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USE OF HETEROTHALLIC MAT DELETION STRAINS OF *FUSARIUM GRAMINEARUM* AS TEST MATES IN CROSSES TO EVALUATE THE GENETICS OF PATHOGENICITY AND FITNESS

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USE OF HETEROTHALLIC MAT DELETION STRAINS OF *FUSARIUM GRAMINEARUM* AS TEST MATES IN CROSSES TO EVALUATE THE GENETICS OF PATHOGENICITY AND FITNESS

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Agriculture, Food and Environment
at the University of Kentucky

By
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Lexington, Kentucky
Director: Dr. Lisa Vaillancourt, Professor of Plant Pathology
2022

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ABSTRACT OF THESIS

USE OF HETEROTHALLIC MAT DELETION STRAINS OF *FUSARIUM GRAMINEARUM* AS TEST MATES IN CROSSES TO EVALUATE THE GENETICS OF PATHOGENICITY AND FITNESS

Fusarium Head Blight (FHB), caused by *Fusarium graminearum sensu stricto* and other members of the *F. graminearum* species complex (FGSC), is a plant disease that occurs on cereal crops worldwide. FHB causes yield losses not only by reducing grain weight, but also by contaminating the kernels with dangerous trichothecene mycotoxins, especially deoxynivalenol (DON). There is a high degree of genotypic and phenotypic variation among pathogen species and strains, but current FHB risk assessment models and treatments do not account for pathogen diversity. Therefore, it is difficult to predict what will happen if a new, potentially more aggressive variant is introduced, or if changes in the environment favor one genotype over another. *Fusarium graminearum* is homothallic, and self-fertility is regulated by the complex MAT1 locus that encodes two genes called *MAT1-1-1* and *MAT1-2-1*. Previous studies have demonstrated that deletion of either gene produces an obligately heterothallic strain that can only outcross with a strain of the opposite type. The goal of my thesis research was to screen a collection of independent *MAT1-1-1* and *MAT1-2-1* (MAT) deletion mutants to identify appropriate test maters that could be used to cross with wild type (WT)

strains and facilitate genetic analyses of traits of interest. Because the deletion strains engage only in heterothallic mating, it solves the problem of identifying outcrossed perithecia. The ideal mating tester strain should be phenotypically similar to the WT in pathogenicity and toxigenicity, and should also grow normally in culture, be highly female-fertile, and produce abundant ascospore progeny that exhibit normal marker segregation patterns. Many of the deletion strains, especially the *MAT1-2-1* deletions, were significantly less pathogenic and fit compared with their WT progenitor strain PH-1. Strains also varied widely in female fertility and levels of interfertility with other mutant and WT strains. Two highly female-fertile *MAT1-1-1* deletion strains that had WT levels of pathogenicity, toxigenicity, and fitness were used in test crosses with several other strains. These included a *MAT1-2-1* deletion strain with reduced fitness and pathogenicity, and several WT strains including PH-1, another strain of *F. graminearum ss.* (Gz3639), and *F. meridionale*, another member of the FGSC that can cause FHB. Antibiotic resistance, MAT alleles, chemotypes, CAPs markers, and fertility all had expected 1-1 segregation patterns in the crosses and expected linkage relationships. These mating tester strains can be used in the future to identify novel genetic markers associated with fitness and pathogenicity that could be incorporated into multi-locus genotyping assays to monitor and predict population shifts.

KEYWORDS:.

Fusarium Head Blight, Wheat Scab, Mycotoxin

Gabdiel Emmanuel Yulfo-Soto

June 14th 2022

Date

USE OF HETEROTHALLIC MAT DELETION STRAINS OF *FUSARIUM GRAMINEARUM* AS TEST MATES IN CROSSES TO EVALUATE THE GENETICS OF PATHOGENICITY AND FITNESS

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August 4th, 2022
Date

DEDICATION

To all those who supported me all these years.

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CHAPTER 1. INTRODUCTION AND BACKGROUND

1.1 *Fusarium graminearum*, a Fungal Pathogen of Cereals.

Fusarium graminearum Schwabe causes important diseases of cereal crops worldwide. Fusarium Head Blight (FHB) of wheat and other small grains, and Gibberella Ear Rot (GER) of maize, are the most economically important (Goswami & Kistler, 2004; Kazan, Gardiner, & Manners, 2012; McMullen, Jones, & Gallenberg, 1997; Trail, 2009). *Fusarium graminearum* causes yield loss by reducing grain weight and by contaminating the grain with dangerous mycotoxins (Chen, Kistler, & Ma, 2019; A. Desjardins & Proctor, 2007; Munkvold, 2017).

Fusarium graminearum produces several different mycotoxins including B-trichothecenes nivalenol (NIV); deoxynivalenol (DON); and acetylated DON derivatives 3ADON and 15ADON: the A-trichothecene NX-2: and mycoestrogens including zearalenone (ZEA) (Chen et al., 2019; A. Desjardins & Proctor, 2007; A. E. Desjardins, 2006; A. Kelly et al., 2016; Liang et al., 2014; Munkvold, 2017; E. Varga et al., 2015). DON is the main mycotoxin produced by most FHB isolates (Aoki, Ward, Kistler, & O'donnell, 2012; Przemieniecki, Kurowski, & Korzekwa, 2014; van der Lee, Zhang, van Diepeningen, & Waalwijk, 2015). DON is a virulence factor for FHB in wheat, promoting movement of the pathogen through the rachis and bleaching of the wheat head (A. E. Desjardins et al., 1996). DON deletion mutants caused disease in inoculated spikelets, but they did not spread in wheat heads (G-H Bai, Desjardins, & Plattner, 2002; Jansen et al., 2005; Proctor, Hohn, & McCormick, 1995). They were also less pathogenic to maize ears (Harris et al., 1999). DON-contaminated grain causes poor weight gain and gastrointestinal problems in animals. In humans DON can cause nausea, vomiting, convulsions, anorexia, cytotoxicity, or abnormalities in immune system function (Berek, Petri, Mesterhazy, Téren, & Molnár, 2001; Pestka, 2010; Sudakin, 2003; Wu, Groopman, & Pestka, 2014). The amount of DON in wheat products for human consumption cannot be more than 1 ppm (U.S. Food and Drug Administration, 2010).

1.2 Fusarium Head Blight of Wheat and Small Grains: Symptoms and Disease Cycle.

The most noticeable symptom of FHB in wheat is bleaching (loss of the normal green color) of the spikes during and after flowering (Wiese, 1978). The bleaching can begin on any spikelet, and progress both up and down the spike. In awned wheat varieties, the awns of bleached spikelets fan out horizontally, in contrast to the vertical awns of healthy wheat heads. Infected grains are often shriveled and discolored, and weigh less than normal (Atanasov, 1920). Symptomatic grains are called “tombstones”.

Under favorable conditions (20-30°C and ≥ 90% humidity), signs in the form of orange-pink sporulation can be seen on bleached spikelets (Ayers, Pennypacker, Nelson, & Pennypacker, 1975; De Wolf, Madden, & Lipps, 2003; Wiese, 1978). Blue-black perithecia can also be found on the dead spike tissues and on host debris. The melanized perithecia survive the winter on the plant residue (Fernandez, Huber, Basnyat, & Zentner, 2008; Pereyra & Dill-Macky, 2008; Sutton, 1982) and produce ascospores that serve as primary inoculum in the spring (Parry & Jenkinson, 1995; Shaner, 2003; Yuen & Schoneweis, 2007) (**Figure 1.1**). Ascospores are forcibly ejected from the perithecia and can be carried long distances through the air (W. G. Fernando, Miller, Seaman, Seifert, & Paulitz, 2000; Maldonado-Ramirez, Schmale III, Shields, & Bergstrom, 2005). The spores infect and colonize the flowers after they land on wheat heads during anthesis (Adams, 1921; Andersen, 1948; Arthur, 1891; Atanasov, 1920; McKay & Loughnane, 1945; Pugh, Johann, & Dickson, 1933; Takegami, 1957) (**Figure 1.1**). Macroconidia are eventually produced on the diseased tissues, and this secondary inoculum is dispersed by wind or splashing water (Atanasov, 1920).

Fusarium graminearum can also infect flowers of other hosts including hemp (Yulfo-Soto et al., 2022) and maize (Christensen & Wilcoxson, 1966; Kruger, 1976; Mortimore & Gates, 1969; Shurtleff, 1980). Spores that land on maize silks can germinate and grow down the silk channels to the rachis (cob) to establish an

ear infection, reducing yields and causing mycotoxin contamination of the grain (Hesseltine & Bothast, 1977; Koehler, 1942) (**Figure 1.1**). After harvest, perithecia can survive on maize stubble left in the field (Burgess & Griffin, 1968; Gordon, 1952, 1959; Hoffer, Johnson, & Atanasoff, 1918; Shurtleff, 1980; Warren & Kommedahl, 1973). Infected maize is a major source of spores for infection of wheat in North America (Dill-Macky & Jones, 2000; Keller, Waxman, Bergstrom, & Schmale III, 2010; Kuhnem, Del Ponte, Dong, & Bergstrom, 2015; Maldonado-Ramirez et al., 2005; Schmale III, Bergstrom, & Shields, 2006; Schmale III, Leslie, et al., 2006; Schmale III, Shah, & Bergstrom, 2005).

1.3 Disease Modeling and Risk Assessment

Several major epidemics of FHB in the U.S. cost wheat growers around $3 billion during the 1990s (McMullen et al., 1997; Nganje, Kaitibie, Wilson, Leistritz, & Bangsund, 2004; Windels, 2000). Since then, FHB has received more research attention and this has resulted in the development of improved disease forecasting models, better fungicides and application technologies, and new sources of resistance in wheat and barley (W. D. Fernando, Oghenekaro, Tucker, & Badea, 2021; McMullen et al., 2012) (McMullen et al., 2012; Torres et al., 2019). Despite these advancements, management of FHB, especially mycotoxin contamination of grain, remains challenging. Mycotoxin levels are not always correlated with disease symptoms, and disease severity and toxin accumulation depend on many factors related to the host genotype, biotic and abiotic micro- and macroenvironments, and disease management protocols (Gui-Hua Bai & Shaner, 1996; Carter, Rezanoor, Desjardins, & Nicholson, 2000; Gale et al., 2011; Guo, Fernando, & Seow-Brock, 2008; Machado, 2020; C. Mirocha, Abbas, Windels, & Xie, 1989; Nicolli, Machado, Spolti, & Del Ponte, 2018; Firas Talas & Bruce A McDonald, 2015). We know that the fungi causing FHB in North America are genetically diverse, but our understanding of the role of pathogen variation in disease outcomes is still very limited. All these factors contribute to the unpredictability of disease and toxin levels from year to year. More data on the

roles and interactions of all these variables could help to improve our ability to predict the impact of FHB epidemics.

The disease triangle tells us that there are three components that interact to influence disease outcomes: host genotype, including the degree of genetic resistance; environmental factors, including both biotic and abiotic components; and pathogen genotype, including aspects related to the degree of virulence and fitness (**Figure 1.2**). This information can be used to assess risk of losses from FHB. The Fusarium Head Blight Prediction Center Fusarium Risk Tool (U.S. Wheat and Barley Scab Initiative) uses geographic location, weather forecast, crop type (including degree of resistance) and crop growth stage to assess regional FHB risk. Another tool, known as FusaProg (Musa, Hecker, Vogelgsang, & Forrer, 2007), assesses risk of DON contamination based on a variety of factors including previous crops, soil and debris management, host resistance, weather conditions and growth stage (W. D. Fernando et al., 2021). These tools are based on data from historical records of associations between host and environmental factors and disease and mycotoxin severity (De Wolf et al., 2003). Historical long-term climate data sets and climate driven crop disease models have also been used successfully to predict low and high FHB risk years in Brazil (Del Ponte, Fernandes, Pavan, & Baethgen, 2009). There is evidence that pathogen genotype also has a significant influence on FHB disease outcome: segregating populations produced from a cross of two similar strains across three different environments had high heritability estimates for aggressiveness and DON production (0.5-0.7), suggesting a major role for pathogen genotype and strong potential for selection and adaptation (Cumagun & Miedaner, 2004). Interactions between pathogen progeny strains and environment explained 30% of the variation in aggressiveness, and 20% of the variation in levels of DON (Cumagun & Miedaner, 2004). Risk assessment tools do not currently consider pathogen genotype, mainly because we have relatively little historical association data for this factor. Generating such data will require better tools for tracking pathogenicity factors among fungal populations.

1.4 Pathogen Diversity and Distribution

In North America, FHB and GER are both caused mostly by *F. graminearum* sensu stricto (ss.), which is divided into at least three subpopulations (NA1, NA2, and NA3) (A. C. Kelly et al., 2015; A. C. Kelly & Ward, 2018; van der Lee et al., 2015). The most common and diverse subpopulation, NA1, produces mainly 15ADON. The NA2 population, which produces primarily 3ADON, is smaller than NA1 but has been expanding its range through the Eastern U.S. and Canada. The NA3 population was identified recently in the upper Midwest and Canada and includes primarily strains with the NX-2 chemotype (A. C. Kelly et al., 2015; A. C. Kelly & Ward, 2018; Liang et al., 2014). In North America, FHB can also be caused more rarely by *F. graminearum* ss. with a NIV chemotype, by other FSASC phylospecies including *F. asiaticum*, *F. gerlachii*, *F. louisianense*, and *F. boothii* (Anderson et al., 2020; Gale et al., 2011), and even by members of other species complexes (Berek et al., 2001). Population genetics studies of *F.* graminearum has shown that the different members of the North American population can also outcross with each other, producing recombinant lines (A. C. Kelly & Ward, 2018). There is evidence that adaptive selection related to host preference and physical or biotic environmental factors is a major factor driving shifts in dominance among populations of *F. graminearum* causing FHB (Anderson et al., 2020; A. C. Kelly & Ward, 2018; J. Lee, Kim, et al., 2012; Qu et al., 2008; Spolti, Del Ponte, Dong, Cummings, & Bergstrom, 2014; Suga et al., 2008; Valverde-Bogantes et al., 2020; Ward et al., 2008; Xu et al., 2021).

