. GENETICS AND GENOMICS USED TO STUDY GENOTYPE-PHENOTYPE ASSOCIATIONS AMONG MEMBERS OF THE *FUSARIUM GRAMINEARUM* SPECIES COMPLEX

Abstract

In this final research chapter of my dissertation, I explored the use of genomics and genetics to characterize *F. graminearum* and *F. meridionale*, as an extension of my previous epidemiological studies. The work in this chapter addressed three major questions. First, I analyzed and compared single nucleotide polymorphisms (SNPs) across whole genome sequences of representative isolates of the two species, to determine whether the phenotypic divergence I had observed between them was associated with genotypic divergence. My results clearly demonstrated that *F. graminearum* and *F. meridionale* are genotypically divergent, and furthermore that host origin does not appear to play a major role in population structure for either species. Second, I used these SNPs as markers to identify potentially introgressed regions, to determine whether the two species have undergone sexual recombination in the field in Brazil. The results provided strong evidence for both intraspecies and interspecies recombination. Interestingly, a likely introgression of *F. graminearum* sequence on chromosome 2, including part of the TRI gene cluster that is responsible for production of mycotoxin, was detected in a Brazilian *F. meridionale* strain that had produced exceptionally high levels of DON in wheat heads. The introgressed region of approximately 160 Kb included about 90 genes, more than half of which have been implicated in pathogenicity. Finally, I determined whether SNP association mapping could be used to study the inheritance of pathologically-significant traits in controlled crosses. For this study, I used progeny from a cross between two strains of *F. graminearum* that had been conducted by a former graduate student. The results revealed an association between aggressiveness and a recombination hotspot on chromosome 2. An introgressed sequence of approximately 320 kb that co-segregated with high levels of aggressiveness included 110 predicted proteins, 40% of which were reportedly associated with pathogenicity, and 10% of which were predicted to be secreted, including two putative effector proteins. The work described here provides a demonstration of the value of a genetic approach to augment and extend our epidemiological studies of these pathogens, and serves as a basis for future analysis of selective forces relevant to mechanisms of divergence in FGSC.

KEYWORDS: *Fusarium graminearum* s.s., *Fusarium meridionale*, recombination hotspots, gene flow.

5.1 Introduction

In this dissertation, I have addressed the question of host dominance among strains of FGSC on wheat versus maize in Brazil. In Chapter 4, I used principal component analysis based on quantification of several pathogenicity- and fitness-related traits to demonstrate that phenotypes of co-localized populations of *F. graminearum* and *F. meridionale* in Brazil are statistically divergent. Several traits were found to be correlated with aggressiveness of these species to wheat versus maize, including ascospore production, toxin production, and growth rate. Populations of *F. graminearum* on wheat versus maize also diverged in some characters, including fertility and aggressiveness to wheat, and production of DON. Overall, however, there was no evidence for partitioning associated with the host of origin: instead, the main effect was due to species. In this chapter, I explored the use of genomics to characterize and evaluate genetic diversity between and within members of the two species, and to compare genetic associations with the phenotypic associations I characterized in Chapter 4. Several published studies have investigated the genetic structure of FGSC to better understand the connection between genotypic and phenotypic variation at the population level (Gale et al., 2011; Lee et al., 2012; Talas et al., 2011). My first question for this final research chapter was: can the phenotypic divergence I observed between the two species be linked to genotypic divergence? Phylogenetic species in FGSC are identified by a multigene approach that examines and compares only six different sequences, including parts of the TRI gene cluster that is involved in production of trichothecene mycotoxins (Ward et al., 2008). Such a small number of sequences may not reflect the full extent of genetic divergence among and within members of these two phylogenetic species. I chose to apply a whole-genome approach focused on analysis of single nucleotide polymorphism (SNP) markers to

estimate the degree of genotype diversity among strains of *F. graminearum* and *F. meridionale* from the Brazilian populations. Recent work using a similar whole genome approach showed evidence that population-specific selection pressures have left distinct signatures in the genomes of North American *F. graminearum* isolates (Kelly and Ward, 2018).

If there is a high level of genotype divergence between *F. meridionale* and *F. graminearum* could imply a lack of gene flow between these species due to isolation (i.e., genetic drift). Isolation could be due to mating barriers, or it could be due to differential selection and adaptation among subpopulations (Nosil et al., 2009). Some members of the FGSC can engage in interspecies outcrosses in the laboratory, e.g. *F. graminearum* with *F. asiaticum* or *F. meridionale*, but generally fertility levels are low and segregation ratios are skewed (Bowden and Leslie, 1999; Fuentes-Bueno, 2012; Summerell and Leslie, 2011; Jurgenson, et al., 2002). It remains unclear whether these species outcross in the field. Thus, my next question was about the presence of gene flow within and between populations of *F. meridionale* and *F. graminearum* in Brazil. Using whole genome SNP analysis, I conducted an analysis to detect evidence for introgression, to provide insight on the ability of *F. meridionale* and *F. graminearum* to undergo sexual recombination in the field.

If the evidence suggests that *F. meridionale* and *F. graminearum* do recombine in the field, and thus that they belong to a single biological species, that means it would be possible to use a genetic approach to test whether traits that appear to be under positive selection are heritable and actually confer specific adaptive advantages. A genome-wide association study (GWAS) would be helpful as a downstream application in which I could

associate particular markers with particular phenotypes, as previously described for genes harboring SNPs associated with fungicide resistance and aggressiveness in *F. graminearum* (Voss et al. 2010, Cumagun and Miedaner 2004, Talas et al., 2016).

Several studies have shown that recombination rates vary significantly across the *F. graminearum* chromosomes, with recombination "hotspots" interspersed with regions where recombination is less frequent. This is associated with a so-called "two-speed genome", in which some regions, enriched in genes under positive selection that are potentially related to pathogenicity, evolve more quickly than others (Laurent et al., 2018; Talas and McDonald, 2015; Wang et al. 2017). It has been suggested that recombination at these hotspots produces variants with adaptive advantages, contributing to the rapid evolution of more aggressive populations under selection. (Cuomo et al. 2007; Laurent et al., 2018; Talas and McDonald, 2015, Wang et al. 2017). Crosses of different *F. graminearum* strains in the laboratory can produce transgressive strains that are more aggressive and toxigenic than the parents (Cumagun et al., 2004a, b; Cumagun and Miedaner, 2004). However, an association of these strains with recombination hotspots has never been directly demonstrated. For the third part of this chapter, I used SNP mapping to show a link between aggressiveness and a recombination hotspot among progeny of a cross that had been made by a former graduate student between two strains of *F. graminearum*. The ability to conduct controlled crosses and develop recombination maps for pathogen species improves our ability to predict patterns of gene flow among natural populations, an important consideration for epidemiological studies designed to improve deployment of host resistance and chemical controls for disease management.

5.2 Material and Methods

5.2.1 Fungal Isolates and Culture

Nine Brazilian isolates of *F. meridionale*, and six of *F. graminearum*, isolated from either maize or wheat were chosen for this study. These isolates were representative of the larger group that was analyzed in the previous chapters of this dissertation (Table 5.1). Four additional genomes were included in the analysis for this chapter. Although a genome assembly was available for the strain Gz3639 (Cuomo et al., 2007), it had very low coverage and was of poor quality, so I generated a new genome assembly for this strain. All isolates were routinely cultured on Mung bean agar (40 g of dried mung beans/liter, placed in boiling distilled water for 23 min or until ~50% of the seed pericarps split, followed by filtering through two layers of cheesecloth, and adjustment to 1 liter followed by addition of 15 g of agar) (Evans et al., 2000) at 23°C under continuous fluorescent light. Isolates were never subcultured more than once.

5.2.2 DNA extraction

To isolate fungal DNA, 80 ml aliquots of YEPD media (20 g dextrose, 10 g Bacto® peptone, 3 g yeast extract per L) in 250 ml glass flasks were inoculated with a 5 x 10^5 macroconidial suspension obtained from an actively growing culture of each isolate on MBA. Inoculated flasks were incubated for 3-5 days at 23 °C with agitation (250 rpm). The mycelial mat was collected by vacuum filtration, frozen in liquid nitrogen and lyophilized. Lyophilized tissue was pulverized with a sterile plastic pestle. The pulverized tissue was mixed with 4 ml of CTAB extraction buffer (20 mls 1 M Tris pH 7.0; 28 mls 5

M NaCl; 4 mls 500 mM EDTA pH 8; 2 g CTAB per 100 mls) and incubated at 65 °C for 30 min. After the samples cooled to room temperature, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, and the sample was rolled on the orbital mixer table for 5 min, followed by centrifugation at 6000 rpm for 15 min. The upper aqueous phase was removed to a new tube and the PCI extraction was repeated, followed by an extraction with chloroform. The upper aqueous phase was removed to a new tube and the DNA was precipitated with 1 volume of isopropanol. The samples were centrifuged for 10 min at 13,000 rpm to pellet the DNA. The pellet was washed twice with 70% ethanol. After the ethanol washes, the DNA pellet was dried for 10 minutes in a transfer hood, then dissolved in 100 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) + 10 µg RNase A (10 mg/ml) at room temperature.

5.2.3 Genome sequencing and assembly

DNA concentration was measured with a Qubit fluorometer, and DNA was sent to the Duke Center for Genomic and Computational Biology (Durham, NC) for preparation of libraries and Illumina sequencing. Libraries containing 300 bp sheared DNA inserts were constructed and the Illumina NextSeq 500 platform was used to generate 150 bp paired-end reads at ~ 50X coverage.

Genomes were assembled with a custom bioinformatics pipeline "BioPipe" (https://github.com/hain222/bio-pipe), followed by use of the Velvet assembler (Zerbino and Birney 2008) via VelvetOptimiser (https://github.com/tseemann/VelvetOptimiser). The parameters used were: start kmer value, 99; end kmer value, 149; step size, 4; and with

the shortPaired setting. A summary of the genome assembly information and statistics are presented in (Table 5.2).

5.2.4 Whole-genome alignment and tree building

The new genome assemblies were compared to one another, and to several reference genome assemblies for both species from other locations (Table 5.1), with a custom pipeline "iSNPcaller" (https://github.com/drdna/iSNPcaller). The protocol includes blast alignment of repeat-masked genomes to generate a pairwise distance matrix. Repetitive sequences were masked by subjecting each genome to blast analysis against itself. Blast reports were pre-screened using a threshold of 1e-200 to filter out aligned regions containing hidden paralogs, or regions that did not uniquely align in each pairwise comparison, before SNP calling. SNPs were then identified for each pairwise comparison and scaled by the total number of nucleotides aligned after excluding repetitive and duplicate regions. This produced a distance metric of SNPs per megabase of uniquely aligned DNA. The resulting distance matrix was input into MEGAX (version 10.1.7) (Kumar et al., 2018) and a similarity cladogram was generated by using the Neighbor Joining program and default parameters.

5.2.5 Haplotype similarity analysis

Chromosome-level haplotypes were generated by mapping SNPs from iSNPcaller to a fully assembled reference genome of the PH-1 strain of *F. graminearum* (King et al., 2015) using custom scripts, followed by data conversion into a format compatible with

Choromopainter (https://people.maths.bris.ac.uk/~madjl/finestructure-old/chromopainter_info.html) (Lawson et al., 2012). The chromopainter files were used as inputs to custom scripts that performed sliding-window analyses of pairwise haplotype similarity in an all-by-all comparison (window size, 200 SNPs, step size, 40). The final sliding window data were imported into a Shiny app (R Core Team 2019: Chang et al., 2019) that runs custom R code for plotting haplotype similarity between any given reference strain and all strains belonging to a set of user-selected host populations/species (https://github.com/drdna/ShinyHaplotypes). Potential recombination blocks were further investigated by manual comparisons with the PH-1 assembly of chromosome 2 (NCBI accession HG970333.1). First, each contig of the genome of interest was individually compared to the PH-1 genome by using BLASTN. Homologous contigs were annotated by submitting them for analysis by FGENESH via the Softberry web interface (softberry.com). Finally, the NCBI PH-1 genome browser was used to manually identify and compare the nucleotide sequence and proteins encoded within the introgressed region from PH-1 and the strain of interest. Individual proteins were further characterized by BLASTP, or via other resources including the PHI database for genes implicated in pathogenicity (http://phi-base.org); Signal-P 5.0 (http://www.cbs.dtu.dk/services/SignalP/) and Phobius (http://phobius.sbc.su.se/) for prediction of subcellular localization; EffectorP 2.0 (effectorp.csiro.au); and the *F. graminearum* genome databases available from the Joint Genome Initiative Mycocosm site (https://mycocosm.jgi.doe.gov/Fusgr1/Fusgr1.home.html) and from EnsemblFungi (http://fungi.ensembl.org/Fusarium_graminearum/Info/Annotation). Other resources included :OmicsDB::Pathogens (https://pathogens.omicsdb.org/); the Fusarium Comparative Genomics Platform (http://genomics.fusariumdb.org/); the AntiSMASH

metabolite cluster predictor (https://fungismash.secondarymetabolites.org/#!/start), Fungidb (https://fungidb.org/fungidb/); and the *F. graminearum* mutant database (https://docs.google.com/spreadsheets/d/1ZJXGqvqKi2jVUCkQmxeGvfgf7BKLX1l1-AR4WqerG0Q/edit#gid=362556506).

5.2.6 Bulk Segregant analysis of marker association with aggressiveness and toxigenicity

A set of 20 transgressive progeny that had been recovered from a cross between two strains of *F. graminearum*, PH-1 and Gz3639 (Bec, 2011), were subjected to a bulk segregant analysis to identify chromosome regions that co-segregated with high versus low levels of aggressiveness. Genomic DNA was prepared as above from two groups of 10 strains representing the least, and the most aggressive towards the susceptible wheat line Pioneer 2555. The pooled DNA samples were sequenced by using Illumina paired-end sequencing at the Texas A&M AgriLife Genomics & Bioinformatics Center (College Station, TX).

Raw reads were aligned against the PH-1 reference genome (King et al., 2015) by using TopHat2 (Trapnell et al., 2009). This tool uses the Bowtie2 alignment engine to map reads to the genome assembly. First, BAM files were sorted using Samtools version 1.7 (Li et al., 2009). Samtools mpileup utility was used to extract information on nucleotide variations between sequence samples and the reference genome to generate the '.bcf' output files. Variant calling was performed using bcftools utility of Samtools program. The final dataset was imported to R and the proportion of reads that mapped to the reference versus the alternative allele were calculated per SNP position. The final dataset was used to produce figures showing the overall proportion of reads per SNP position that came from

the reference genome (PH-1) or were assumed to have come from the alternative parent, Gz3639.

PCR was used to validate a potential recombination hotspot on Chromosome 2. Cleavable Amplified Polymorphic Sequences (CAPS) markers were generated by using a list of SNPs differentiating the low and high pools from the PH-1 reference genome. Five unlinked SNPs that comprised restriction sites were identified, and a ~500 bp segment spanning each selected SNP site was amplified by using primer sets that were designed for each region (Table 5.4). The thermocycling protocol consisted of initial denaturation for 1 min at 95°C; followed by 40 cycles of 30 sec denaturation at 95°C, 20 sec annealing at the temperature specified in Table 4 for each marker, and 1 min extension at 72°C; and one extension cycle for 7 min at 72°C. Ten μL of each PCR amplicon was used for each restriction reaction. Restriction reactions used Invitrogen restriction enzymes *Hind*III, *Pst*I and *Xho*I according to the manufacturer's instructions at 37°C for 2 h. Restriction reactions were separated on a 1% agarose gel for analysis.

I produced a new assembly of the Gz3639 genome for this study to identify the specific contigs that matched the PH-1 region of interest on Chromosome 2. Identification of homologous contigs, and manual comparison and characterization of genes and proteins, was performed as described above.

5.3 RESULTS

5.3.1 Whole-genome alignment and tree building

Genomes of six representative *F. graminearum* isolates and nine *F. meridionale* isolates from maize and from wheat were sequenced and assembled (Table 5.2). Assemblies were aligned, and the number of SNPs was compared, to produce a whole genome tree (Table 5.3, Fig. 5.1). The neighbor-joining tree built using "total-genome" pairwise distances (Table 5.3) revealed two main clusters that were consistent with the phylogenetic species *F. graminearum* and *F. meridionale*. Brazilian strains of each species grouped together with reference isolates of the same species from North America and from Nepal, respectively (Fig. 5.1). The Nepalese strains of *F. meridionale* occupied a branch within the cluster separate from the Brazilian *F. meridionale* strains. The North American *F. graminearum* strains also occupied a branch apart from the Brazilian strains of *F. graminearum*, indicating that populations of both species are geographically structured at a global scale, although there was no evidence for more localized subpopulations. There was also no evident relationship with the host of origin for either species.

5.3.2 Haplotype similarity analysis

For both *F. graminearum* and *F. meridionale*, isolates were characterized by an uneven distribution of haplotype divergence. Thus, the ends of each of the chromosomes were generally divergent, and there were two or three additional highly polymorphic regions per chromosome interspersed with much more conserved regions (Figure 5.2 and Figure 5.3). In both cases, individual strains were mosaics of chromosome segments

inherited from strains that have different evolutionary histories. These data suggest that there has been extensive admixture, probably due to outcrossing, within the Brazilian *F. graminearum* and *F. meridionale* populations.

A likely introgression of *F. graminearum* DNA was detected in *F. meridionale* strain 10, which originated from maize in Ponta Grossa, Brazil. The block was on chromosome 2 and appears to be introgressed from a *F. graminearum* strain that was very similar to the North American strain PH-1 (Fig. 5.4). Contigs from the genome assembly for strain 10 were mapped to chromosome 2 of PH-1, and one contig was identified that spanned the relevant region, from 5,400,000 to 5,560,000 bp (Fig. 5.5). Contig 111 was 98% identical to this part of PH-1 chromosome 2, with about 1900 SNPs and 358 small indels across the entire 160 Kb (Fig. 5.6). There were no major rearrangements or inversions. This sequence in PH-1 is predicted to encode 91 genes, including all but the first three genes of the trichothecene metabolite cluster (*TRI*4, *TRI*6, *TRI*5, *TRI*10, *TRI*9, *TRI*11, *TRI*12, *TRI*13, and *TRI*14). Several genes downstream of the cluster, including a transcription factor (FGSC_03551) (Son et al., 2011), are co-regulated and may also be involved in mycotoxin production (Puri et al., 2016). Twenty-two proteins were predicted to be secreted, and 18 were predicted to be effectors. Comparisons with the PHI database showed that 48 of the 91 proteins had functions in pathogenicity. These included a chitin deacetylase (FGSG_03544) expressed during early infection that may be important for blocking host recognition (Puri et al., 2016), a triacylglycerol lipase named FGL5 (FGSG_03583) (Nguyun, 2008), and a regulator of G-protein signaling named FgFlbB (FGSG_03597) (Park et al. 2012). Knockout mutations in each of these genes reportedly resulted in reductions in pathogenicity of *F. graminearum* to wheat.

I next identified contigs that matched this region of PH-1 from strain 17 of *F. meridionale*, which doesn't have the introgression and is similar to the rest of the Brazilian *F. meridionale* strains. The sequence of strain 17 was more divergent from PH-1, including almost 4000 SNPs across the approximately 150 Kb of DNA that could be aligned (Fig. 5.6). More than a quarter of the SNPs (1261) occurred in the region encompassing the TRI genes that spans only 16.6 Kb, or about 11% of the total alignment. Nonetheless, the TRI proteins of strain 17 were very similar to the PH-1 proteins, ranging from 98% to 100% identity at the amino acid level. Most other proteins were similarly conserved, but a few were more divergent (ranging from 76-84% identity). These included an unnamed protein that is induced during early infection of wheat heads (FGSG_12416), an integral membrane protein (FGSG_03561), an efflux pump protein (FGSG_03571), and a predicted effector (CEF78399). Knockout mutations of FGSG_12416 and the effector both resulted in reductions in aggressiveness to wheat, according to the PHI database. Mutations in the other two had no effect.