At least sixteen different phylogenetic species (phylospecies) in the *F. graminearum* species complex (recently renamed as the *F. sambucinum* species complex, FSASC) (Laraba, McCormick, Vaughan, Geiser, & O'Donnell, 2021) can cause FHB (Aoki et al., 2012; Del Ponte et al., 2022; O'Donnell, Kistler, Tacke, & Casper, 2000; O'Donnell et al., 2008; O'Donnell, Ward, Geiser, Kistler, & Aoki, 2004; Sarver et al., 2011; Starkey et al., 2007; Valverde-Bogantes et al., 2020; van der Lee et al., 2015; Yli-Mattila et al., 2009). The phylospecies can be identified by sequencing a portion of the translation elongation factor 1 gene (TEF1), or by using

a multilocus genotyping (MLGT) PCR assay involving probes that detect single nucleotide polymorphisms (SNPs) in six different loci, including the trichothecene biosynthetic genes (*TRI* genes) which can predict mycotoxin profiles, aka chemotypes (Alexander, McCormick, Waalwijk, van der Lee, & Proctor, 2011; Bec et al., 2015; T. Lee, Han, Kim, Yun, & Lee, 2002; E. Varga et al., 2015). The MLGT assay has been used to determine species and chemotype of FSASC collections in multiple studies worldwide (Aoki et al., 2012; Del Ponte et al., 2022). Phylogenetic species have also been shown to interbreed and produce recombinants (O'Donnell et al., 2000).

1.5 Genetics of Pathogenicity and Virulence in *F. graminearum*

Fusarium graminearum is homothallic, but it crosses efficiently in the laboratory (R. L. Bowden & Leslie, 1998) and there is also evidence that it outcrosses frequently in the field (Talas, 2016; F. Talas & B. A. McDonald, 2015) (A. C. Kelly & Ward, 2018). Different FSASC phylospecies are also capable of interbreeding, and natural hybrids have been reported (Boutigny et al., 2011; O'Donnell et al., 2000). Wild grasses and other hosts are sources of additional strains and phylospecies that can contribute genetic diversity to the FHB population via recombination (Michael R Fulcher, Winans, Benscher, Sorrells, & Bergstrom, 2021; M. R. Fulcher, Winans, Quan, Oladipo, & Bergstrom, 2019; Gale et al., 2011; Lofgren et al., 2018; Sarver et al., 2011; Wegulo, Baenziger, Nopsa, Bockus, & Hallen-Adams, 2015). Some alleles will confer adaptive advantages, while others may reduce fitness in different environments. Aggressiveness, fungicide sensitivity, and mycotoxin production are all quantitative traits that are influenced by multiple loci (Fingstag et al., 2019; M. R. Fulcher et al., 2019). Genetic recombination can generate transgressive highly aggressive progeny (Bec, 2011; Bissonnette, Kolb, Ames, & Bradley, 2018; Goswami & Kistler, 2004). Except for the *TRI* genes, relatively few of the loci impacting aggressiveness and toxigenicity of *F. graminearum* have been characterized, and interactions among them, as well as their roles in competition and fitness, are still poorly understood.

Right now, it is hard to predict what will happen when something changes in the environment or when a new strain or species is introduced.

F. graminearum genomes have regions of highly polymorphic DNA interspersed with more conserved regions (Cuomo et al., 2007). The highly polymorphic regions contain many pathogenicity-associated genes and appear to be changing more rapidly than the more conserved regions that tend to contain housekeeping genes. Recombination across the four chromosomes of *F. graminearum* is not random, and physical maps do not match genetic linkage maps as the highly polymorphic regions are also associated with recombination hotspots (Cuomo et al., 2007; Gale L.R., 2005). This is known as a "two speed genome" (Laurent et al., 2018). Enhanced recombination rates among pathogenicity genes may allow pathotypes to evolve more quickly (Voss, Bowden, Leslie, & Miedaner, 2010).

1.6 Developing A Protocol for Genetic Analysis of *F. graminearum*

Genetic and genomic analysis can be used to monitor allelic shifts in FSASC (Fall et al., 2019; A. C. Kelly & Ward, 2018), and the use of the MLGT primers to track species and chemotype distributions is an excellent example of the possibilities of marker technology. However, the focus so far has been on relatively few markers mostly associated with the trichothecene (TRI) gene cluster. Our long-term goal is to modify the MLGT assay to include markers for other important loci that play major roles in pathogenicity and toxigenicity, which could allow us to better incorporate predictions of the contribution of the pathogen genotype into risk assessment models. It would also let us monitor and track populations and help us to predict how they may evolve in the future.

My goal in my thesis research was to develop mating tester strains that can be used for making controlled genetic crosses of *F. graminearu*m. These crosses could be used to map and identify markers that co-segregate with traits of interest. Crosses of *F. graminearum* can be performed *in vitro* under laboratory conditions (R. L. Bowden & Leslie, 1998), but the challenge is identifying the minority of

outcrossed perithecia from among the majority of selfed perithecia. One way to solve this problem is to use a heterothallic strain that is unable to self. Deletion of the *MAT1-1-1* or *MAT1-2-1* mating type genes causes self-sterility and produces obligately outcrossing strains (J. Lee, Lee, Lee, Yun, & Turgeon, 2003). Fertile perithecia produced by a deletion strain in a cross can only result from outcrossing. Ideal tester strains will be highly female-fertile with a variety of other strains, have normal fitness and pathogenicity, and produce progeny that segregate normally for various phenotypic and genotypic markers. In this thesis I report my findings after evaluating these traits for a group of independent MAT deletion strains produced in the *F. graminearum* PH-1 strain background.

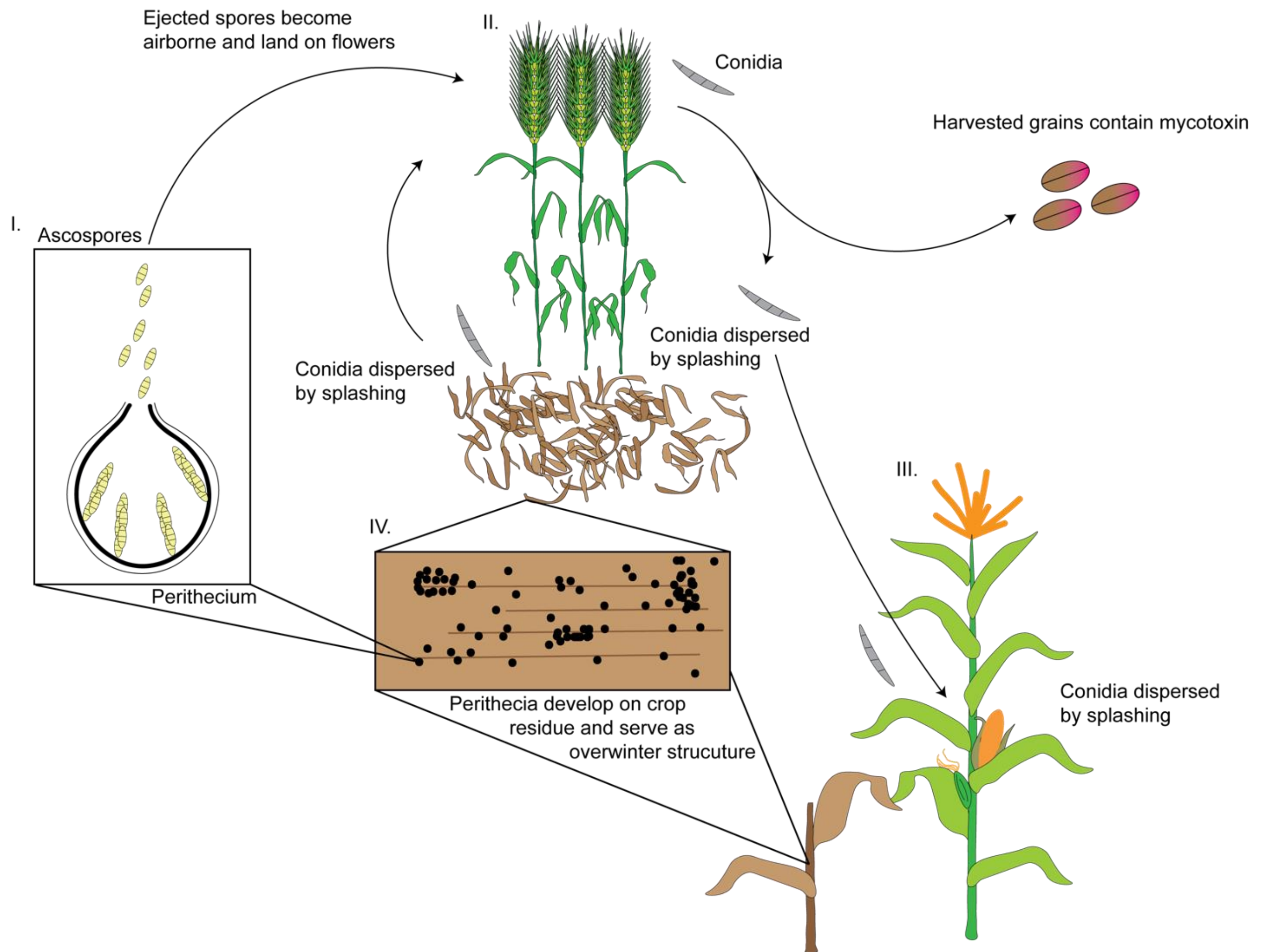


Figure 1.1 Fusarium Head Blight and Gibberella Ear Rot Disease Cycle. (I) Primary inoculum, ascospores, are ejected from the overwintering perithecia, and become airborne. (II) The ascospores land on flowering wheat heads and establish infections after colonizing the flowers, causing bleaching of flower spikes. Wheat grains develop poorly and accumulate mycotoxins including deoxynivalenol (DON). (II) Warm and humid conditions favor fungal sporulation on infected tissue. Pink-orange masses contain the secondary inoculum, conidia, that are dispersed by splashing water onto neighboring wheat plants and other hosts, like maize. (III) Conidia can land on extruded maize silks and germinate down the silk channels, eventually colonizing developing kernels, while depositing mycotoxins. (IV) Perithecia develop on infected plant residues that remain in the fields and provide primary inoculum the following season.

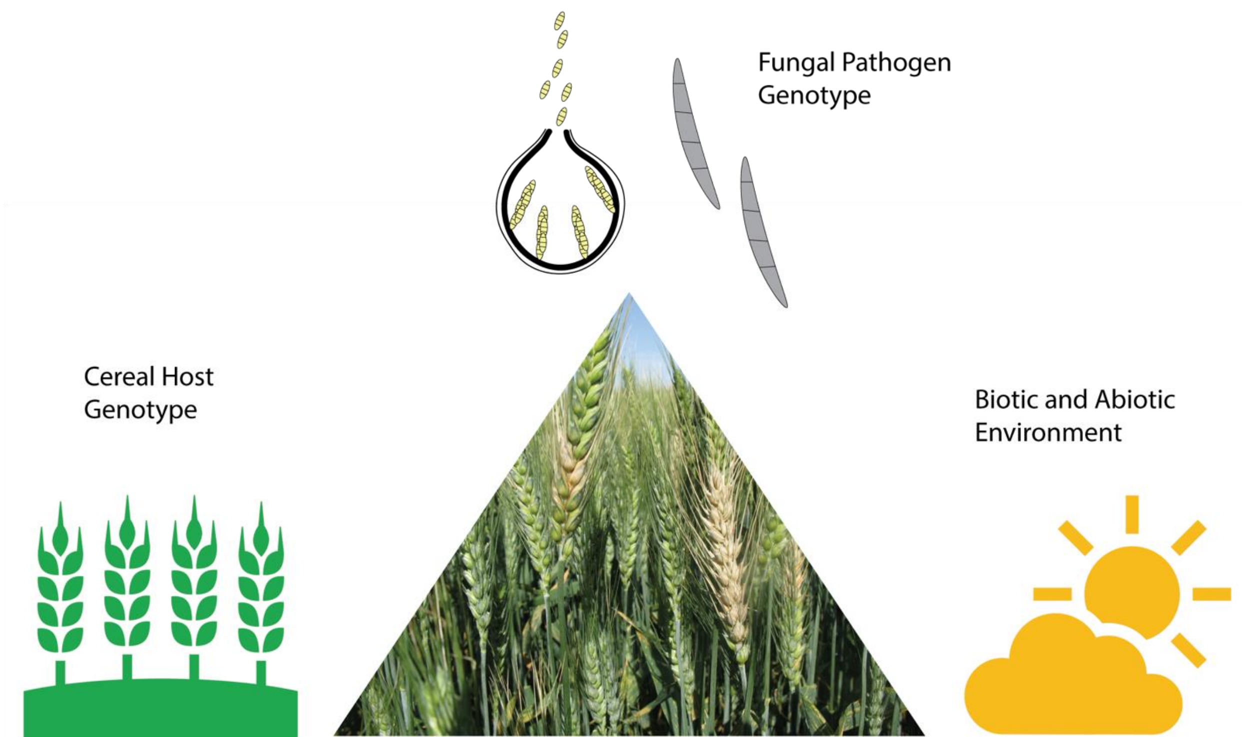


Figure 1.2 The Plant Disease Triangle includes all the factors that influence Fusarium Head Blight.
Current risk assessment models mainly consider cereal host genetics and environmental factors. The influence of the fungal pathogen genotype is mostly not included.

CHAPTER 2. FEMALE FERTILITY AND INTERFERTILITY OF *MAT1-1-1* AND *MAT1-2-1* DELETION STRAINS.

2.1 Introduction

Mating and sexual development in Ascomycetes are controlled by a group of mating type genes housed within the complex MAT1 locus (Casselton, 2008). The MAT1 locus has two alternative allelic forms called MAT1-1 and MAT1-2 (Turgeon & Yoder, 2000). These allelic forms are more generally known as idiomorphs since they share no homology. The MAT1-1 idiomorph is comprised of three genes; *MAT1-1-1*, *MAT1-1-2*, and *MAT1-1-3*; while MAT1-2 includes *MAT1-2-1* (**Figure 2.1**). These genes play different important roles in sexual development. The *MAT1-2-1* gene encodes an HMG domain protein, and the *MAT1-1-1* gene encodes an alpha-factor domain protein (Klix et al., 2010). These two proteins act as DNA-binding transcription factors, and are both essential for fertility (Klix et al., 2010). They interact as heterodimers to produce an active transcription factor that switches on multiple genes that are involved in sexual development (Jacobsen, Wittig, & Pöggeler, 2002; Metzenberg & Glass, 1990; Staben & Yanofsky, 1990).