5.3.3 Bulk Segregant analysis of marker association with aggressiveness and toxigenicity

For this analysis, I mapped SNPs to DNA that was isolated from pools of 10 progeny that represented the most, and least aggressive among 96 total progeny of a cross between *F. graminearum* strains PH-1 and Gz3639. In the absence of bias, I would theoretically see a 50:50 distribution of SNPs from both parents in both pools. However, if genes from one parent provided a selective advantage, in this case for aggressiveness, I expected to see that parent over-represented among those genes in the highly aggressive pool relative to the

low pool. In observing SNP distributions for the two pools, I saw that the distributions generally did not have the expected 50:50 representation. Instead, numerous regions of the chromosomes were biased toward one parent (especially PH-1) for both pools. For example, almost the entire length of chromosome 3 seems to have been inherited from PH-1 by progeny in both pools. This could be due to contamination of the ascospore progeny with ascospores or conidia of PH-1. However, two CAPs markers were shown to follow Mendelian segregation patterns among the progeny pools (Bec, 2011), which argues against that possibility.

There were three regions that seemed to be associated with high versus low levels of aggressiveness, two on chromosome 2 and one on chromosome 4 (Fig. 5.7, gray rectangles). In each case, the highly aggressive strains had inherited these regions from the Gz3639 strain. I investigated more closely one introgressed Gz3639 region (~320 kb), located on chromosome 2 (from 6,470,806 to 6,823,783 bp) and associated with a recombination hotspot. This region was near the previously identified introgression in *F. meridionale* strain 10, but it did not overlap it. Five unlinked SNPs, one at each flanking region and three within the region of interest, segregated at similar ratios as the observed in the bulk analysis for the highly aggressive progeny (Fig. 5.8; Table 5.5). These findings confirmed that this region in the more highly aggressive strains was indeed biased towards the Gz3639 parent.

Contigs from the newly produced assembly of Gz3639 were mapped to this region, and three overlapping contigs were identified that spanned it (Fig. 5.9). This region does not include the trichothecene metabolite cluster (Fig. 5.7). The first crossover point was located in a highly polymorphic region between a thioredoxin gene and a gene encoding a

probable pimeloyl-ACP methyl ester carboxylesterase (6,470,806 to 6,474,246 bp in chromosome 2, Fig. 5.10A), while the second crossover point was associated with a large inversion at position 6,823,783 bp (Fig. 5.10B).

The recombined region included 110 predicted proteins. There were about 1773 SNPS and several small indels that differentiate the two strains in this region. About half the Gz3639 proteins were identical to the PH1 versions. SNPS and indels have produced one or more amino acid changes in the remainder (no frameshifts were observed). All but six proteins were at least 95 percent identical. About 40% of the genes were potentially pathogenicity associated, based on PH1 database matches and about 10% were secreted proteins. Two predicted effector proteins were present in Gz3639 but not in PH1. Both of these effectors were conserved in other *Fusarium* strains or species.

5.4 DISCUSSION

My primary objective in this chapter was to apply genetics and genomics approaches to study potential associations between phenotypes and genotypes of isolates of two co-existing members of the FGSC in Southern Brazil. The delineation of species within *F. graminearum* has been the subject of much discussion, and some controversy, for at least two decades. There are at least 16 phylogenetic species that comprise the FGSC (O'Donnell et al., 2004; O'Donnell et al., 2008; Sarver et al., 2011; Starkey et al., 2007; Yli-Mattila et al., 2009). Since 2004, when lineages were elevated to species rank (O'Donnell et al., 2004), some researchers have questioned the significance of these phylogenetic species, given the fact that they can all cause the same grain diseases. Furthermore, members of different phylogenetic species are interfertile, and thus belong to

the same biological species as defined by Mayr (1942): “Species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups”. It has been suggested that the *F. graminearum* species complex might be in the early stages of speciation. Nonetheless, intrinsic reproductive isolation appears to be minimal, with patterns of cross fertility that are more strain-specific than lineage-specific (Fuentes-Bueno, 2012; Bowden and Leslie, 1999; Jurgenson et al., 2002). This lack of intrinsic reproductive barriers suggests to some that these lineages diverged as a result of geographic isolation and have been brought together more recently through global trade (Fuentes-Bueno, 2012).

Summerell (2019) in his review about *Fusarium* status, expresses little doubt that the FGSC members are phylogenetically distinct taxa, and that some of those differences reflect differences in biology, toxin production, and biogeography. He raises the possibility that some taxa may be hybrids, and points to the existence of a small number of apparent natural interspecific/interlineage hybrids that have been detected in the field (Boutigny et al., 2011; O’Donnell et al., 2000). For example, strain NRRL28721 was suggested to be a natural hybrid between *F. meridionale* and *F. asiaticum* based on multilocus analysis (O’Donnell et al., 2004, Starkey et al., 2007). More recently though, a whole genome analysis definitively placed this strain within *F. meridionale* (Walkowiak et al., 2016), indicating that the hybridization is limited in scope and encompasses only the regions that are used for multilocus phenotyping. My own results in this chapter confirmed the placement of this strain within *F. meridionale* and suggests that the prior evidence for natural hybridization based on multilocus markers may actually be due to an introgression block or blocks. Clearly, analyses based on whole genomes are superior to MLGT assays

if our goal is to fully describe the degree and effect of hybridization and/or introgression among members of the FGSC. For example, a recent study used genomic data from a large collection of more than 100 strains to address questions about population structure and genetic targets of selection within the *F. graminearum* population causing FHB in North America (Kelly and Ward, 2018). These authors presented evidence that a divergent population that produces NX-2 mycotoxin was endemic and may have originated in wild grasses (Lofgren et al., 2017). They concluded that this population had experienced a recent expansion, and implicated selection as the main force driving this. Signatures of selection were found in genes related to host infection, niche competition, and environmental adaptation including fungicide resistance and mycotoxin-associated aggressiveness. Genomic tools will be very important in enabling us to address these critical questions, and consider implications related to competition, selection, and adaptation among pathogen populations in the field.

My results in this chapter and in Chapter 4 demonstrated that *F. graminearum* and *F. meridionale* are divergent in both phenotype and genotype, implying the existence of sympatric or allopatric isolation during their evolutionary history. Very little structure was imposed by host species, indicating that the relative host dominance we observe on maize versus wheat in Brazil is a result, rather than a cause, of species-related divergence. There was some evidence that the species are geographically structured on a continental scale, although the sample size is too small to be very confident in this conclusion. Although clusters of closely related Brazilian strains were observed within each species, there was no correlation with collection locations, suggesting that the populations of both species are regional in Brazil. Two Brazilian *F. meridionale* strains (05 and 09) were divergent from

the rest but very similar to one another, even though they had come from different locations and from different hosts. This suggests a possibility of an introduction of a different genotype into the region from another location. Clearly there is a need to analyze a much more extensive sample of strains of both species from across the region to further evaluate these conclusions.

Genome-wide comparisons revealed a high degree of polymorphism among the strains within both species in Brazil, and this might be related to the high level of phenotypic diversity I also observed among individual strains. Polymorphisms were clustered rather than randomly distributed across the chromosomes. Several studies have reported a similar "two-speed" genome structure in *F. graminearum*, in which some regions enriched in potential pathogenicity genes that appear to be under positive selection are more polymorphic and change more quickly than others (Laurent et al., 2018; Talas and McDonald, 2015; Wang et al. 2017). I observed an increase in haplotype divergence near the ends of the four chromosomes, and also at two or three internal regions, separated by more highly conserved sequences. Those findings agree with previous reports where the highest density of polymorphic sites was found in regions near subtelomeres and in similar internal locations, especially on Chromosomes 1, 2 and 4 (Kelly and Ward, 2018; Cuomo et al., 2007; Walkowiak et al., 2015; Laurent et al., 2018). The significance of the two speed genome in pathogen adaptation is still unclear, but polymorphic regions have been correlated in a small number of studies with recombination hotspots, and this has led to the suggestion that recombination plays a role in "shuffling" these groups of polymorphic genes, leading to the generation of new genotypes with novel pathogenic capabilities that can be subject to selection (Cuomo et al., 2007).

In addition to intraspecies recombination, my analysis also provided strong evidence for interspecies recombination and gene flow between *F. graminearum* and *F. meridionale* in Brazil. Closer investigation of a likely introgression comprising about 160 Kb on Chromosome 2 from *F. graminearum* into one *F. meridionale* isolate (id. 10) revealed that it included a portion of the trichothecene metabolism cluster that is involved in the production of DON. Interestingly, this strain was one of only two (I didn't sequence the genome of the other) that produced DON in wheat heads, as I reported in Chapter 4. Even though it produced as much or more DON as most of the *F. graminearum* strains, id. 10 was much less aggressive than those strains and was instead more similar to the other *F. meridionale* strains. This suggests that factors other than DON are necessary for high levels of aggressiveness to wheat. The introgressed region includes more than 40 other genes that are implicated in pathogenicity, including eight predicted effectors, several genes that are known to be co-expressed with the TRI cluster, and many others that are expressed during early infection of wheat (Puri et al., 2016). Although the introgressed region is quite polymorphic at the nucleotide level between *F. graminearum* and *F. meridionale*, the proteins are much more conserved, with only five having less than 95% identity.

Associations between loci and phenotypes can be identified on a much larger scale with appropriate markers. For example, (Talas et al., 2016) used a genome-wide association study (GWAS) to identify a QTL that is strongly associated with azole sensitivity in *F. graminearum*. Genome data facilitate the identification and tracking of appropriate markers for this type of analysis. It would be interesting in the future to conduct similar studies on *F. graminearum* and *F. meridionale* related to traits e.g. fungicide

sensitivity, sexual fertility, host aggressiveness, and toxigenicity, that vary between the two. These studies would help us understand the role of this variation in determining host dominance and population structure across different environments.

Although correlative studies like GWAS are valuable, they are even more powerful when they can be combined with controlled crosses in which patterns of co-segregation of traits can be evaluated among progeny. Although laboratory crosses between *F. graminearum* and *F. meridionale* have been made (Leslie, personal communication), I was unfortunately unsuccessful in numerous attempts to make interspecies crosses among my strains. As I showed in Chapter 4, and others have also reported (Fuentes-Bueno, 2012), *F. meridionale* is much less fertile than *F. graminearum*. The relative infertility of *F. meridionale* made it hard to make either intra- or interspecies crosses, although I did see increases in the numbers of perithecia when I paired some *F. meridionale* strains with one another.

Unlike *F. meridionale, F. graminearum* is highly fertile and relatively easy to cross. A former graduate student in the laboratory had made a cross between two strains of *F. graminearum* and evaluated 98 progenies for aggressiveness on a susceptible variety of winter wheat (Bec, 2011). She identified transgressive progeny that were significantly more aggressive and toxigenic than either parent. I conducted a bulk segregant analysis using DNA pools from the ten most aggressive, and ten least aggressive strains from this cross. I then applied SNP genotyping in order to identify chromosomal regions that appeared to be associated with high levels of aggressiveness and toxigenicity. I identified at least three such regions: in each case the highly aggressive progeny had disproportionately inherited sequences from the more aggressive and toxigenic Gz3639

parent. I investigated one of these regions more closely, because it was associated with a recombination hotspot. This is the first direct evidence relating a hotspot to changes in pathogenicity among the recombinant progeny. Interestingly, this region was on the same arm of chromosome 2 as the introgression I described above in *F. meridionale* strain id. 10. This arm of chromosome 2, and chromosome 2 in general, has been shown to be particularly enriched in pathogenicity related genes, including the TRI cluster and multiple secreted effectors (King et al., 2015, Walkowiak et al., 2016). In this case, the TRI cluster was not included, but there were numerous genes implicated in pathogenicity that diverged between the two strains, as well as a PKS secondary metabolite cluster that produces orsellinic acid, and several predicted effectors that also might be important and would be interesting to explore further. Overall, this work showed the potential value of a genetic approach in identifying novel genes that may be involved in traits of interest.

The work in this final research chapter is preliminary, but it serves as a demonstration of the application of genetic recombination analyses to dissect the potential for the production of more highly adapted strains via recombination. It provides a basis for future studies of the selective forces relevant to mechanisms of adaptation and divergence within and between these species. A better understanding of the mechanisms that regulate host- and location-specific variations in FGSC species composition would help to improve disease and mycotoxin management strategies, given that different species within the complex differ in fungicide sensitivity and aggressiveness to wheat and maize (Spolti et al. 2012b; Nicolli et al. 2018; Machado et al. *unpublished*, Mendes et al. 2018).

5.5 TABLES

Table 5.1. Description of the *Fusarium* isolates used in this study.

Species	Strain ID	Host	Year	Location City	Location State/ Country	GenBank Accession	Reference
Fgra	197	wheat	2010	Coxilha	RS	TBD	This Dissertation
Fgra	07	wheat	2010	Estação	RS	TBD	This Dissertation
Fgra	199	wheat	2011	Ijuí	RS	TBD	This Dissertation
Fgra	15	maize	2011	Bom Jesus	RS	TBD	This Dissertation
Fgra	205	maize	2011	Vacaria	RS	TBD	This Dissertation
Fgra	207	maize	2011	Bom Jesus	RS	TBD	This Dissertation
Fmer	02	wheat	2009	Coxilha	RS	TBD	This Dissertation
Fmer	05	wheat	2011	Sertão	RS	TBD	This Dissertation
Fmer	198	wheat	2010	Água Santa	RS	TBD	This Dissertation
Fmer	08	wheat	2009	Santa Bárbara do Sul	RS	TBD	This Dissertation
Fmer	09	maize	2011	Marechal Cândido Rondon	PR	TBD	This Dissertation
Fmer	10	maize	2011	Ponta Grossa	PR	TBD	This Dissertation
Fmer	17	maize	2011	Paranapanema	SP	TBD	This Dissertation
Fmer	200	maize	2011	Ponta Grossa	PR	TBD	This Dissertation
Fmer	204	maize	2011	Palmeira	PR	TBD	This Dissertation
Fmer	NRRL 28721	maize		NA	Nepal	GCA_001717825.1	Walkowiak et al. 2016
Fmer	NRRL 28723	maize		NA	Nepal	GCA_001717855.1	Walkowiak et al. 2016
Fgra	Gz3639	wheat		NA	Kansas, USA	TBD	This Dissertation
Fgra	PH1	maize		NA	Michigan, USA	GCA_900044135.1	King et al. 2015

[a] For Brazilian isolates, species and trichothecene genotype identified using the multilocus genotype method (Ward et al. 2008). Fgra = *F. graminearum* with 15-acetyl-deoxynivalenol genotype, Fmer = *F. meridionale* with nivalenol genotype.

[b] Brazilian isolates were obtained either from maize kernels (Kuhnem et al. 2016) or symptomatic wheat heads (Del Ponte et al. 2015) in previous surveys.

[c] Brazilian states: RS = Rio Grande do Sul, PR = Paraná, SP = São Paulo.

Table 5.2. Genome and assembly statistics of *Fusarium* Isolates used in this study.

Strain ID	Species	Host	Genome Size (Mb)	N50 (Mb)	Reference
197	Fgra	wheat	37.0	1.86	This study
07	Fgra	wheat	42.1	0.05	This study
199	Fgra	wheat	36.4	0.61	This study
15	Fgra	maize	36.8	0.21	This study
205	Fgra	maize	43.2	0.78	This study
207	Fgra	maize	36.5	1.24	This study
02	Fmer	wheat	36.9	1.64	This study
05	Fmer	wheat	43.1	0.15	This study
198	Fmer	wheat	36.7	0.76	This study
08	Fmer	wheat	36.8	1.40	This study
09	Fmer	maize	37.2	0.23	This study
10	Fmer	maize	36.8	1.74	This study
17	Fmer	maize	36.8	1.72	This study
200	Fmer	maize	36.8	1.08	This study
204	Fmer	maize	36.8	1.26	This study
NRRL 28721	Fmer	maize	36.5	0.11	Walkowiak et al. 2016
NRRL 28723	Fmer	maize	36.4	0.41	Walkowiak et al. 2016
Gz3639	Fgra	wheat	36.5	1.12	This study
PH1	Fgra	maize	38.1	9.39	King et al. 2015

Table 5.3. Pairwise distances (number of differences per kilobase) calculated from analysis of pairwise BLAST alignments between repeat-masked genomes.

	NRRL 28723	**10**	**207**	**NRRL 28721**	**197**	**15**	**5**	**17**	**200**	**198**	**2**	**9**	**199**	**8**	**Gz3639**	**PH1**	**205**	**204**
NRRL28723																		
10	1770																	
207	18418	18756																
NRRL28721	2175	3116	19764															
197	18425	18763	1804	19771														
15	18427	18766	2180	19773	2188													
5	3179	3517	18532	4525	18539	18541												
17	1823	1617	18809	3169	18816	18819	3570											
200	1826	1620	18812	3172	18819	18822	3573	1570										
198	1848	1815	18834	3194	18841	18844	3595	1868	1871									
2	1759	1552	18745	3105	18752	18754	3506	1502	1329	1804								
9	3216	3554	18569	4562	18576	18578	37	3607	3611	3633	3543							
199	18396	18734	2149	19742	2156	1865	18510	18787	18790	18812	18723	18547						
8	1800	1767	18786	3146	18794	18796	3548	1820	1823	1802	1756	3585	18764					
Gz3639	18466	18805	4666	19813	4673	4675	18581	18858	18861	18883	18794	18618	4643	18835				
PH1	18433	18771	4632	19779	4639	4641	18547	18824	18827	18849	18760	18584	4610	18801	2934			
205	18567	18906	2540	19914	2548	2550	18681	18959	18962	18984	18894	18719	2518	18936	4815	4781		
204	1910	1943	18896	3256	18904	18906	3657	1996	1999	2021	1932	3695	18874	1974	18945	18911	19046	
7	17201	17540	1962	18548	1969	1971	17315	17593	17596	17618	17528	17353	1940	17570	3449	3415	2111	17680

Table 5.4. List of Cleaved Amplified Polymorphic Sequences (CAPS) markers used to confirm the introgressed region of Chromosome 2 in PH1 and Gz3639 progenies.

Marker	Restriction Enzyme	Primer sequence (5' -> 3')	Annealing temperature	Fragment size	Restriction site	Reference
CAPS_*Hind*III	*Hind*III	ATCTCGGCACCTTTTTCCTT TGAACGAGGGCTAGCAACTT	57°C	232	6444819	This study
CAPS2_*Pst*I	*Pst*I	GCTTGAGAAACCACTGGCAA CCTGTGATGAATGCGACCAG	62°C	542	6576167	This study
CAPS2_*Xho*I	*Xho*I	TTCCCTGCGAACTCTCAAGT TTGTTGGAGCTGATGCTCAC	62°C	207	6693819	This study
CAPS_*Xho*I	*Xho*I	AAGCATGATGTTTGGCGCAT AAGCATGATGTTTGGCGCAT	58.8°C	661	6774043	This study
CAPS2_*Hind*III	*Hind*III	TAGTCAGTCGCCTCACATCC CGGATCTGTCTCACACTCGA	62°C	528	6883759	This study

Table 5.5. Segregation analysis of Cleaved Amplified Polymorphic Sequences (CAPS) markers used to validate a potential recombination region in Chromosome 2 in the high pool progreny from a cross between PH1 and Gz3639 strains.

Progeny	Markers[a]				
	CAPS_*Hind*III	CAPS2_*Pst*I	CAPS2_*Xho*I	CAPS_*Xho*I	CAPS2_*Hind*III
High Pool					
1517	-	-	-	-	-
1624	+	+	+	+	+
1122	+	+	+	+	+
1621	-	-	-	-	-
1601	-	-	-	-	-
1602	+	-	-	-	-
1607	-	-	-	-	-
1622	+	+	+	+	+
1220	-	-	-	-	-
1614	-	-	-	-	+
PH1:Gz3639	4:6	3:7	3:7	3:7	4:6

[a] (+): region from PH1 parent and (-) from Gz3639 parent.