Ascomycetes can either be homothallic (self-fertile) or heterothallic (self-sterile) (Bölker, 1998; Casselton & Olesnicky, 1998; Coppin, Debuchy, Arnaise, & Picard, 1997; Fraser & Heitman, 2004; Heitman, 2006). Heterothallic species have either a MAT1-1 or a MAT1-2 locus, and they require another individual with the complementary locus to mate. Homothallic species usually have both loci and will self-fertilize most of the time, but they can also outcross at a reduced frequency (R. L. Bowden & Leslie, 1998; Coppin et al., 1997; Taylor, Jacobson, & Fisher, 1999). *Fusarium graminearum* is a homothallic fungus, meaning that it has the capability to self-fertilize without the need of a partner (Eide, 1935). Using strains with mutations in nitrate utilization genes, Bowden and Leslie showed that *F. graminearum* could be crossed in the laboratory and that the progeny segregated in the expected haploid ratios (Robert L Bowden & Leslie, 1992). *Fusarium graminearum* also has the capability to cross with other related phylogenetic

species in the *F. sambucinum* species complex (FSASC). These phylogenetic species could contribute to the genetic diversity of field populations of *F. graminearum* through recombination (Michael R Fulcher et al., 2021; M. R. Fulcher et al., 2019; Gale et al., 2011; Lofgren et al., 2018; Sarver et al., 2011; Wegulo et al., 2015).

Obligately heterothallic *F. graminearum* strains were first developed by Dr. Jungkwan Lee, who reported that: (i) the deletion of either *MAT1-1-1* or *MAT1-2-1* resulted in self-sterility; (ii) *mat1-1-1* and *mat1-2-1* deletion mutants can cross with the wild type; (iii) *mat1-1-1* and *mat1-2-1* deletion strains can cross with each other; and (iv) normal segregation of unlinked markers can be observed in heterothallic matings (J. Lee et al., 2003). Other work on MAT gene deletions has been done by the Dr. Jin-Rong Xu lab, who noted that their heterothallic mutants produced dark-pigmented sterile protoperithecia that were smaller than the fertile perithecia produced by the wild type (Zheng et al., 2013). Deletion of each of the four MAT genes individually showed that none of them were essential for production of the protoperithecia, but all were required for normal development of ascogenous hyphae, asci, and ascospores, and for perithecial maturation and enlargement (Zheng et al., 2013). Fertile perithecia were produced when *mat1-2-1* deletion strains were used as the male parent, and the *mat1-1-1* deletions were used as the female. However, no perithecia were formed when the *mat1-1-1* deletions were male, and the *mat1-2-1* deletions were female.

Sexual development in *F. graminearum* plays an important role in the disease cycle of FHB. The perithecia function as overwintering structures (Cook, 1981). The melanized perithecia survive on infected residue until spring, when ascospores are forcibly discharged and carried by wind to wheat flower heads, initiating an FHB epidemic (Trail, Xu, Loranger, & Gadoury, 2002). When strains with deletions of the entire MAT1 locus were used to colonize maize stalk pieces placed on the ground between rows of wheat, they were not able to cause epidemics in the field (A. Desjardins et al., 2004). However, when conidia produced asexually by these strains were used to inoculate wheat flowers directly in the greenhouse, there were no significant differences from the wild type in

aggressiveness (A. Desjardins et al., 2004). It was suggested that the lack of pathogenicity in the field was due mainly to an inability to produce ascospores that could be launched sufficiently high in the air to be carried to the flowers, emphasizing the importance of the ascospores as primary inoculum for FHB epidemics.

The report by Desjardins et al. (2004) showed that a deletion of the entire MAT1 locus did not affect pathogenicity to wheat in the greenhouse. A more recent study found that deletions of the individual *MAT1-1-1* and *MAT1-2-1* genes were also unaltered in pathogenicity to wheat in the greenhouse (Zheng et al., 2013). Thus, there is a possibility to use MAT deletion strains in heterothallic matings to study the impact of other genes on wheat pathogenicity and toxigenicity by using a classical genetics approach. Because *F. graminearum* is self-fertile, the heterothallic deletion mutants offer a significant advantage for such studies since the only fertile perithecia they will produce must be the result of outcrossing. This removes the necessity to screen through large numbers of selfed perithecia to identify the small number of outcrossed ones.

In the Vaillancourt Lab, *MAT1-1-1* and *MAT1-2-1* gene deletions, as well as deletions of the entire MAT1 locus, were made in the PH-1 genome-sequenced strain by Dr. Sladana Bec (Bec, 2011) The genes were replaced by the Hygromycin B antibiotic resistance gene by using a split-marker or intact marker double-crossover strategy (Bec, Yulfo-Soto, & Vaillancourt, 2021). The main goal of my thesis research was to identify the best mating tester strains from among a group of independent deletion mutants, and to develop a standardized mating protocol for future genetic studies. The ideal heterothallic *F. graminearum* tester strain should grow well and produce plenty of conidia in culture, should have normal pathogenicity and toxigenicity to wheat and to other hosts, should be highly female fertile with most or all mating partners, and should produce abundant ascospore progeny that display expected patterns of segregation and recombination. In this first chapter, I describe morphological variation in growth and fertility among independent deletion strains *in vitro*.

2.2 Materials and methods

2.2.1 Fungal strains and growth conditions.

Strain PH-1 was obtained from Dr. Frances Trail. MAT gene deletion strains were constructed earlier (Bec et al., 2021). Strains in this study are described in (**Table 2.1**). All fungal strains were routinely grown at 23°C with constant light (Sylvania F032/741/ECO). Gene deletion strains were single-spored and stored on silica gel at -20°C or -80°C (Tuite, 1969, after Perkins, 1962). Strains were started on PDA for 5 days, before collecting colonies with sterile toothpicks and culturing on sporulation inducing media. Production of asexual spores (macroconidia) was done on mungbean agar (MBA) (40 g mungbean and 10 g Bacto® Agar per L) and/or in carboxymethylcellulose (CMC) liquid media cultures at 250 rpm, both at 23°C for 10 days. Mungbean agar was prepared by boiling 40 g of mungbeans in 1 L of water for about 23 min or until the beans began to split. The boiled beans were filtered through a double layer of cheesecloth, the liquid was measured, and water was added to 1 L. Ten grams of Bacto® Agar were added before autoclaving. For cultures on mungbean agar, 2 mL of sterile water was applied to the surface of the Petri plate and the spores were released by rubbing with a sterile plastic micro-pestle. Asexual spore suspensions from mungbean and CMC cultures were filtered through a double layer of sterile cheesecloth to remove mycelia and collected in a sterile 50 mL Falcon tube. Spores were counted by using a hemocytometer. For use of inoculum, spores were centrifuged at 3333 x g then washed once in sterile water and resuspended to adjust to the necessary concentration. Production of sexual spores (ascospores) was done on carrot agar media (CAM) (Klittich & Leslie, 1988). CAM was prepared by peeling and chopping 400 g of carrots into 1-inch pieces and autoclaving in 1 L of water. After cooling, the carrots were pureed in a food processor. Water was added to the puree up to 1 L, and 15 g Bacto® Agar were added before autoclaving. CAM plates were inoculated in the center with 10 µL of a spore

suspension (1 x 10^5 per mL) and cultured at 23°C for 7 days before induction (see below).

2.2.2 Mycelial growth assays.

Cultures were initiated from silica stocks that had been stored at -20°C or -80°C. Once growth was apparent, a small swab of mycelia was transferred to PDA agar media and cultured for 5-7 days at 23°C with constant light (Sylvania F032/741/ECO). Plugs (3 mm in diameter) were taken from the edges of the colony and transferred to the centers of Petri plates containing PDA. The cultures were kept in the dark at room temperature. Mycelial growth was measured after 7 days for three replications of each strain. The experiment was performed once. The cultures were photographed with a ruler for scale. Colony diameter was determined by imposing two perpendicular lines over the colony using the line feature of Fiji (version 2.0.0-rc-69/1.52p). The measurements were recorded, and radial growth was determined by dividing the average diameter in half.

2.2.3 DNA extraction.

To isolate fungal DNA, five ml of YEPD media (20 g dextrose, 10 g Bacto® peptone, 3 g yeast extract per L) in a 50 ml glass tube was inoculated with an 8-mm plug of an actively growing culture and incubated for 5-7 days at room temperature with agitation (250 rpm). The mycelial mats were harvested by filtration, frozen at -80°C for one hour, and lyophilized in a freeze-drier. Lyophilized tissue was pulverized with a metal spatula and 100 - 200 mg was transferred to a 15 mL tube. Lyophilized tissue was suspended in a 1.4 mL lysis buffer (0.5 M NaCl, 1% SDS, 10 mM Tris HCl, Ph7.5, 10 mM EDTA) for 30 minutes at 65°C. The solution was subsequently extracted with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) for an additional 30 min at 65°C. The resulting slurry was centrifuged for 20 min at 4,440 x g to separate the phases. The aqueous phase (500-600 µL) was transferred into a clean Eppendorf tube and precipitated with 0.1

volume of 3M sodium acetate and 0.7 volume isopropanol. The samples were centrifuged for 15 min at 14,430 x g to pellet the DNA. The pellet was washed once with 70% ethanol. After the ethanol washes, the DNA pellet was air dried for 10 minutes in a transfer hood, then dissolved in 50 mL 0.05 %DEPC water and 5 μL of 10 mg/mL RNaseA at room temperature.

2.2.4 PCR amplification.

Primers used in this study are shown in (**Table 2.2**). I designed the multiplexing primers for *MAT1-1-1* and *MAT1-2-1* by using the program Primer-BLAST (Ye et al., 2012). Multiplex PCR reactions consisted of a 20 μl total reaction volume including 2 μl of 10X PCR buffer, 1.6 μl of 25 mM MgCl2, 2 μl of 10 mM dNTP mix, 1 μl of each of the four primers (10 nM), and 1 μl of template DNA (5-10 ng/μL), 8.7 μl of water and 0.7 μl of Taq polymerase. A strain of DH5a containing the pTAQ Taq-polymerase gene was provided by Dr. Pradeep Kachroo, and preparation of the enzyme used the method described in (Desai & Pfaffle, 1995). The thermocycling parameters were as follows: initial denaturation for 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 20 sec, and extension at 72°C for 1 min with a final extension for 7 min at 72°C. The PCR amplicons were separated on a 0.9% agarose gel in 1 X TAE.

2.2.5 Crossing procedure and perithecial production.

MAT deletion strains were crossed by depositing 10 μL spore suspension (1 X 10^5 per mL) of each strain equidistant from each other on CAM in a 60mm Petri Plate using a method modified from (R. L. Bowden & Leslie, 1998). After 4 days of incubation at 23°C, perithecial production was induced by applying 1 mL of 2.5% Tween 60 to the surface of each plate, and gently rubbing the culture with a sterile glass rod to flatten it and remove excess mycelia. Rubbing the aerial mycelia ensured distribution of the spores of both strains across the whole plate. Following

induction, the plates were incubated at 23°C with constant fluorescent light until perithecial maturation.

2.2.6 Quantifying perithecial production numbers.

Three 3 mm plugs of agar containing perithecia were collected with a cork borer from each side of the CAM crossing plate (**Figure 2.2**). Each plug was individually photographed (Leica MC170 HD) under a dissecting microscope (Zeiss Stemi 2000-C). Perithecia on each plug image were counted by using the Counting application in Fiji (version 2.0.0-rc-69/1.52p).

2.2.7 Spore counts.

Ten microliters of a spore suspension (1 x 10^5 per mL) of each deletion strain were placed in the center of a MBA plate and cultured at 23°C with constant light (Sylvania F032/741/ECO). After 7 days, 2 mL of sterile water was added to the plates and a plastic pestle was rubbed across the surface to release the spores. One mL of the spore suspension was collected into a 1.5 mL Eppendorf and the spores were counted by using a hemocytometer. This experiment was done twice. The first experiment had three replications and the second one had two.

2.2.8 Data Analysis

All experiments were conducted as a completely randomized design. Data were visualized and analyzed by 95 % Confidence Intervals (CI) and by Scott Knott. CI was done by using 'mean_cl_boot' from the 'Hmisc' package, which implements basic nonparametric bootstrapping to obtain confidence limits for a population of means without assuming a normal distribution (Harrell Jr & Harrell Jr, 2019). CI was performed for multiple experimental replications. If confidence limits of mutants overlapped with the wild type PH-1, these were considered similar. For the Scott Knott, data from multiple replicated experiments were

combined. The Scott Knott was used to group the isolates according to the means of measurements and counts (Jelihovschi, Faria, & Allaman, 2014).The overall means of measurements (growth), and counts (conidia, perithecia) of the strain groups were also compared by using the Tukey test, with $\alpha = 0.05$, after performing an analysis of variance. All analyses were run in R (R Core Team 2019).

2.3 Results

2.3.1 Genetic characterization of heterothallic *F. graminearum* strains.

Five independent *mat1-1-1* deletion strains, and six *mat1-2-1* deletion strains, were evaluated for my study. The deletion strains were previously confirmed by Southern blots (Bec et al., 2021). Deletions were affirmed by using PCR multiplex markers for the individual MAT genes. The five *mat1-1-1* deletion strains produced amplicons only with the *MAT1-2-1* gene primers, and the six *mat1-2-1* deletion strains produced amplicons only with the *MAT1-1-1* gene primers. Two strains with deletions of the entire MAT locus were not amplified by either primer. The PH-1 wild type strain amplified both markers (**Figure 2.3**).

2.3.2 Mycelial growth of strains.

The independent deletion strains varied in their appearance on PDA medium (**Figure 2.4**). Four of the six *mat1-2-1* deletion strains produced an excess of tightly matted aerial mycelium that could be very easily removed from the agar, in comparison with the PH-1 progenitor strain. This phenotype was designated "velvet".

The deletion strains were compared for radial growth after 7 days. The data for each of the strains is provided in (**Table 2.3**). Overall, averages of the *mat1-1-1* and *mat1-2-1* deletion mutant classes were not significantly different from one another, or from PH-1 (**Table 2.4**). Confidence intervals of two individual *mat1-2-1* and three *mat1-1-1* deletions strains overlapped with PH-1, while the other strains

grew faster than the wild type (**Figure 2.5**). Four statistically different groups were defined among the individual strains by using the Scott Knot groupings test (**Figure 2.6**). One of the *mat1-2-1* deletion strains (2_5) grew significantly faster than the rest, with an average radial growth of 4.70 cm. One of the *mat1-1-1* deletions, (1_3) was the sole occupant of the second group, with an average growth of 4.23 cm. The PH-1 progenitor strain belonged to the slowest-growing group, along with one *mat1-1-1* deletion strain and most of the *mat1-2-1* deletion strains.