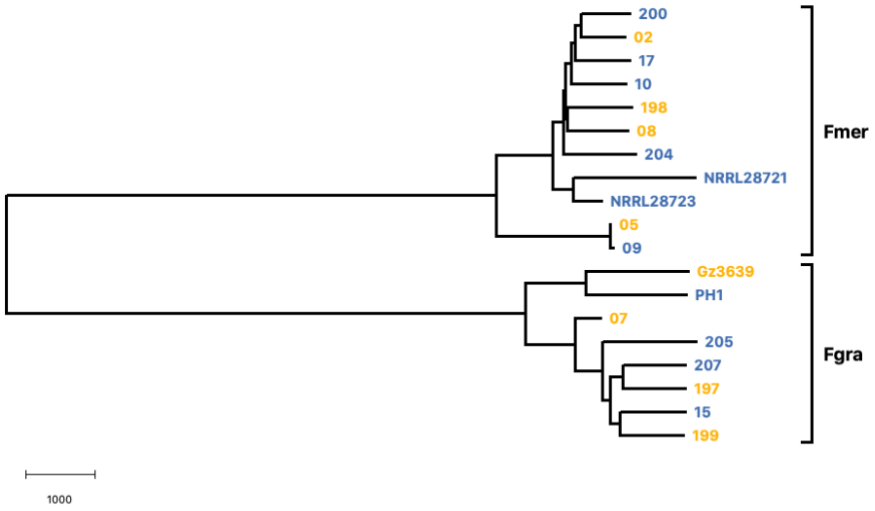

Figure 5.1. Neighbor-joining distance tree using pairwise distances (number of differences per kilobase) calculated from analysis of pairwise BLAST alignments between repeat-masked genomes of *Fusarium graminearum* (Fgra) and *F. meridionale* (Fmer) isolates. Isolates obtained from maize kernels are shown in blue whereas isolates obtained from symptomatic wheat heads are presented in yellow.

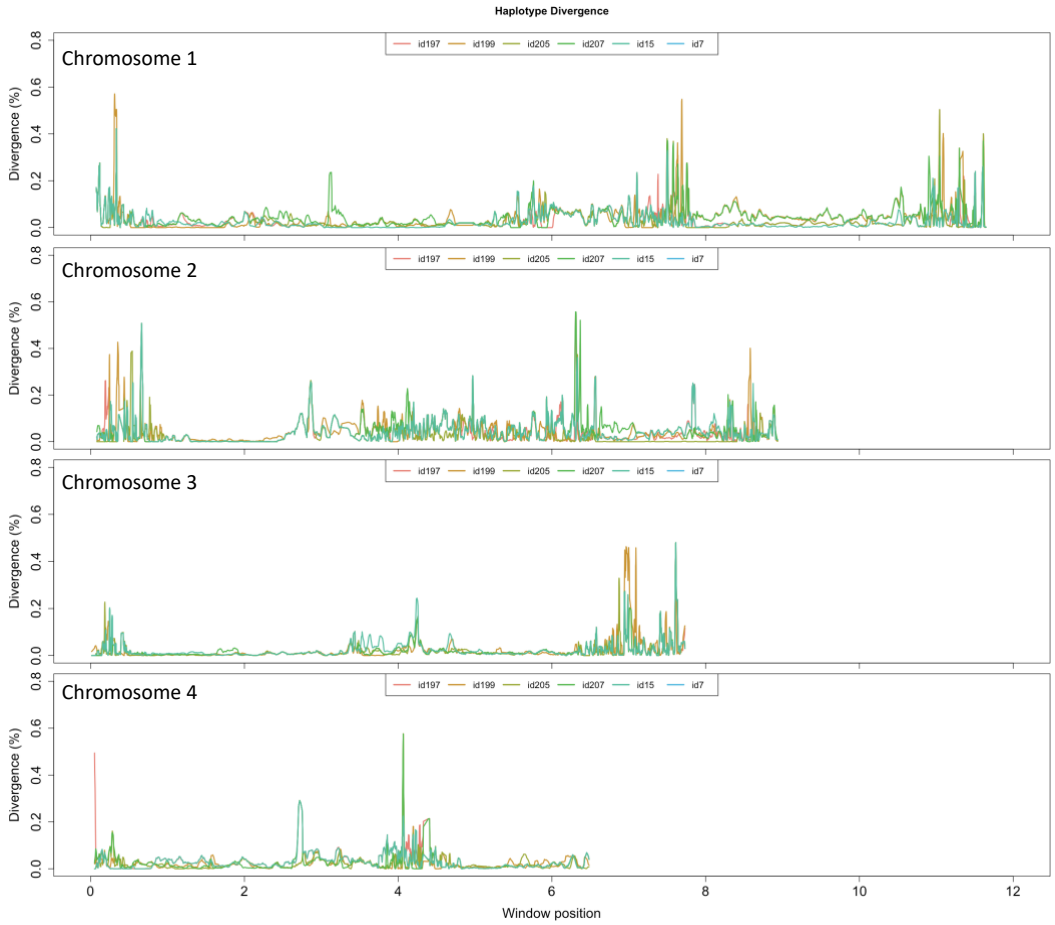

Figure 5.2. Haplotype divergence across the genome of six *Fusarium graminearum* Brazilian strains. Strain id. 197 was compared with all of the other strains to make this plot. The patterns of polymorphism were similar no matter which strain was used for the comparison. Plot lines were color-coded to identify individual strains as described in Table 1.

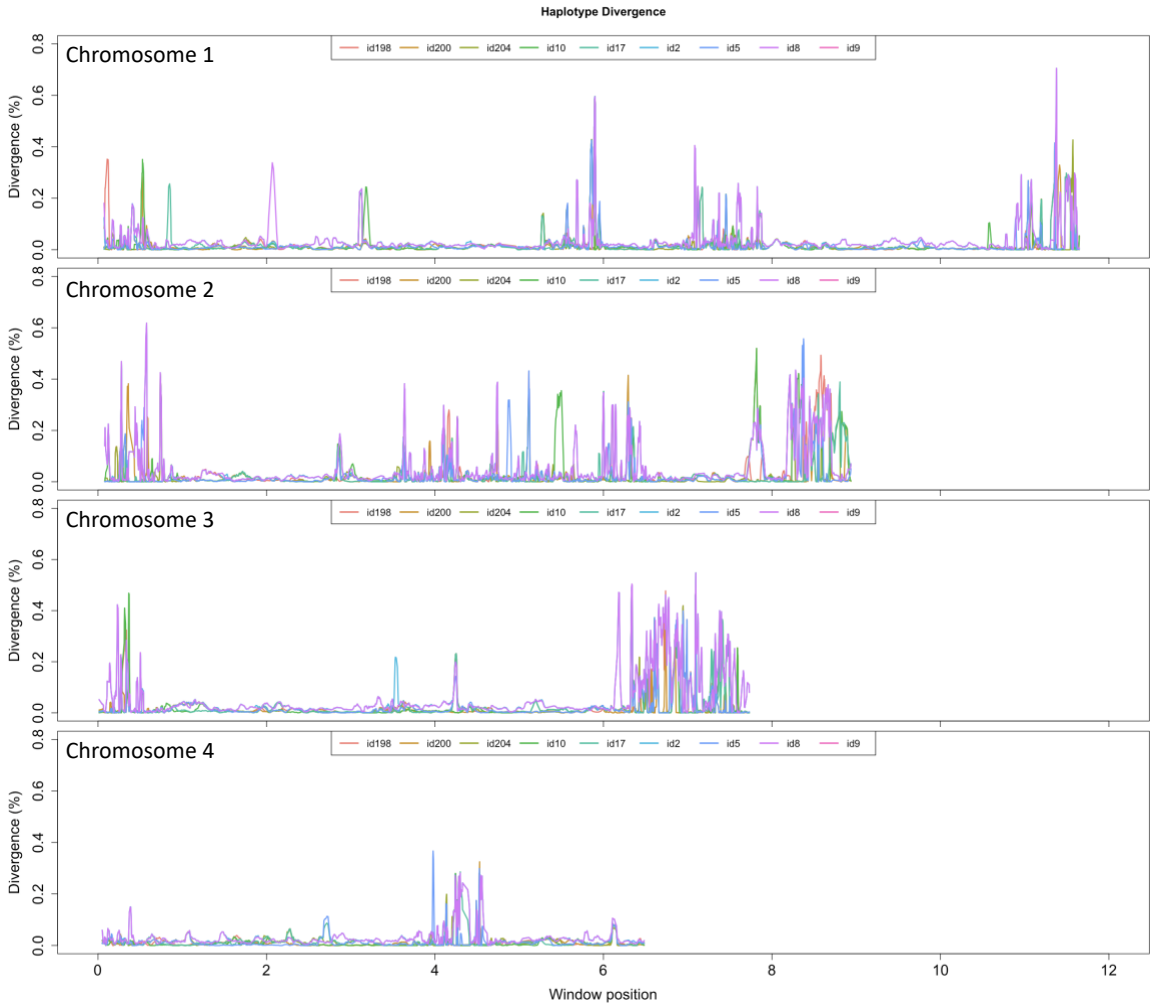

Figure 5.3. Haplotype divergence across the genome of nine *Fusarium meridionale* Brazilian strains. Strain id. 2 was compared with all of the other strains to make this plot. The patterns of polymorphism were similar no matter which strain was used for the comparison. Plot lines were color-coded to identify individual strains as described in Table 1.