2.3.3 Spore counts.

Spore production by MAT deletion strains and the wild type PH-1 strain were compared in two experiments. Experiments were analyzed separately. One outlier data point (2_5) was excluded in experiment two. The number of spores per mL ranged between 0 and 5 x 10^7 per mL in the first experiment, and from 0 to 8 x 10^5 in the second (**Table 2.3**). Confidence intervals of four strains in the first experiments (2_6, 1_5, 1_3, and 1_2) and six in the second experiment (2_4, 2_2, 1_5, 1_3, 1_2, 1_1), overlapped with PH-1 (**Figure 2.7**). In the first experiment, the Scott Knott test identified two significantly different groups, with one group that included both *mat1-1-1* (1_1, 1_2) and *mat1-2-1* (2_2) deletions producing significantly more spores than the second group that contained the PH-1 strain and the remainder of the deletion strains (**Table 2.3**). In the second experiment there were no significant differences observed among the individual strains by the Scott Knott test (**Table 2.3**). Overall averages of spore production by the three groups (*mat1-1-1* deletions, *mat1-2-1* deletions, and PH-1) did not differ statistically (**Table 2.4**). No spores were produced by the *mat1-2-1* deletion strain 2_3 in either experiment.

2.3.4 Female fertility of heterothallic strains.

The female fertility of the individual *F. graminearum* MAT deletion strains and of the wild type PH-1 was assessed based on their ability to produce

protoperithecia (which were smaller and contained no asci or ascospores) or fertile perithecia (**Table 2.3**). The *mat1-1-1* and *mat1-2-1* deletion strains produced only sterile protoperithecia on CAM, compared to the wild type PH-1 that produced fertile perithecia. The *mat1-1-1* deletion strains, on average, produced more protoperithecia than the *mat1-2-1* deletion strains **(Table 2.4)**. Wild type PH-1 produced fertile perithecia that were less abundant than the protoperithecia produced by the deletion strains. Confidence intervals of two strains in the first experiment (2_6, 1_5), and three in the second (2_6, 2_4, 1_4) overlapped with PH-1 (**Figure 2.8**). In both experiments, strains 2_6, 2_4, 1_5, 1_3, 1_2 and 1_1 were more female fertile than PH-1. Scott Knott analysis determined 4 groups (**Figure 2.9**). Three *mat1-2-1* deletion strains produced very few protoperithecia, compared to the other *mat1-2-1* strains and the *mat1-1-1* deletion strains. Two of the strains that produced few protoperithecia had the velvet phenotype.

2.3.5 Interfertility of heterothallic strains.

Different combinations of strains produced significantly different numbers of fertile perithecia on both sides of the crossing plates (**Table 2.3**). Production of fertile perithecia varied depending on which strains were involved in the cross (**Figure 2.10**). The *mat1-1-1* strains produced larger numbers of fertile perithecia overall than the *mat1-2-1* strains **(Table 2.4)**. The *mat1-1-1* strains also produced a higher number of fertile perithecia in crosses than the WT strain PH-1 **(Table 2-4)**. Confidence intervals of one strain in the first experiment (1_5), and four in the second experiment (2_6, 2_4, 1_5, 1_3), overlapped with PH-1 (**Figure 2.11**). In both experiments, strains 1_4 and 1_1 were more female fertile than PH-1. Scott Knott groupings showed five significantly different groups among the individual strains based on interfertility (**Figure 2.12**).

2.4 Discussion

The amount of morphological variation that I observed among this group of independent deletion transformants, all produced in the same PH-1 background, was somewhat surprising. Many of the transformants grew faster than the wild type, but there was no association with the deletions themselves, as members of both groups were also found that were not significantly different from the wild type. Four of the *mat1-2-1* deletion strains produced a non-typical mycelial morphology compared to the wild type. Strains 2_3, 2_4, and 2_5 produced a white, thick, velvety mycelium, which was different from the dark red-yellow mycelia typical of the *mat1-1-1* deletion strains and PH-1. The 2_6 strain also sectored frequently to the velvety mycelial type. This phenotype was only observed on the *mat1-2-1* deletion strains. Sladana Bec, a former student in the Vaillancourt lab who originally produced these deletion strains, did not note the presence of this phenotype, and her photo records show that these strains looked more like the wild type when she was working with them. The velvet phenotype I observed could be a result of mutation. Fungi that are frequently subcultured have been reported to undergo strain degradation, which includes phenotypic changes like loss of virulence or morphological changes (Butt, Wang, Shah, & Hall, 2006; Duncan & Bu'Lock, 1985; Shah, Wang, & Butt, 2005). The PH-1 strain of *F. graminearum* is reportedly prone to this degradation (F. Trail, J. Leslie personal communication). There are several mechanisms that give rise to these instabilities, including alteration of gene expression due to transposable elements, infection by dsRNA viruses and/or chromosomal polymorphisms (Chu et al., 2002; Fowler & Mitton, 2000; Frank Kempken & Kück, 1998; F Kempken & Kück, 2000). Although the velvet phenotype was only seen in the *mat1-2-1* deletion strains, some *mat1-2-1* deletion strains were normal, indicating that the deletion itself was not directly responsible for the degradation. However, it is possible that the *mat1-2-1* deletion indirectly influences the likelihood of degradation and, if true, that would be an interesting and novel association. This question requires further study.

The strains varied widely in their production of macroconidia as well, but these differences were mostly not significant, and not associated with the type of deletion. There was a high degree of variability among the replications, including several in each case where no spores were detected with the hemocytometer. There was also a lot of variation between the two experiments. Spore production can be influenced by many factors including light, media, temperature, and strain, and the variability could be related to any of these, as well as to variation in spore recovery. The hemocytometer is quite unreliable when spore numbers are low. A better way to do the experiment would have been to concentrate the spores by spinning them down and resuspending them in a smaller volume before counting. This experiment should be repeated in the future. The velvet strains generally produced low numbers of conidia, including one (2_3) that never produced conidia at all. Degraded strains often produce fewer conidia, and faster mycelial growth at the expense of spore production may be selected in culture (Cooper & Sweeney, 1986; Kawakami, 1960; Lord & Roberts, 1986; Shah et al., 2005; Wang, Skrobek, & Butt, 2003).

The *mat1-1-1* deletion strains overall were more female fertile than the *mat1-2-1* strains and the PH-1 wild type. The 2_6 strain stood out as the only individual *mat1-2-1* deletion strain that produced perithecia at the same rate as the wild type. The ability to outcross varied, depending on the partner, but some of the *mat1-1-1* strains did appear to be more fertile overall in outcrosses than others, and these would be most suitable as test maters. The velvet *mat1-2-1* strains were the least female-fertile and produced few or even no protoperithecia either alone or in matings. The same mycelial phenotype was observed for the velvet strains on CAM, and the mycelia was easily removed from the surface. This could have influenced their performance in the interfertility experiments because if the strain does not produce any spores, and if the mycelia is removed from the surface, then there will be no fertilization occurring.

Previous literature has not mentioned such extreme differences among independent transformants (H.-K. Kim, Cho, Lee, Lee, & Yun, 2012; J. Lee et al., 2003; Zheng et al., 2013). However, after talking with several people in the field, it

seems that this type of variation is not uncommon and is generally believed to result from off-site mutations that occur during the process of transformation (J. Xu, F. Trail, personal communication). The MAT deletion strains I used in my experiment contain off-site insertions of the transforming DNA (Bec et al., 2021) and this could have caused additional mutations that affected these quantitative phenotypes.

Overall, the *mat1-1-1* deletion strains were morphologically like the PH-1 wild type, grew faster, produced adequate conidia, and were more female fertile than the *mat1-2-1* deletion strains. Thus, the *mat1-1-1* deletion strains are the best choice for test maters, based on these assays. In my next chapter I will examine the pathogenicity and toxigenicity phenotypes of these mutant strains.

Table 2.1 List of strains used in this study.
Strain labels that begin with 1 are *mat1-1-1* deletion strains, while strains that begin with 2 are *mat1-2-1* deletion strains.

Strains					
Code	Name (Bec, 2011)	Relevant MAT Genotype	Transformation Protocol	Colony type	Hygromycin B Resistance
0_1	mat1 sm5	*mat1-1-1/mat1-2-1*	split-marker	Flat	R
0_2	mat1 sm16	*mat1-1-1/mat1-2-1*	split-marker	Flat	R
1_1	mat111 sm1	*mat1-1-1/MAT1-2-1*	split-marker	Flat	R
1_2	mat111 sm5	*mat1-1-1/MAT1-2-1*	split-marker	Flat	R
1_3	mat111 sm12	*mat1-1-1/MAT1-2-1*	split-marker	Flat	R
1_4	mat111 sm19	*mat1-1-1/MAT1-2-1*	split-marker	Flat	R
1_5	mat111 sm20	*mat1-1-1/MAT1-2-1*	split-marker	Flat	R
2_1	mat121 sm1	*MAT1-1-1/mat1-2-1*	split-marker	Flat	R
2_2	mat121 sm6	*MAT1-1-1/mat1-2-1*	split-marker	Flat	R
2_3	mat121 sm16	*MAT1-1-1/mat1-2-1*	split-marker	Velvet	R
2_4	mat121 sm21	*MAT1-1-1/mat1-2-1*	split-marker	Velvet	R
2_5	mat121 WC5	*MAT1-1-1/mat1-2-1*	whole cassette	Velvet	R
2_6	mat121 sm7	*MAT1-1-1/mat1-2-1*	split-marker	Flat	R
WT	PH-1 FT2	*MAT1-1-1/MAT1-2-1*	Not applicable	Flat	S

Table 2.2 List of primers used in this study.
These primers were used together in one reaction for the MAT Multiplex PCR.

Gene of Interest	Direction	Amplicon	Primer Sequence 5' - 3'
MAT1-1-1	F	MAT1-1-1 internal probe forward	TCGAGGAAACTCTTGCCTTA
	R	MAT1-1-1 internal probe reverse	CGAGGACCATGTTACCAAAG
MAT1-2-1	F	MAT1-2-1 internal probe forward	CAGGGTTGAGTTCGGAAAGC
	R	MAT1-2-1 internal probe reverse	TCCAGCATCGTCCAAGAACT

Table 2.3 Summary of data for individual *mat1-1-1* and *mat1-2-1* deletion strains.
Significantly different groups were determined using Scott Knott. Different superscript letters indicate significantly different mean values. P <0.01, α = 0.05.

	Mean Growth (cm)	Spore Experiment 1	Spore Experiment 2	Selfed Female Fertility	Female Fertility in Crosses
Strain		Mean Concentration (spore/mL)	Mean Concentration (spore/mL)	Mean Number of Perithecia or Protoperithecia	Mean Number of Perithecia
1_1	2.80^{d}± 0.212	5E+07^{a}± 2E+07	5E+05^{a}± 0	302^{a}± 200	171^{a}± 73
1_2	3.17^{c}± 0.04	5E+07^{a}± 3E+07	7E+05^{a}± 5E+05	183^{b}± 115	105^{b}± 85
1_3	4.23^{b}± 0.04	2E+07^{b}± 1E+07	6E+05^{a}± 4E+05	139^{c}± 114	48^{d}± 53
1_4	2.91^{d}± 0.189	3E+07^{b}± 6E+06	4E+05^{a}± 1E+05	119^{c}± 113	102^{b}± 74
1_5	2.85^{d}± 0.104	2E+07^{b}± 1E+07	7E+05^{a}± 2E+05	119^{c}± 116	71^{c}± 65
2_1	3.17^{c}± 0.429	2E+07^{b}± 6E+06	4E+05^{a}± 1E+05	21^{d}± 27	7^{e}± 21
2_2	3.17^{c}± 0.254	5E+07^{a}± 2E+07	8E+05^{a}± 4E+05	4^{d}± 10	6^{e}± 9
2_3	3.69^{c}± 0.63	0E+00^{b}± 0	0E+00^{a}± 0	2^{d}± 6	8^{e}± 24
2_4	3.32^{c}± 0.07	1E+06^{b}± 1E+06	5E+05^{a}± 0	175^{b}± 140	31^{d}± 65
2_5	4.71^{a}± 0.438	2E+07^{b}± 8E+06	3E+05^{a}± NA	1d± 1	2^{e}± 5
2_6	2.66^{d}± 0.199	2E+07b± 1E+07	8E+05^{a}± NA	96^{c}± 126	48^{d}± 50
WT	2.70^{d}± 0.117	2E+07^{b}± 0	7E+05^{a}± 2E+05	55^{d}± 47	55^{d}± 47

Table 2.4 Summary table for results of MAT deletion strain characterization.
Letters indicate the difference is significant at α= 0.05 (significant differences from the wild type, with P values all <0.001, are highlighted).

Strain Class	Mycelial Growth	Spore Production	Perithecia* (Selfed)	Perithecia (Crossed)
PH-1 (WT)	2.89[a] ± 0.248	2E+07[a] ± 2E+07	55[b] ± 47	55[b] ± 47
mat1-1-1	4.13[a] ± 1.57	3E+07[a] ± 2E+06	172[a] ± 151	99[a] ± 82
mat1-2-1	4.89[a] ± 2.17	2E+07[a] ± 2E+07	50[b] ± 101	17[c] ± 40
MAT1	NA	NA	NA	NA

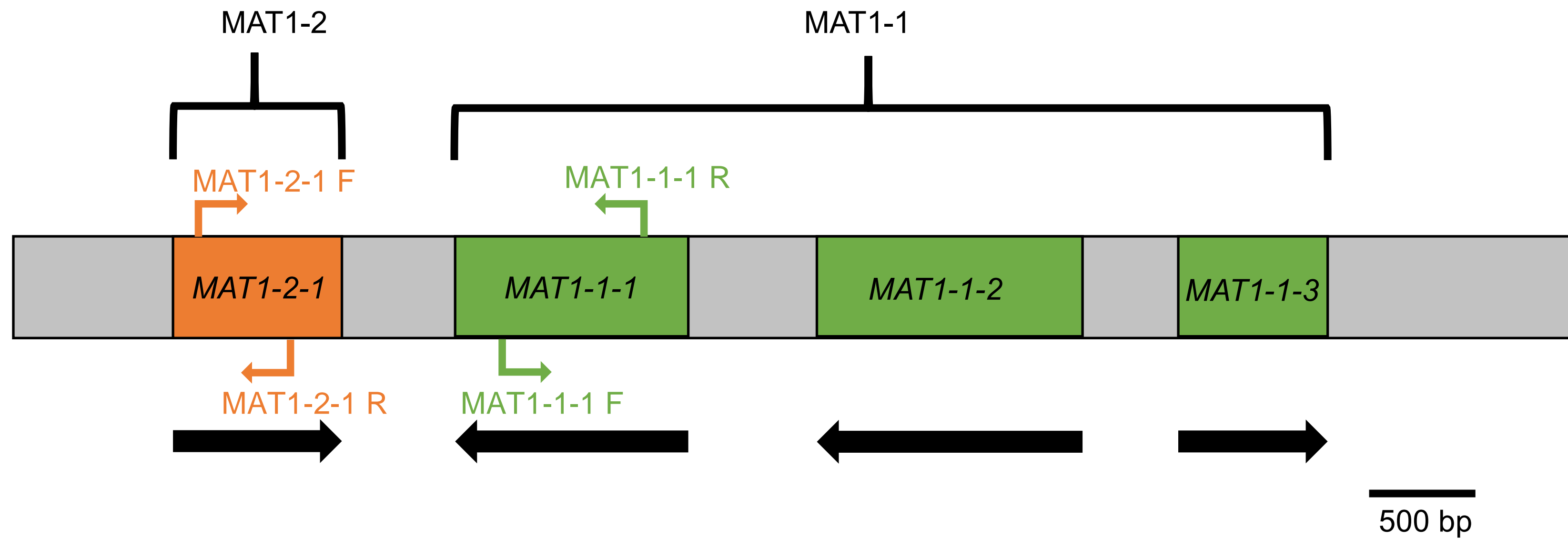


Figure 2.1 MAT locus of *Fusarium graminearum*, including the MAT1-1 (green) and the MAT1-2 (orange) idiomorphs.
Bold arrows beneath the figure indicate 5' to 3' orientation of each gene. Primer binding sites for *MAT1-2-1* and *MAT1-1-1* are indicated by the orange and green arrows.