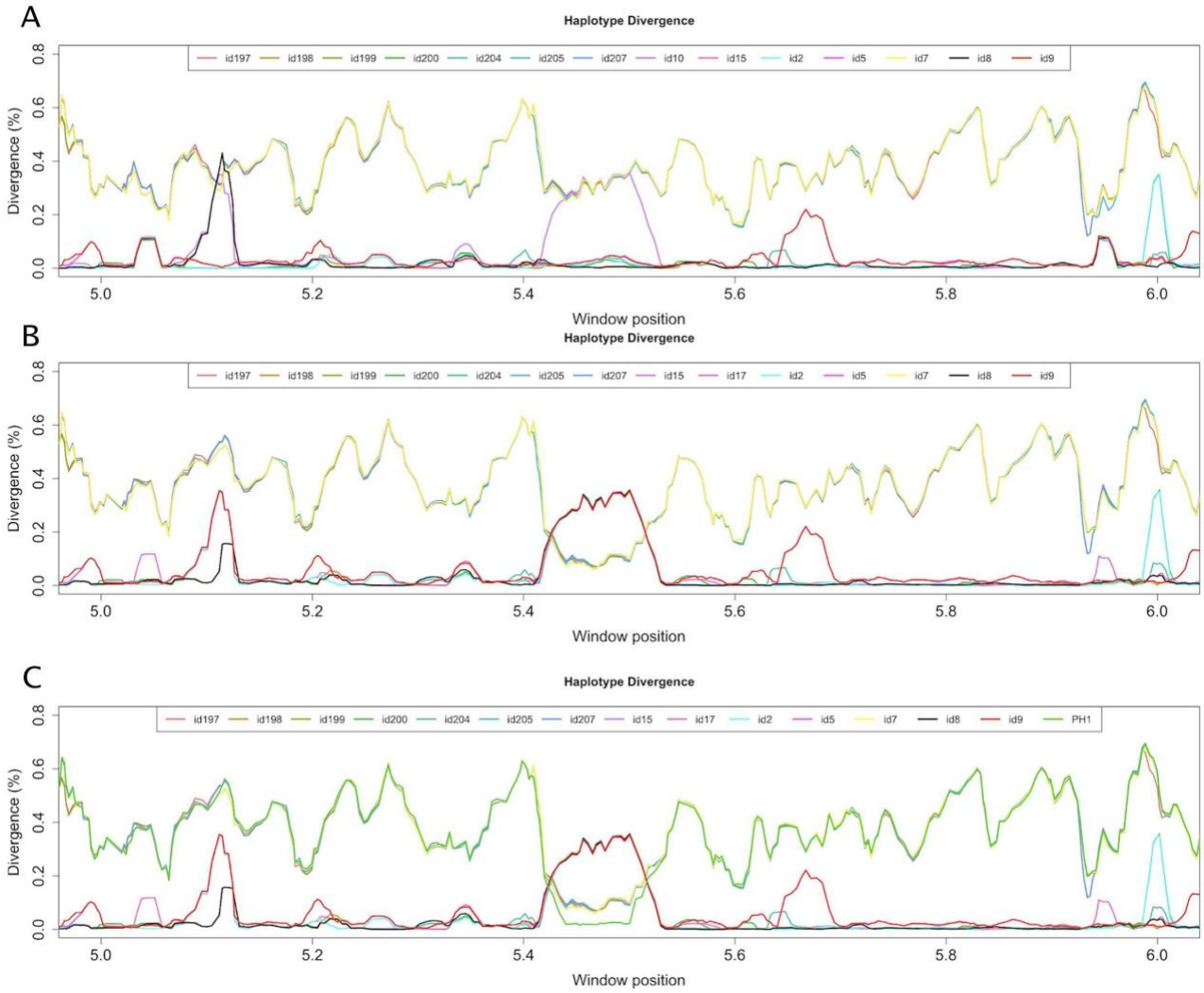

Figure 5.4. Haplotype divergence across the Chromosome 2 of a *Fusarium meridionale* Brazilian strain (id. 10) showing a potential introgressed region from approximately positions 5.4 to 5.55 Mb. Plot lines were color-coded to indicate to six *F. graminearum* (ids. 197, 199, 205, 207, 15, 7) and nine *F. meridionale* strains (ids. 198, 200, 204, 10, 17, 2, 5, 8, 9). In Plot A, strain id. 17 has been compared with all the other Brazilian strains and we can clearly see the introgression as a purple line, where strain id. 10 matches the *F. graminearum* strains. In Plot B, strain id. 10 has been used for the comparison. The region of low similarity to the other *F. meridionale* strains, and increased similarity with *F. graminearum* strains, is clearly visible. In Plot C, the North American Strain PH-1 has been added, and we can see that *F. meridionale* id. 10 is more similar to PH-1 in this region than it is to the Brazilian strains of *F. graminearum*.

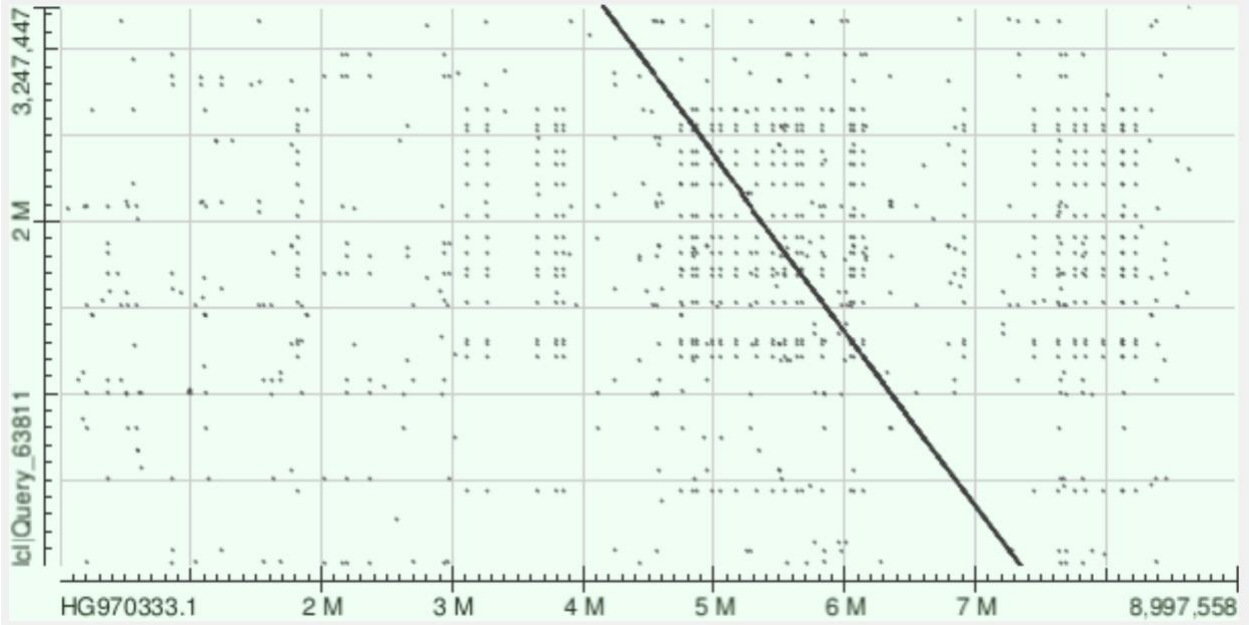

Figure 5.5. Blast reports of *F. meridionale* strain (id.10) contig 111 against the PH1 reference genome. The introgression region runs from about 5.4 Mb through 5.6 Mb. Note the presence of numerous repetitive sequences in this area, beginning at approximately 4.5 M and ending at around 6.2 Mb. The TRI cluster is located at approximately 5.4 Mb.

Figure 5.6. Alignments of *F. meridionale* strain 10 (A) and 17 (B) with *F. graminearum* strain PH-1 (Query) in the region of the introgression block (5.5 Mb to 5.6 Mb).

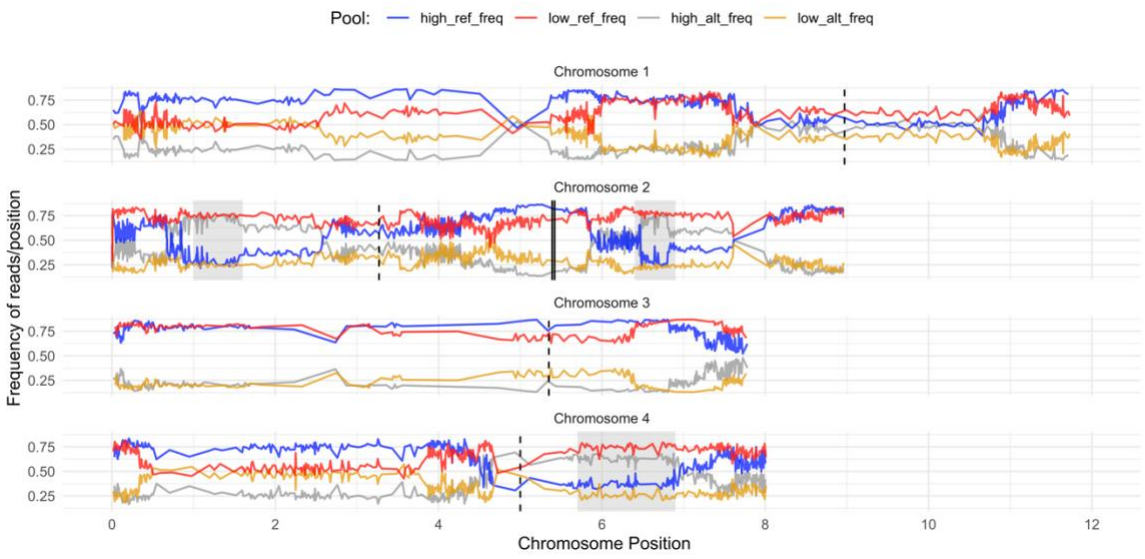

Figure 5.7. Bulk segregant analysis of SNP marker association with aggressiveness using two progeny pools, one consisting of the ten most aggressive strains, and one of the ten least aggressive. Regions highlighted by the gray boxes are potential introgressed regions in Chromosome 2 and Chromosome 4. Vertical black lines in Chromosome 2 represent the position of the TRI cluster in PH1. Dashed lines represent the centromere region in each chromosome.