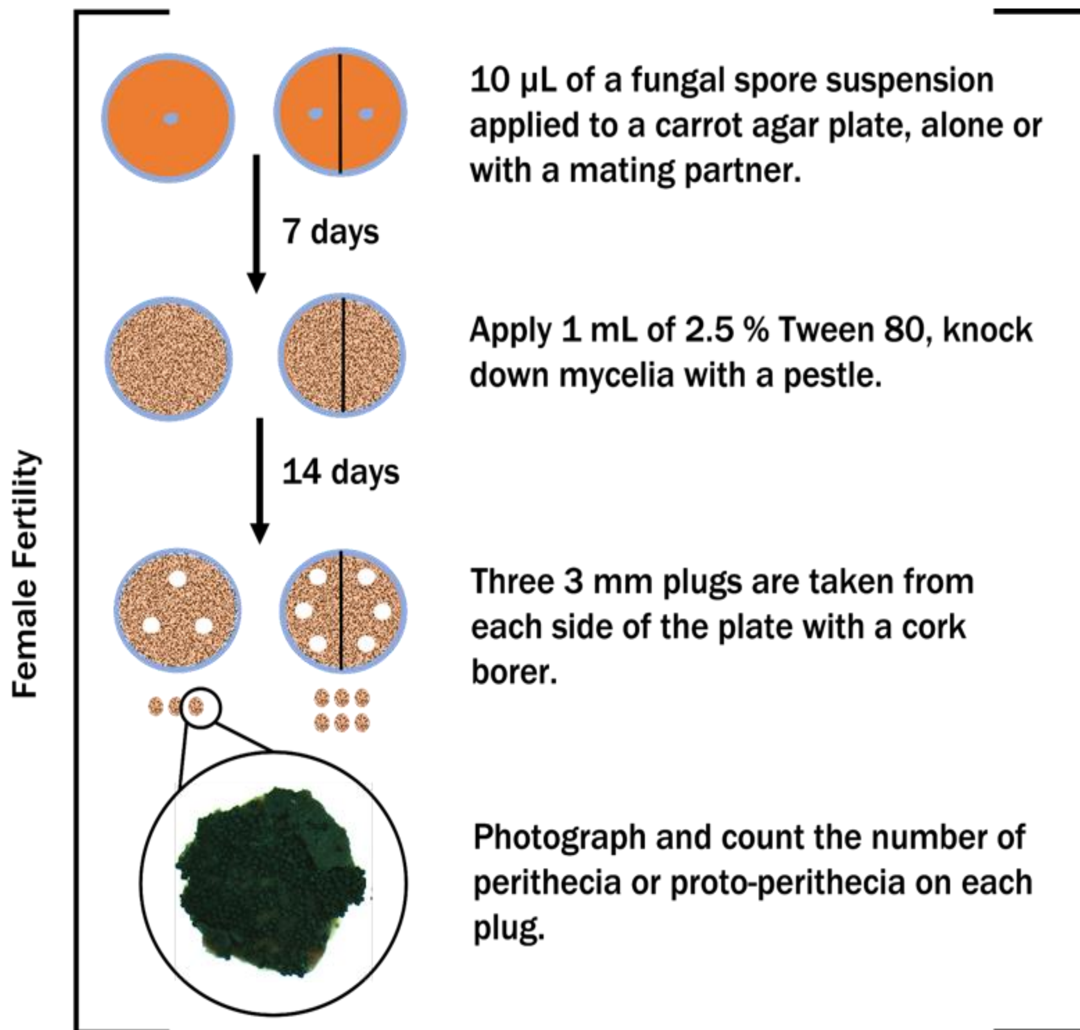


Figure 2.2 Visual Flow Chart for Female Fertility Analysis.
The left side of the arrow are the steps for selfed female fertility assays, while the right side shows the steps for crosses between two isolates.

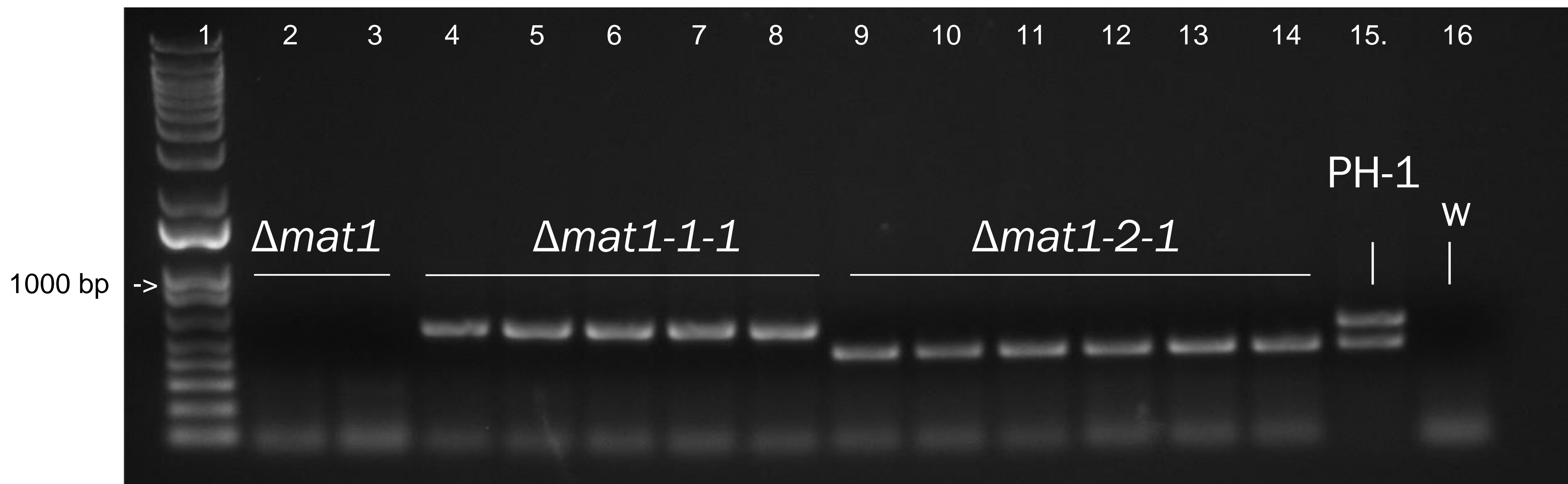


Figure 2.3 Amplification of *MAT1-1-1* and/or *MAT1-2-1* in a multiplex PCR reaction.
Lane 1: 1 Kb ladder; lanes 2-3, whole MAT deletion strains (0_1, 0_2); lanes 4-8, *mat1-1-1* deletion strains (1_1, 1_2, 1_3, 1_4, 1_5); lanes 9-14 *mat1-2-1* strains (2_1, 2_2, 2_3, 2_4, 2_5, 2_6); lane 15: PH-1; lane 16: PCR water control.

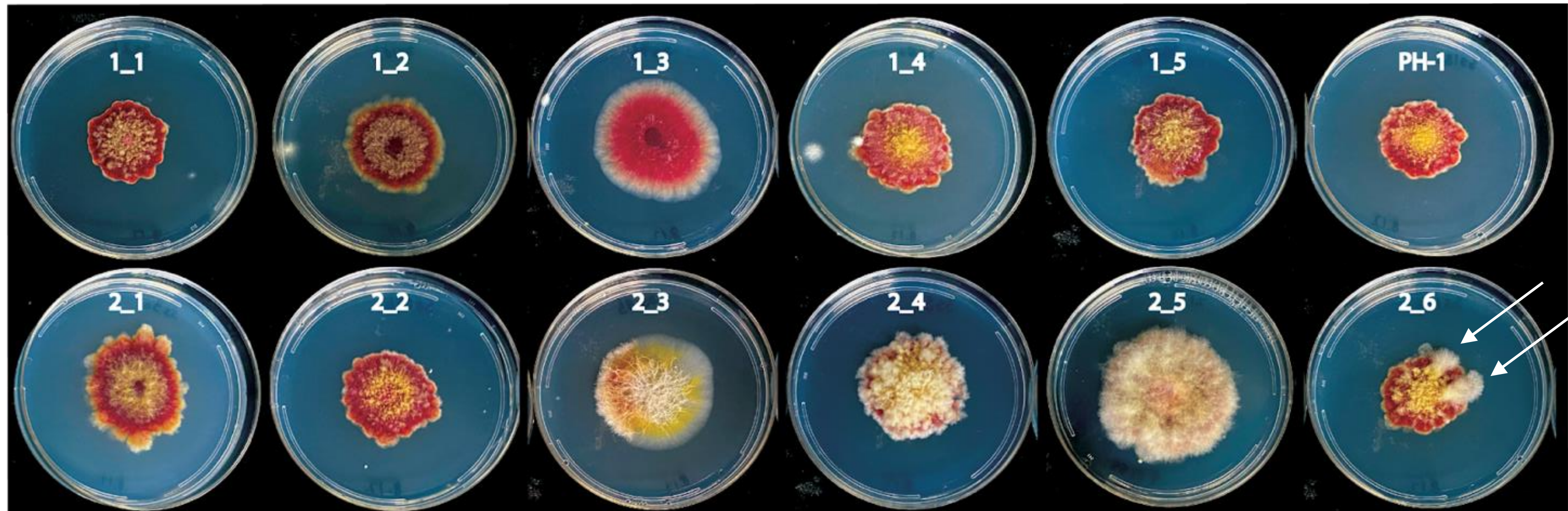


Figure 2.4 *Fusarium graminearum* wild type and MAT deletion strains after 7 days of growth on PDA. Strain labels that begin with “1” are *mat1-1-1* deletion strains, while strains that begin with “2” are *mat1-2-1* deletion strains. PH-1 is the wild type progenitor strain for all the transformants. Strains 2_3, 2_4, and 2_5 have the velvet phenotype, while strain 2_6 shows instability and velvet sectors (white arrows).

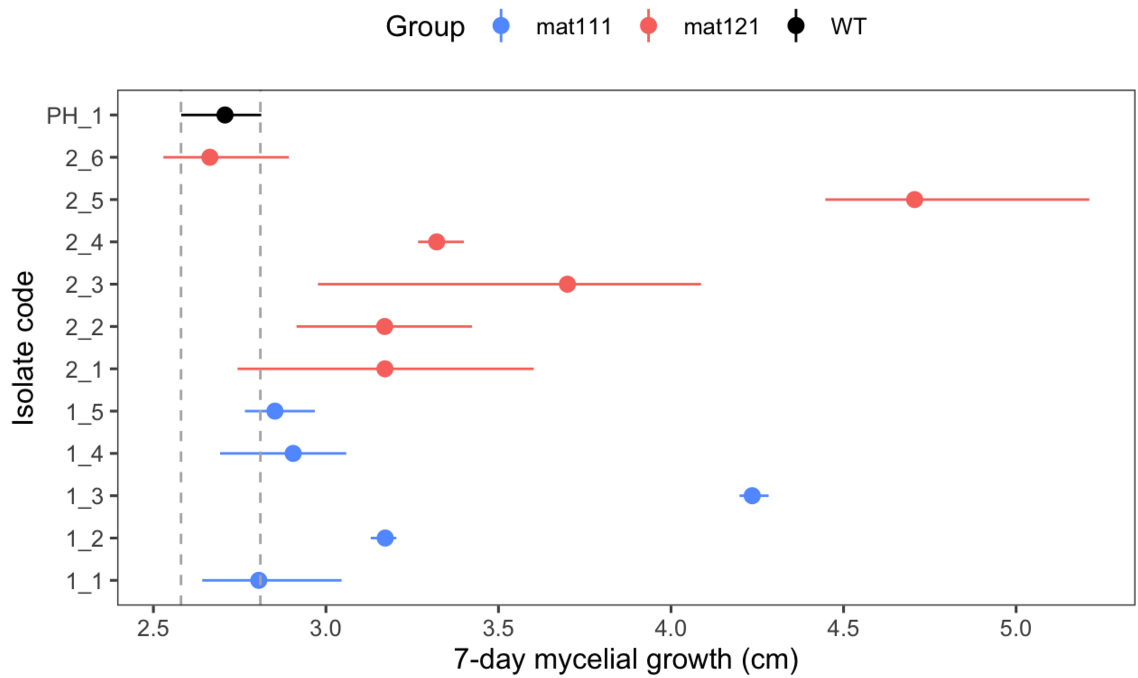


Figure 2.5 Average radial growth (cm) (95% confidence interval [CI]) of wild type and MAT deletion strains *of Fusarium graminearum* 7 days after inoculation on PDA.
Points represent mean growth for each strain. Dashed lines correspond to PH-1 CI. Strains that overlap with PH-1, behave similarly to PH-1.

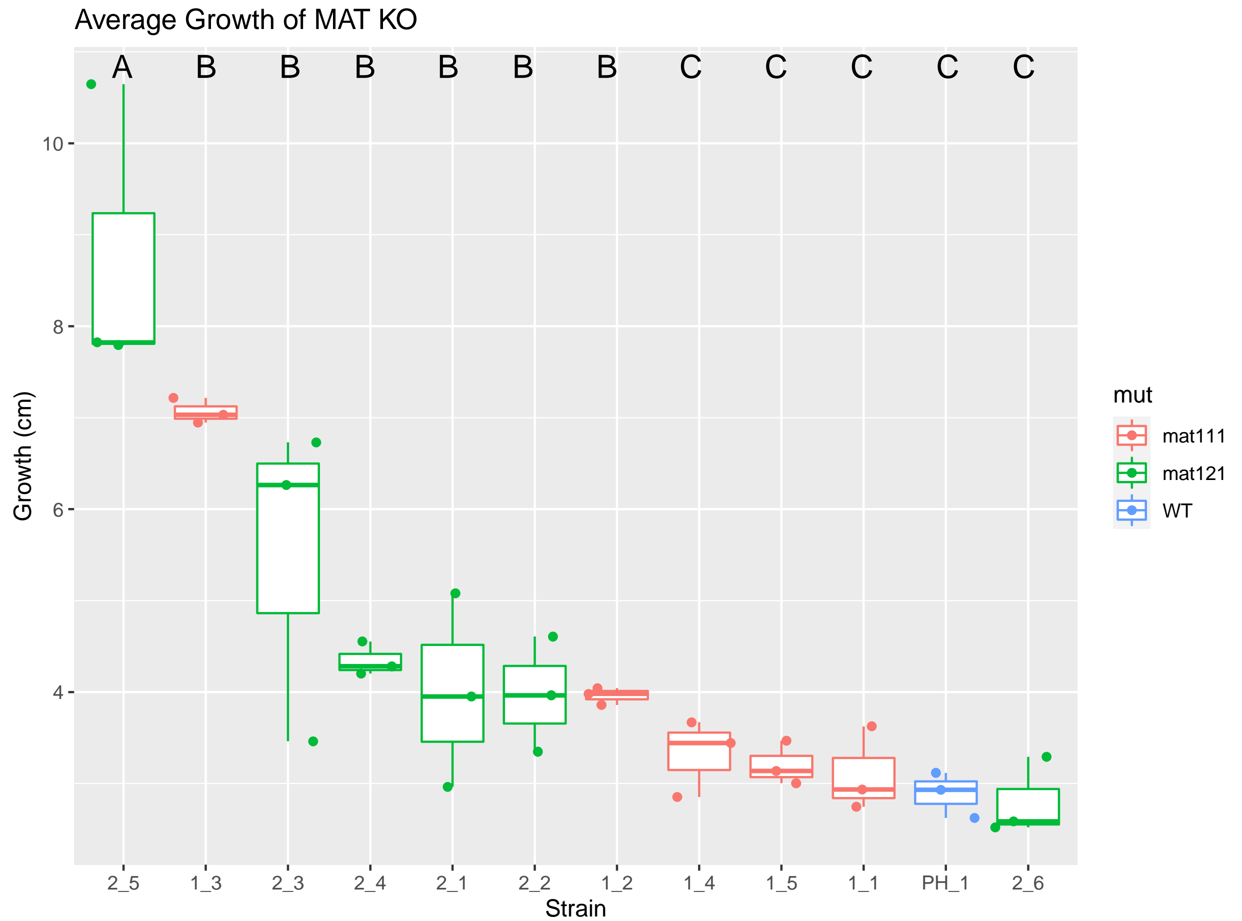


Figure 2.6 Average radial growth (cm) of wild type and MAT deletion strains *of Fusarium graminearum* 7 days after inoculation on PDA.
Each dot shows a single data point. The horizontal bars inside the boxes indicate the mean value of that treatment. The letters on top of the bars represent the significant groupings determined by Scott Knott ($P < 0.001$, $\alpha = 0.05$).

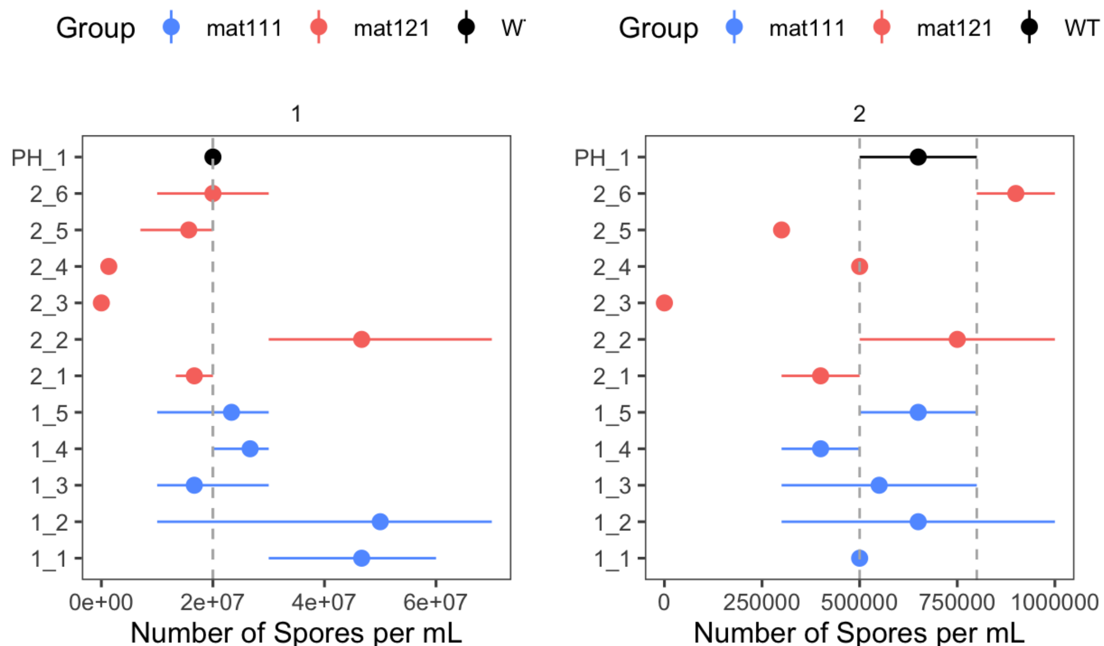


Figure 2.7 Average spore count [95% confidence interval (CI)] of wild type and MAT deletion strains *of Fusarium graminearum* for two experiments. Points represent mean spore count for each strain. Dashed lines correspond to PH-1 CI. Strains that overlap with PH-1, behave similarly to PH-1.

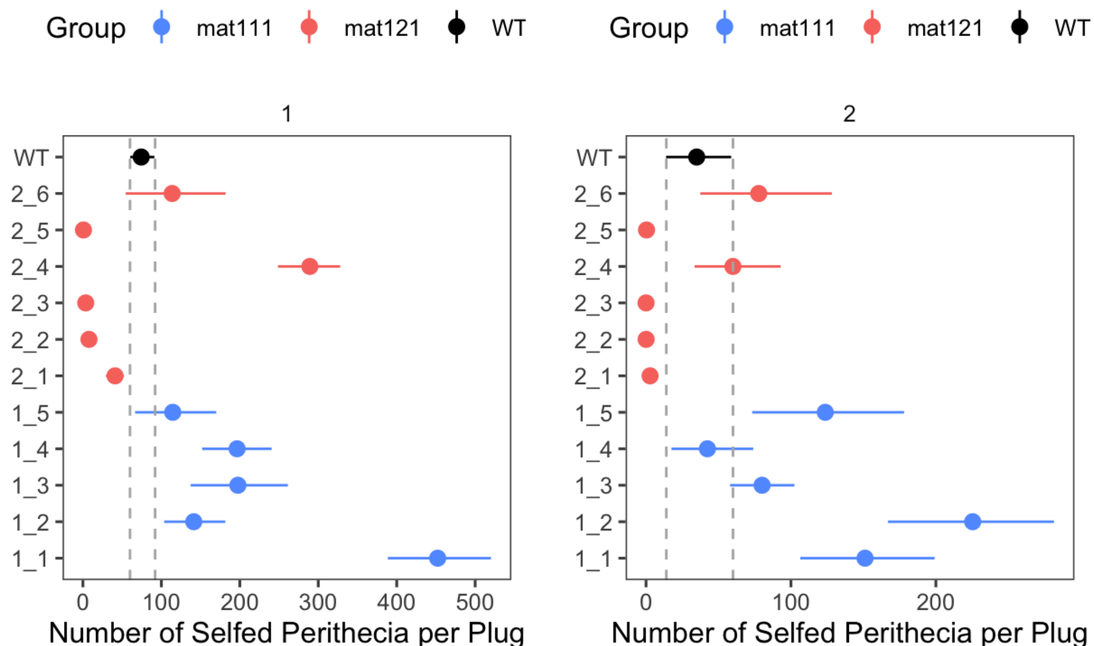


Figure 2.8 Average number of selfed perithecia [95% confidence interval (CI)] of wild type and MAT deletion strains *of Fusarium graminearum* for two experiments.
Points represent mean number of selfed perethecia for each strain. Dashed lines correspond to PH-1 CI. Strains that overlap with PH-1, behave similarly to PH-1.

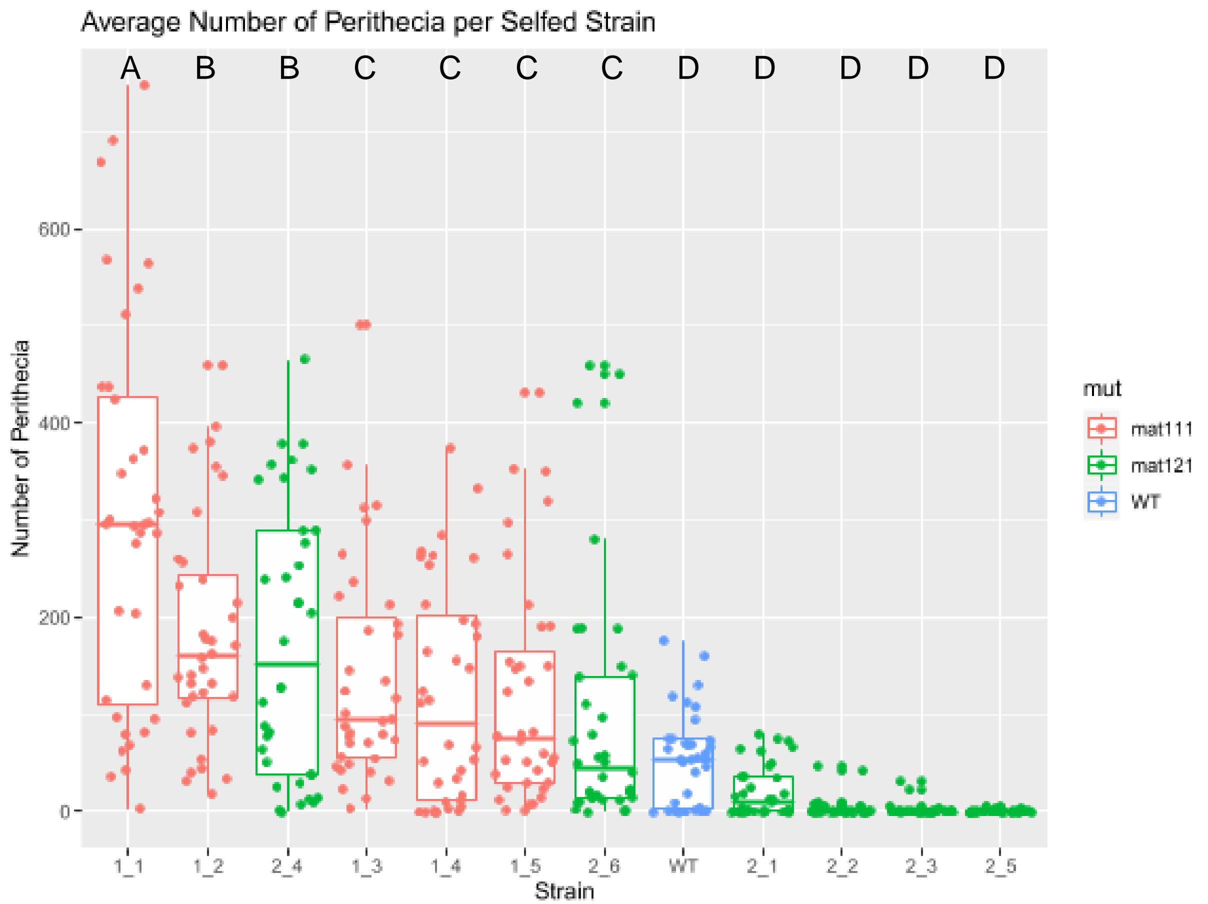


Figure 2.9 Average number of fertile perithecia (PH-1) or protoperithecia (deletion strains) per plug from individual MAT deletion strains.
Each dot shows a single data point. The horizontal bars inside the boxes indicate the mean value of that treatment. The letters on top of the bars represent the significant groupings determined by Scott Knott ($P < 0.001$, $\alpha = 0.05$).