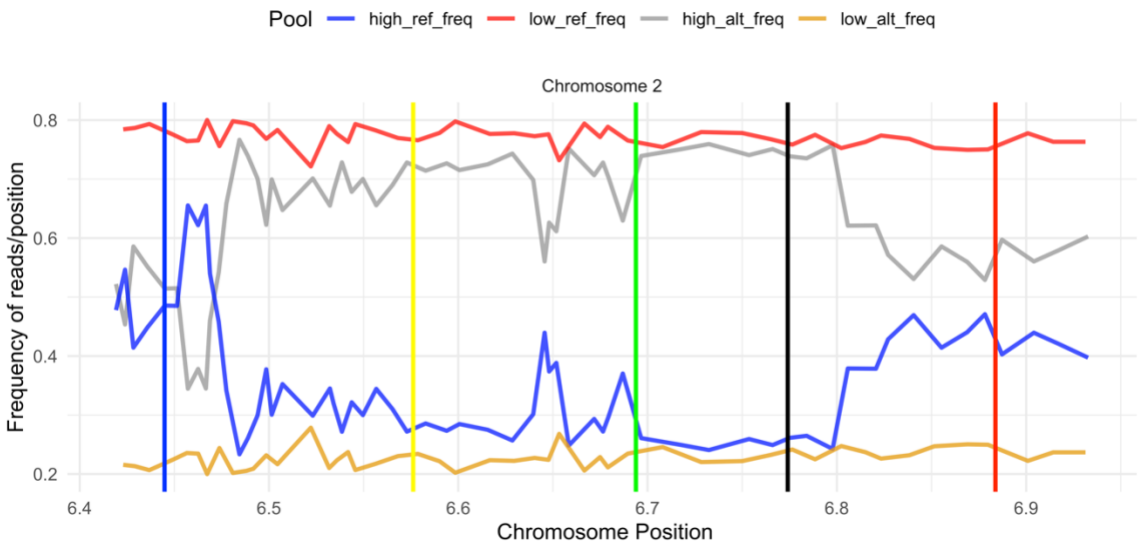

Figure 5.8. Position of Cleaved Amplified Polymorphic Sequences (CAPS) markers used to validate a potential recombination region in Chromosome 2. Vertical lines represent the position for CAPS markers. Blue: CAPS_*Hind*III; Yellow: CAPS2_*Pst*I; Green: CAPS2_*Xho*I; Black: CAPS_*Xho*I; Red: CAPS2_*Hind*III.

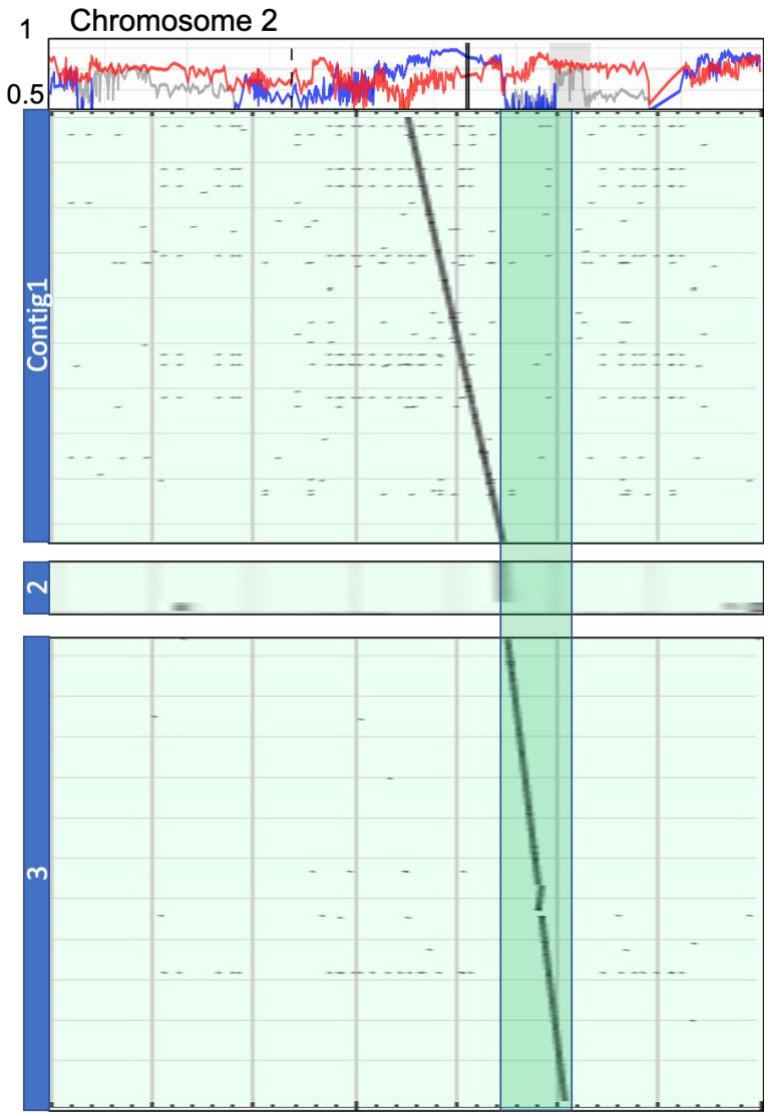

Figure 5.9. Blast reports of Gz3639 contigs against PH1 reference genome. Dashed line represents the centromere, and double line is the TRI cluster.

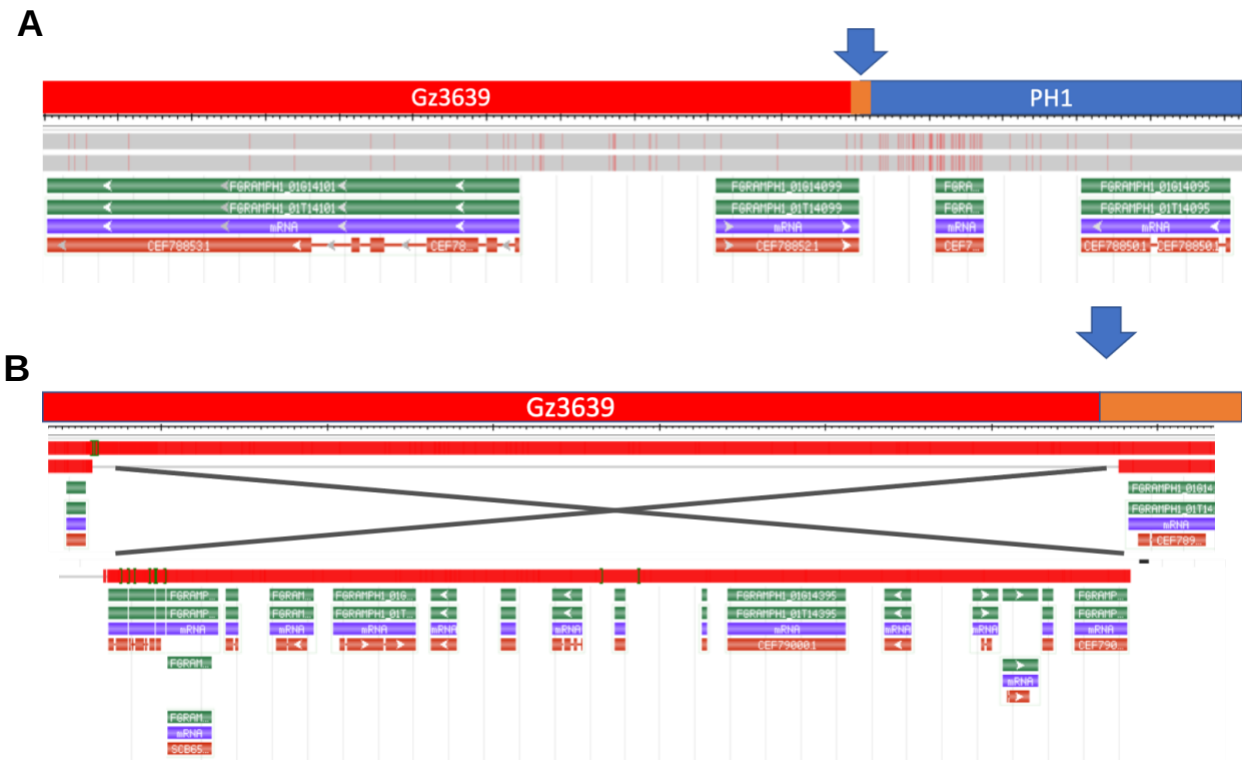

Figure 5.10. Location of the first (**A**) and second crossover point (**B**) in Chromosome 2 obtained from a cross between PH1 and Gz3639 strains. Arrows indicate the approximate crossover points.

CHAPTER 6. CONCLUDING REMARKS

This dissertation describes my work as a Dual Degree Doctoral/PhD student at the Universidade Federal de Viçosa and the University of Kentucky. I used a combination of epidemiological and genetic approaches to address the question of dominance in Brazil of *F. meridionale* on maize, and of *F. graminearum* on wheat. I began with a hypothesis that host dominance was related to relative aggressiveness of the two strains on wheat versus maize. Previous studies from the Del Ponte group and others had already provided evidence that *F. graminearum* was more aggressive than *F. meridionale* and other members of the FGSC on wheat, but similar studies on maize had not been done. The results of the field studies I reported in my second and third chapters supported my hypothesis, demonstrating that, on average, *F. meridionale* is about twice as aggressive, and more competitive on maize ears than *F. graminearum*. The B-trichothecene mycotoxin NIV was produced by *F. meridionale* strains inoculated onto maize ears, which was a very important observation since NIV is highly toxic but not yet regulated in maize food or feed products in Brazil. My studies show that it is very important to change that.

In my study, I included a very large number of individual strains of both species, and I also evaluated them individually. It is more common in field pathogenicity experiments to look at relatively few strains, or to pool them, as I did in the first chapter of my dissertation. Although results in both chapters supported the hypothesis, showing that on average *F. meridionale* was more aggressive than *F. graminearum*, the results in Chapter 3 revealed that there was a lot of intraspecies variation in aggressiveness, and that the ranges actually overlapped. That suggests that aggressiveness is not the only factor that is important in determining species and population structure in wheat and maize in Brazil.

I set out to explore additional potentially important factors in Chapter 4, in which I applied a multivariate PCA analysis based on characterization of a variety of other pathogenicity and fitness-related phenotypes. I showed that *F. graminearum* and *F. meridionale* could be differentiated statistically, based on a combination of these traits. The most important for differentiating the two species were reproductive fitness, growth rate in culture and on maize silks, and aggressiveness to wheat. The primary factor associated with dominance of *F. meridionale* in maize was its faster vegetative growth rate. It is possible that this provides *F. meridionale* with a competitive advantage in maize when both species are present, as implied by my results on maize ears reported in Chapter 2. In the future, it will be very important to do more co-inoculation studies on a broader range of host tissues to study this question of competitive advantage in more detail. My results confirmed previous reports that *F. graminearum* is more aggressive on wheat, and more fit regarding its sexual or asexual reproduction *in vitro*. Although the species identity was much more important in structuring the pathogen populations, I did find some evidence that the host (wheat versus maize) imposes selection pressures that also have some effect. For example, isolates of *F. meridionale* were more aggressive overall than isolates of *F. graminearum* on maize ears, but when only wheat strains were considered, the difference was not significant. This suggests the presence of differential selection due to host, either selection for more aggressive *F. meridionale* strains by maize, or of more aggressive *F. graminearum* strains by wheat, or a combination of the two. My evidence also suggests that wheat selects for higher numbers of ascospores and higher levels of mycotoxin. Given that aggressiveness to maize ears was correlated with levels of both DON and NIV in my study, populations

of *F. graminearum* in regions where both maize and wheat are grown might become more aggressive to maize in time, due to selection by the wheat for higher mycotoxin levels.

In my final research chapter, I undertook a genomic analysis of the *F. meridionale* and *F. graminearum* strains that provided further insights. The genome data were consistent with the phenotypic data, showing that the two species are divergent, and that the host (wheat versus maize) and local origin doesn't play a major role in structuring the populations of either species. Significantly, the genome data provided clear evidence for gene flow, both within and between species. One strain of *F. meridionale* had an introgression from *F. graminearum* that incorporated a portion of the TRI mycotoxin cluster. Remarkably, this strain was also one of only two *F. meridionale* strains that produced more DON than NIV in wheat heads, although this was insufficient to allow it to be an aggressive pathogen of wheat. This finding indicated that there are factors other than DON that are important for aggressiveness to wheat. Analysis of segregation patterns among progeny of a laboratory cross of *F. graminearum* strains supported this, as it revealed a novel block of genes on Chromosome 2 associated with high levels of aggressiveness that did not include the TRI cluster.

Genome analysis and bioinformatics were new for me and it was quite a challenge for me to learn and apply these methods, since my background is primarily in epidemiology. It is important to emphasize that the genetics and genomics analyses represent preliminary work, but the methods and findings here provide an important foundation for future studies focused on understanding the diversity and evolution of FGSC members and the potential role of the host and the crop environment in structuring pathogen populations.

Overall, my research findings have provided valuable new information regarding the importance of FGSC diversity on different hosts for both maize and wheat breeders. For the growers, my work implies that FGSC members have the potential to differently infect maize and wheat, increasing the risk of NIV contamination, and with important implications for the durability of management involving host resistance or fungicide treatments. The ability of *F. graminearum* and *F. meridionale* to infect both hosts increases the level of complexity of management strategies, and reiterates the risks of wheat-maize rotations.

I am grateful for the opportunity to be the first student enrolled in the Dual Degree program of UFV and UK. The advantages of this program go far beyond obtaining a Ph.D. degree simultaneously from both institutions. The opportunity to step out of my comfort zone changed everything for me, and I have had so many new and enriching experiences, both personally or professionally. As a student of the Dual Degree program, I enjoyed and benefited from the structure and complementary expertise of both research groups, as well as the types of academic training that each institution provided. The Dual Degree contributed significantly to the advancement of my research, learning and training, and I had the opportunity to develop my research using techniques that I would not have had access to in Brazil. It was a great opportunity for me to work with this international group of professionals who carry out important research on wheat and maize diseases. A particularly positive aspect of the program was the national and international meetings that I had the chance to attend, including meetings of the NC1183 mycotoxins committee, the USWBSI annual meeting, and the APS annual meeting, where I was able to interact with a broader group of Fusarium researchers from around the world. I was so grateful for these

opportunities to meet scientists whose work I had read so many times. I think that one of the biggest challenges of the program for me was to balance the classwork and the research, especially because I had only one year to fulfill the UK course and program requirements. Unfortunately, time is not a good friend of research, as science never goes as expected. People have asked me if it was twice the work, and I would have to say that it was maybe more. However, it was not impossible, especially with the support of so many kind people at both universities who were willing to help. This experience was very challenging for so many reasons, but as Dr. Vaillancourt likes to say, it is a win-win-win situation, and for me, it was definitely a big WIN!

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VITA

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EDUCATION

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ADDITIONAL EDUCATION

2019

Short Term Course: KBRIN 2019 Next Generation Sequencing Workshop (40h).
University of Kentucky, UK, Lexington, U.S.

2017

Short Term Course: Epi-fluorescence and confocal laser scanning microscopy (76h).
Universidade Federal de Lavras, UFLA, Lavras, Brazil

Short Term Course: Scientific Writing Workshop. (8h).
Universidade Federal de Viçosa, UFV, Vicosa, Brazil

Short Term Course: Tropical Fusarium Workshop 2017. (30h).
Universidade Federal de Viçosa, UFV, Vicosa, Brazil

2015

Short Term Course: Real time PCR. (8h).
Universidade Federal de Viçosa, UFV, Vicosa, Brazil

Short Term Course: Light microscopy technique and image obtaining. (4h).
Universidade Federal de Viçosa, UFV, Vicosa, Brazil

2014

Short Term Course: Simulation modeling in agricultural research. (20h).
Universidade Federal de Viçosa, UFV, Vicosa, Brazil

Short Term Course: Molecular Filogeny. (8h).
Universidade Federal de Viçosa, UFV, Vicosa, Brazil

2013

Short Term Course: Use of Autocad software in irrigation. (8h).
Universidade Federal de Viçosa, UFV, Vicosa, Brazil

2012

Short Term Course: Fotografy. (4h).
Universidade Federal de Viçosa, UFV, Vicosa, Brazil

2010

Short Term Course: Pre- and post-harvest pathology. (4h).
Universidade Federal de Viçosa, UFV, Vicosa, Brazil

PROFESSIONAL EXPERIENCE

Postdoctoral researcher, Fundo de Defesa da Citricultura-Fundecitrus, Araraquara, SP, Brazil. 2020-present.

PhD. candidate, University of Kentucky, Lexington, KY, USA. 2018-present.

Doctoral candidate, Universidade Federal de Viçosa, Department of Plant Pathology (March 2016-2020).

Master's student, Universidade Federal de Viçosa, Department of Plant Pathology (March 2014-February 2016).

Scientific Initiation Fellow, Universidade Federal de Viçosa, Department of Plant Pathology (July 2010-February 2014).

Graduate student, Universidade Federal de Viçosa, Department of Plant Pathology (March 2009, February 2014).

AWARDS

Votes of Praise given by the Center of Agricultural Sciences for having stood out among his peers during the course of Agronomy, Universidade Federal de Viçosa. **2014**

ORAL PRESENTATIONS AT CONFERENCES

1. Identification of markers associated with increased levels of aggressiveness and toxigenicity in transgressive progeny from a cross of two genetically and phenotypically similar *Fusarium graminearum* strains. 2018 National Fusarium Head Blight Forum. St. Louis, Missouri, March 12, 2019.

RESEARCH PUBLICATIONS

1. Duffeck, M.R., Alves, K.S., **Machado, F.J.**, Esker, P.D. and Del Ponte, E.M. 2020. Modeling yield losses and fungicide profitability for managing Fusarium head blight in Brazilian spring wheat. Phytopathology 110:370–378.
2. Moreira, G. M., **Machado, F. J**., Pereira, C. B., Neves, D. L., Tessmann, D. J., Ward, T. J. and Del Ponte, E. M. 2020. First report of the *Fusarium tricinctum* species complex causing Fusarium Head Blight of wheat in Brazil. Plant Disease 104:586.
3. Duarte, H. S. S.; Zambolim, L.; **Machado, F. J.**; Rodrigues, F. A.; Porto, H. P. 2019. Comparative epidemiology of late blight and early blight of potato under different environmental conditions and fungicide applications programs. Semina. Ciências Agrárias 40:1805-1818.
4. Nicolli, C. P., **Machado, F. J.**, Spolti, P. and Del Ponte. 2018. Fitness traits of deoxynivalenol and nivalenol-producing *Fusarium graminearum* species complex strains from wheat. Plant Disease 102:1341-1347.

5. **Machado, F. J.**, Nicolli, C. P., Möller, P. A., Arruda, R., Ward, T. J. and Del Ponte, E. M. 2017. Differential triazole sensitivity among members of the *Fusarium graminearum* species complex infecting barley grains in Brazil. Tropical Plant Pathology 42:197-202.
6. **Machado, F. J.**, Santana, F. M., Lau, D., and Del Ponte, E. M. 2017. Quantitative review of the effects of triazole and benzimidazole fungicides on Fusarium head blight and wheat yield in Brazil. Plant Disease 101:1633-1641.
7. Del Ponte, E. M., Pethybridge, S. J., Bock, C. H., Michereff, S. J., **Machado, F. J.**, and Spolti, P. 2017. Standard area diagrams for aiding severity estimation: scientometrics, pathosystems, and methodological trends in the last 25 years. Phytopathology 107:1161-1174.
8. **Machado, F. J.**, Möller, P. A., Nicolli, C. P., Del Ponte, E. M., and Ward, T. J. 2015. First report of *Fusarium graminearum*, *F. asiaticum,* and *F. cortaderiae* as head blight pathogens of annual ryegrass in Brazil. Plant Disease 99:1859.

BOOKS PUBLISHED

1. Colman, A. A.; Barros, A. V.; **Machado, F. J.**; Silva, M. A.; Caires, N. P.; Alves, P. S. Doenças em espécies florestais e fruteiras. Viçosa, 2015

PUBLISHED CONFERENCE PROCEEDINGS

Conference Proceedings (10)

Abstracts (17)

Franklin Jackson Machado