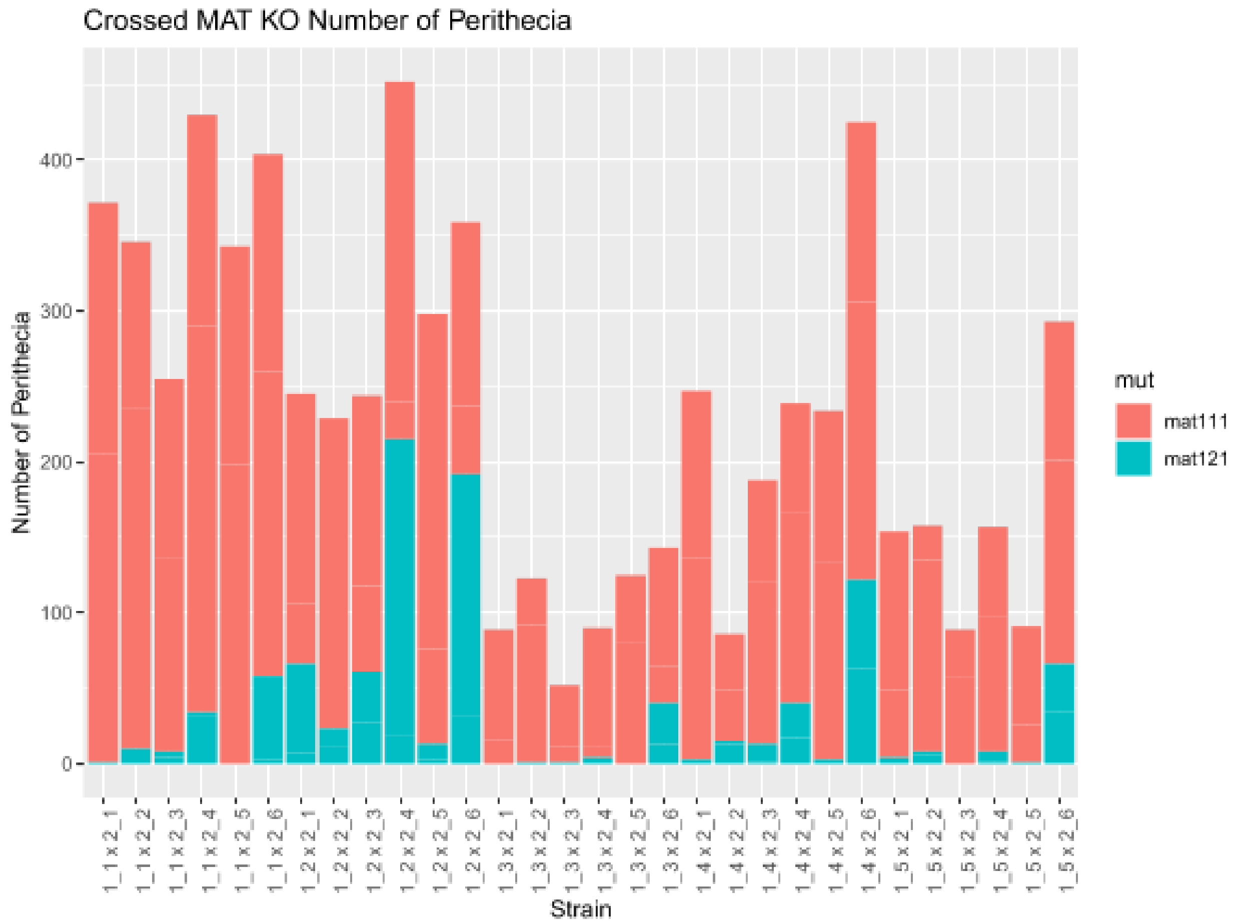


Figure 2.10 Average number of fertile perithecia produced by each MAT deletion strain in different heterothallic crosses.
This shows the average number of perithecia per strain for each cross from both experiments.

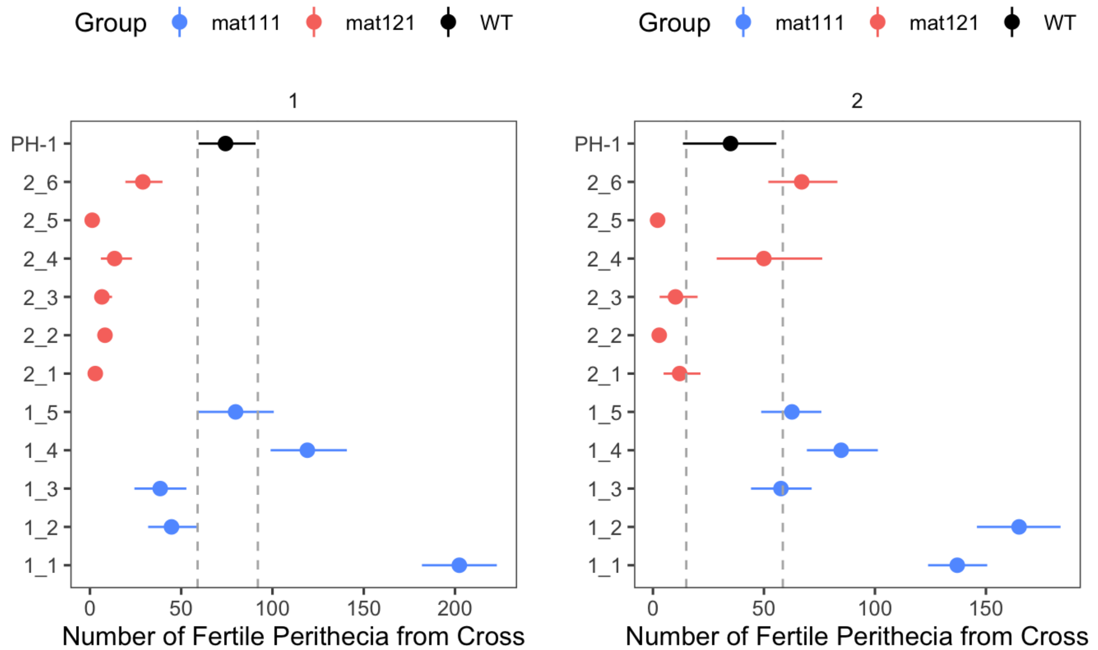


Figure 2.11 Average number of crossed perithecia [95% confidence interval (CI)] of wild type and MAT deletion strains *of Fusarium graminearum* for two experiments.
Points represent mean number of crossed perithecia for each strain. Dashed lines correspond to PH-1 CI. Strains that overlap with PH-1, behave similarly to PH-1.

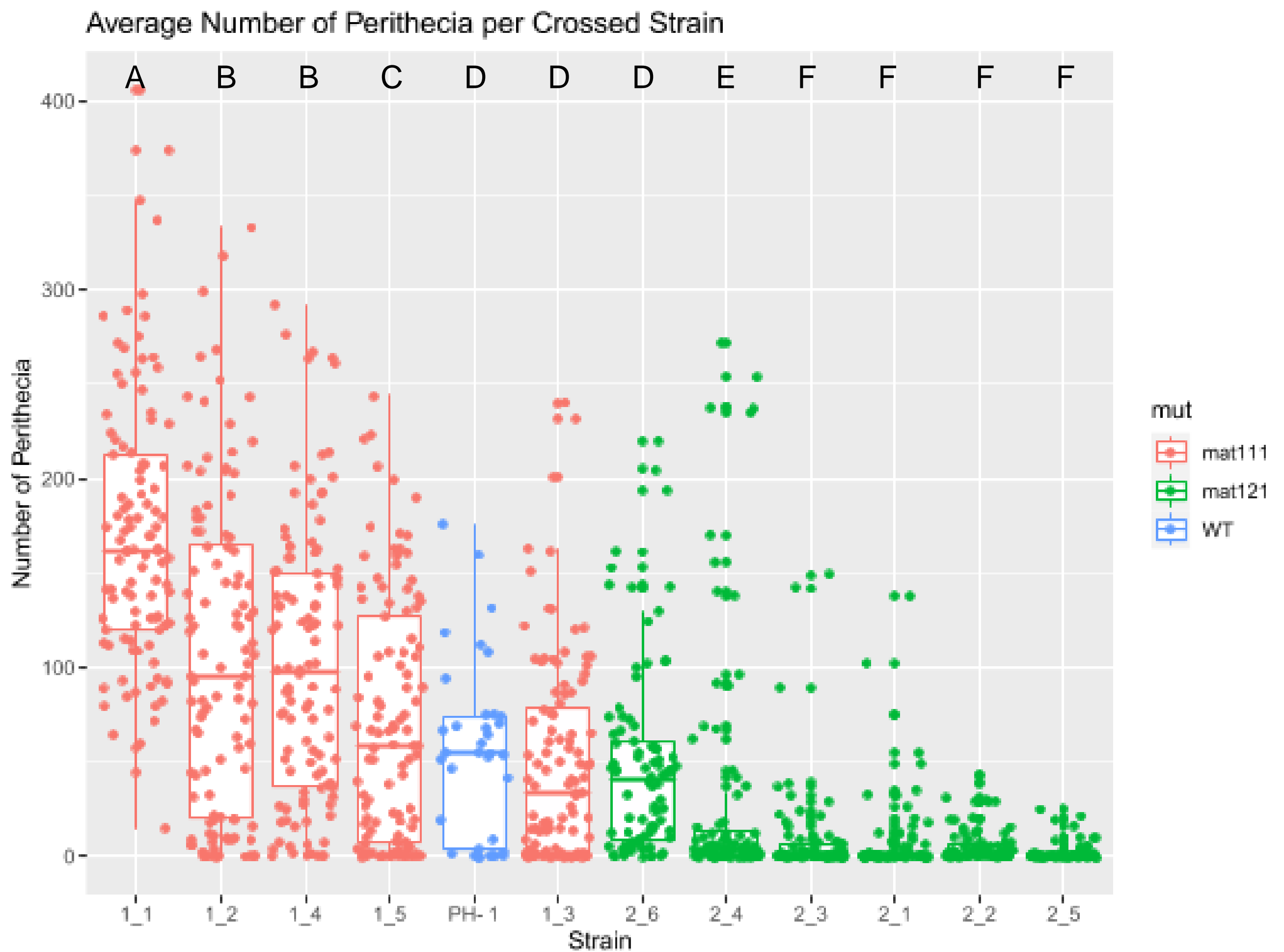


Figure 2.12 Average number of fertile perithecia corresponding to each MAT deletion strain crossed with each other MAT deletion strains.
Each dot shows a single data point. The horizontal bars inside the boxes indicate the mean value of that treatment. The letters on top of the bars represent the significant groupings determined by Scott Knott ($P < 0.001$, $\alpha = 0.05$).

CHAPTER 3. EFFECTS OF *MAT1*, *MAT1-1-1*, AND *MAT1-2-1* DELETIONS ON AGGRESSIVENESS AND TOXIGENICITY ON WHEAT HEADS, AND ON AGGRESSIVENESS TO MAIZE STALKS

3.1 Introduction

The genes residing in the MAT1 locus are master regulators that control development of fruiting bodies and production of sexual spores that serve as primary inoculum of the homothallic Ascomycete fungus *F. graminearum* (R. L. Bowden & Leslie, 1998; Cook, 1981; Yun, Arie, Kaneko, Yoder, & Turgeon, 2000). Previous studies showed that deletion of the entire MAT1 mating locus, or of the *MAT1-1-1* and *MAT1-2-1* mating type genes, results in an inability to produce ascospores by self-fertilization (Bec, 2011; Bec et al., 2021; A. Desjardins et al., 2004; J. Lee et al., 2003). In Chapter 2 of this thesis, I verified that several MAT deletion strains were self-sterile, and that they produce fertile perithecia and ascospores in heterothallic matings. Previous studies have shown that asexual conidia produced by MAT deletion strains induce normal levels of disease when introduced directly into flowering wheat heads in a greenhouse (A. Desjardins et al., 2004; H.-K. Kim et al., 2012; Zheng et al., 2013, Kim et al, 2012.). In contrast, a MAT1 deletion strain did not induce epidemics in a field study when it was introduced on infested maize stalk pieces placed on the ground between the rows. This may have been due to an inability to produce ascospores that could be forcibly ejected high enough into the air to reach and infect the wheat flowers (A. Desjardins et al., 2004). There is also the possibility that the MAT loci play more direct roles in pathogenicity, and that these were more noticeable in the field due to aspects of the environment or host that were not duplicated in the greenhouse studies.

Many Ascomycete fungi seem to have lost the ability to reproduce sexually, but still carry a highly conserved and fully functioning MAT locus (Alvarez-Perez, Blanco, Alba, & Garcia, 2010; Arie et al., 2000; Pöggeler, 2002; Sharon et al., 1996; Turgeon et al., 1995; J. Varga, 2003; Yun et al., 2000). This could support

the idea that the MAT genes have important functions other than in mating. There is also some direct evidence for this. For example, MAT1-1 strains of the heterothallic wheat pathogen *Mycosphaerella graminicola* were more pathogenic than MAT1-2 strains in greenhouse pathogenicity assays (Zhan, Torriani, & McDonald, 2007). The MAT1-1 and the MAT1-2 mating types were equally distributed in the field in most locations, but the frequency was skewed in favor of MAT1-2 in fields that had been treated with fungicide, suggesting that the MAT1-2 mating type may have a role in fungicide tolerance (Zhan, Kema, Waalwijk, & McDonald, 2002). *Fusarium graminearum mat1-1-1* and *mat1-2-1* deletion strains were reportedly 66% and 77% less aggressive to maize stalks, respectively, in comparison with the wild type strain in greenhouse assays (Zheng et al., 2013). In the United States, maize-wheat rotations are common, and maize crop debris is a primary source of ascospore inoculum for FHB (Wegulo, Jackson, Baenziger, Carlson, & Nopsa, 2008). Although the same study indicated that the *mat1-1-1* and *mat1-2-1* deletion strains were unaltered in pathogenicity to wheat heads, results of a single greenhouse pathogenicity assay using our MAT deletion strains on Pioneer 2555 variety hard red winter wheat (HRWW) gave a different result (Bec, 2011). Bec noted a reduction in aggressiveness of the *mat1-1-1* and *mat1-2-1* deletion strains in comparison to the wild type, with the *mat1-1-1* deletions not significantly different from the water controls (Bec, 2011). The role of the MAT genes on pathogenicity of *F. graminearum* to wheat thus remains in question.

An ideal mating tester strain should have normal levels of pathogenicity and toxigenicity if it will be used to study those important traits. Having noted significant variation in morphology and fertility among individual deletion transformants in the previous chapter, my objective in this chapter was to measure the pathogenicity and toxigenicity of the individual MAT deletion transformants on wheat and maize stalks.

3.2 Materials and methods

3.2.1 Fungal Strains, Preparation of Inoculum.

MAT gene deletion strains were constructed earlier (Bec et al., 2021) and are listed in Chapter 2 (**Table 2.1**). All fungal strains were routinely grown at 23°C with constant light (Sylvania F032/741/ECO). Mutant strains were single-spored and stored on silica gel at -20°C or -80°C (Tuite, 1969, after Perkins, 1962). Strains were cultured on PDA for 5 days, before collecting colonies with sterile toothpicks and transferring to sporulation inducing media. Asexual spores (macroconidia) were produced on mungbean agar (40 g mungbean and 10 g Bacto® Agar per L) and/or in carboxymethylcellulose (CMC) shaking cultures (250 rpm), both at 23°C for 10 days. CMC media was prepared by boiling 15 g of carboxymethylcellulose in 500 mL of water until completely dissolved, then adding 1 g of ammonium nitrate, 1 g of potassium dihydrogen phosphate, 0.5 g magnesium sulfate heptahydrate, 1 g of yeast extract, and bringing the volume up to 1 L before autoclaving (Cappellini & Peterson, 1965). Spores were harvested from mungbean agar cultures by applying 2 mL of sterile water to the surface of the Petri plate and rubbing gently with a sterile plastic micro-pestle. Asexual spore suspensions, from mungbean or CMC, were filtered through a double layer of sterile cheesecloth to remove mycelia and collected in a sterile 50 mL Falcon tube. Spores were counted by using a hemocytometer. For use as inoculum, spores were centrifuged at 3330 x g, then washed once in sterile water and resuspended at a concentration of 1×10^5 per mL.

3.2.2 Fusarium Head Blight Pathogenicity Assay.

The moderately resistant hard red spring wheat (HRSW) variety Alsen and the susceptible HRSW variety Wheaton were used for this study. Experiments on Wheaton were done by former students Franklin Machado and Aline de Viera and used here with their permission. Wheat seeds were planted in a mixture of topsoil (Maury silt loam) and PromixBC grown substrate (3:2) in plastic planting cones at a rate of three seeds per Cone-tainer™ (Steuwe and Sons, Inc.). The seeds were

lightly covered with a moist layer of soil mixture. Seeds were germinated in the greenhouse at ambient temperature (25-28°C). After germination, seedlings were thinned to leave one per cone. Wheat plants were grown in a greenhouse with a 14/10 photoperiod, provided by "Hortilux" LU430S/HTL/EN high pressure sodium lights, and ambient temperatures between 25°C-28°C. Plants were fertilized with 150 ppm of N:P:K (20:10:20) fertilizer formulation twice a week, beginning two weeks after transplanting with last fertilization at heading. Flowering typically occurred after 3-4 weeks. At early- to mid- anthesis, a single centrally positioned floret on the primary flowering stem of each plant was inoculated with 10 µL of a 1 x 10^5 per mL spore suspension as described by (Miedaner et al. 2003) and covered with a small plastic bag for 24 hours to increase humidity. The severity of FHB was measured as the number of symptomatic spikelets divided by the total number of spikelets in the head and multiplied by 100 (percentage). Symptom severity was recorded at 7, 10- and 14-days post-inoculation. Each treatment had 15-20 replicates per experiment, and two experiments were performed for each wheat variety. After 14 days, the spikelets were collected and air-dried before processing them for mycotoxin analysis.

3.2.3 DON analysis

Harvested wheat heads from the FHB assay were dried in the greenhouse and kept in a cold room (4°C) until analysis. Mycotoxin production by each of the 14 isolates and the water control was determined by pooling the samples from each experiment, with each bulked sample considered 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, where the amount of DON and its acetylated forms (15ADON and 3ADON), NIV, and ZEA were quantified by using a gas chromatography–mass spectrometry method as described (Fuentes et al., 2005; C. J. Mirocha, Kolaczkowski, Xie, Yu, & Jelen, 1998).

3.2.4 Gibberella Stalk Rot Pathogenicity Assay.

The susceptible hybrid sweet corn line "Golden Jubilee" was used for this study. Three maize seeds were planted in a mixture of topsoil (Maury silt loam) and PromixBC growth substrate (3:2) in plastic 11-inch pots. The seeds were lightly covered with a moist layer of soil mixture. Seeds were germinated in the greenhouse at ambient temperature (25°C-28°C). After germination, seedlings were thinned, leaving two per pot. Maize plants were grown in a greenhouse with a 14/10 photoperiod, provided by "Hortilux" LU430S/HTL/EN high pressure sodium lights, and ambient temperatures between 25-28°C. Plants were fertilized with 150 ppm of N:P:K (20:10:20) fertilizer formulation twice a week beginning two weeks after transplanting, and then daily once they began to produce pollen. At anthesis, the third or fourth internode was punctured to a depth of about 1.27 cm with a sterile needle at a 45° angle. The wounds were inoculated with 100 µL of a 5 x10^5 per ml spore suspension. Wounds were wrapped with parafilm for 24 h. Stalks were harvested after 14 days and photographed after splitting them longitudinally. Lesions were measured by using Fiji (version: 2.0.0-rc-69/1.52p, 2010-2022) and disease severity was expressed as total lesion area (lesion length X lesion width) as a percentage of the total area (length X width) of the internode. Five stalks were inoculated for each strain per experiment, and two experiments were performed.

3.2.5 Data Analysis

All experiments were conducted as a completely randomized design. Data were visualized and analyzed by 95 % Confidence Intervals (CI) and by Scott Knott. CI was done by using 'mean_cl_boot' from the 'Hmisc' package, which implements basic nonparametric bootstrapping to obtain confidence limits for a population of means without assuming a normal distribution (Harrell Jr & Harrell Jr, 2019). CI was performed for multiple experimental replications. If confidence limits of mutants overlapped with the wild type PH-1, these were considered similar. For the Scott Knott, data from multiple replicated experiments were combined. The Scott Knott was used to group the isolates according to the means of

measurements and counts (Jelihovschi et al., 2014).The overall means of measurements (% of disease, number of infected spikelets and ppm of mycotoxin accumulation) of the strain groups were also compared by using the Tukey test, with $\alpha = 0.05$, after performing an analysis of variance. All analyses were run in R (R Core Team 2019).

3.3 Results

3.3.1 Aggressiveness and Mycotoxin Accumulation on Wheat Heads.

Most strains produced typical FHB symptoms on wheat heads, but at 10-14 days post inoculation, disease severity varied among the strains on both wheat lines (**Table 3.1)**. The *mat1-2-1* deletion strains, on average, were less aggressive and less toxigenic than the wild type, but there was no difference between the wild type and the other deletion classes **(Table 3.2)**.

On the susceptible Wheaton, FHB severity ranged from 25% to 80%. All strains were significantly different from the water treatment. Confidence intervals of six strains (2_6, 1_5, 1_4, 1_2, 1_1, 0_2 and 0_1) overlapped with PH-1 in the first experiment, and seven (2_6, 2_2, 1_5, 1_4, 1_2, 1_1 and 0_2) overlapped in the second (**Figure 3.1**). The Scott Knott test defined six significantly different groups at 10 days after inoculation (**Figure 3.2**). For DON measurements, confidence intervals of four strains (2_6, 1_5, 0_2, and 0_1) overlapped with PH-1 in the first experiment, and five (2_2, 1_5, 1_4, 1_2, and 1_1) overlapped in the second (**Figure 3.3**). In the Scott Knott analysis, mycotoxin levels were defined by the same six groups defined by disease severity (**Figure 3.4**).

On moderately resistant Alsen wheat, none of the strains caused disease severities of greater than 25%. Confidence intervals of five strains (2_4, 2_1, 1_5, 1_1, and 0_1) overlapped with the PH-1 in the first experiment, and five (2_6, 2_4, 1_5, 1_1 and 0_1) overlapped in the second (**Figure 3.5**). Two groups were identified by Scott Knott analysis 14 days after inoculation (**Figure 3.6**). The first group included the wild type and at least one member from each class of MAT

deletion mutants. The second group consisted of deletion strains, mostly *mat1-2-1*, but also *mat1-1-1*, grouped with the water control. Mycotoxin data have not been produced yet for the Alsen experiment, although the wheat heads have been preserved.

3.3.2 Aggressiveness on susceptible maize stalks.

Only discoloration of the original inoculated internode was considered for this analysis. Summaries of the data for individual strains are presented in (**Table 3.1**). Overall, the *mat1-1-1* and *mat1-2-1* deletion strain classes were less aggressive to maize stalks, as they were to both varieties of wheat (**Table 3.2**). However, there was no positive or negative correlation between disease severities on the various hosts based on the Pearson's correlation (**Table 3.3**).

Confidence intervals of eight strains (2_6, 2_4, 1_5, 1_4, 1_3, 1_1, 0_2, and 0_1) overlapped with PH-1 in experiment one, and 10 (2_6, 2_5, 2_4, 2_2, 2_1, 1_5, 1_4, 1_3, and 0_2, and 0_1) overlapped in experiment two (**Figure 3.7**). Most of the strains caused necrotic lesions that encompassed more than half of the total internodal area, and some strains (2_6, 1_3, and 1_4) even produced lesions that spread beyond the inoculated internode. Application of the Scott Knott test defined three significantly different groups (**Figure 3.8**). All the MAT1 locus deletion strains, one of the *mat1-2-1* deletion strains, and three of the *mat1-1-1* deletion strains were grouped with the WT in the first group. Other strains were less aggressive than the wild type, including one velvet strain (2_3) that was not significantly different from the water control. The *mat1-2-1* strain 2_3 was also less aggressive on Alsen wheat and grouped with the water.

3.4 Discussion

Earlier studies (Desjardins et al. 2004, Bec 2011) reported that deletion of the entire MAT1 locus had no significant effect on pathogenicity to wheat in greenhouse studies. Bec (2011) reported a significant reduction in aggressiveness

of *mat1-1-1* deletion strains and *mat1-2-1* deletion strains to HRWW, but Zheng et al. (2013) indicated that these deletions had no effect in a variety of HRSW. In my study, deletion of MAT1 and *mat1-1-1* had no overall effect on pathogenicity to wheat, while the *mat1-2-1* deletions were less aggressive on average. However, results for individual strains were more variable. The two MAT1 locus deletion strains were each less aggressive on one of the two varieties of spring wheat, and one of the *mat1-1-1* deletion strains (1_3) was significantly less aggressive than the wild type on both wheat varieties. There was also one *mat1-2-1* deletion strain (2_2) that did not differ from the wild type on both varieties. All strains were less aggressive on Alsen wheat versus Wheaton, as expected, and the *mat1-2-1* strains were also less aggressive on average than the wild type on this variety. The *mat1-2-1* deletion strain 2_4 grouped with the wild type on Alsen, even though it was one of the least aggressive on Wheaton. A correlation analysis suggested that there was no significant relationship among the strains in their aggressiveness to the two wheat varieties or maize. However, it is important to note that the relatively small amount of data here may not allow the analysis to give fully robust results. The fact that at least some strains of each deletion type were not significantly different from the wild type suggests that the deletions themselves have no direct effect on pathogenicity to wheat.

Zheng et al. 2013 reported that deletions of *MAT1-1-1* or *MAT1-2-1* both resulted in a reduction in pathogenicity to maize stalks. My results showed an overall reduction in aggressiveness of the *mat1-2-1* deletion strains, but not of the MAT1 or *mat1-1-1* deletions. However, there were individual strains in both groups of transformants that were less aggressive than the wild type, while some seemed to be more aggressive, even producing lesions beyond the inoculated internode. Unfortunately, the experiment was left longer than ideal, so the more aggressive strains could not be differentiated from one another. This experiment should be repeated with an earlier harvest or use of a maize line that is less susceptible than Golden Jubilee.

One *mat1-2-1* deletion strain, 2_3, was consistently less pathogenic to both wheat and maize. This strain also had the velvet phenotype, was very low in

fertility, and produced very few spores. It is relatively common for degraded pathogenic fungi with a reduced ability to produce spores or fruiting bodies to also lose virulence (Al-Aidroos & Seifert, 1980; Dumas & Papierok, 1989; Hajek, Humber, & Griggs, 1990; Kawakami, 1960; Latch, 1965; Lord & Roberts, 1986; Morrow & Boucias, 1988; Nagaich, 1973; Rockwood, 1950; Samšiňáková & Kalalova, 1983; Schaerffenberg, 1964; Shah et al., 2005; Wang et al., 2003).

In the previous chapter I reported that the independent deletion strains were highly variable in morphology and fertility, and in this chapter, I show that they were also highly variable in their aggressiveness to wheat and maize. The report by (Zheng et al., 2013) evaluated three independent deletions of each type and indicated that they were all similar. Their report does not specify whether all these independent mutant strains were used in their pathogenicity assays, however. Desjardins et al. (2004) tested 38 deletion strains and found no major differences in aggressiveness from the wild type or from each other. They did mention, however, that MAT1 deletion strains from an earlier transformation experiment were less aggressive, linking it to the protoplast batch used. It is difficult to say why this group of transformants I am using is so variable in comparison to these earlier reports. Differences in the protoplasts could be important, since each type of deletion strain was produced with a different batch of protoplasts (Bec, 2011). It may have something to do with the fact that they were produced several years ago and stored in the meantime, so they may have accumulated mutations. It may relate to the use of the split marker method to generate these strains, although this method was also used by Zheng et al. (2013). It may relate to the background strain: Desjardins used the Gz3639 strain that anecdotally is more stable than PH-1 (F. Trail, J. Leslie, personal communication). However, Zheng et al. (2013) also used PH-1. It is possible that other groups have noticed degenerated or abnormal strains among their transformants and simply discarded them without reporting it. Many factors can affect pathogenicity including light, temperature, and nutrient status and our plants may experience differences in these factors compared with other studies that exacerbate differences among strains. Finding the answer to this question will require more work. However, despite the variability among strains in

my study, there are some strains that appear stable, with high fertility, and that are morphologically and pathogenically normal, and these should be suitable as tester strains if they can undergo heterothallic matings and produce progeny with normal marker segregation patterns. This question will be the topic of the next chapter.

Table 3.1 Disease Severities (%) and Mycotoxin Accumulation (ppm) for wild type and MAT mutant strains on two wheat lines.

Significantly different groups were determined using Scott Knott. Different superscript letters indicate significantly different mean values. $P < 0.01$, $\alpha = 0.05$.

	FHB on Wheaton		FHB on Alsen	GSR on Maize
Strain	Mean Severity (%)	Mean Mycotoxon (ppm)	Mean Severity (%)	Mean Severity (%)
0_1	53.15^{c}± 36.09	119.57^{b}± 77.97	30.09^{a}± 30.12	66.89^{a}± 17.53
0_2	76.55^{a}± 24.33	183.40^{a}± 35.1	8.94^{b}± 9.68	74.35^{a}± 13.76
1_1	60.69^{b}± 32.16	118.24^{b}± 50.71	22.61^{a}± 26.88	47.51^{b}± 19.13
1_2	72.68^{a}± 27.17	169.84^{a}± 37.72	6.01^{b}± 3.41	38.70^{c}± 13.05
1_3	43.59^{d}± 30.98	86.02^{c}± 49.81	4.68^{b}± 3.35	59.66^{b}± 31.25
1_4	65.10^{b}± 28.66	148.10^{b}± 46.98	7.18^{b}± 5.98	61.19^{b}± 20.69
1_5	73.99^{a}± 24.26	182.71^{a}± 32.34	28.07^{a}± 23.32	64.44^{a}± 20.04
2_1	14.44^{e}± 12.6	34.23^{d}± 24.39	8.60^{b}± 8.14	35.11^{c}± 18.71
2_2	50.70^{c}± 33.57	114.47^{b}± 40.26	2.93^{b}± 3.56	54.82^{b}± 15.45
2_3	36.41^{d}± 31.58	72.98^{c}± 55.88	4.39^{b}± 3.54	20.18^{d}± 17.58
2_4	20.85^{e}± 16.56	30.41^{d}± 20.61	20.85^{a}± 23.38	66.50^{a}± 7.85
2_5	23.16^{e}± 18.54	40.49^{d}± 31.6	5.44^{b}± 2.72	53.55^{b}± 19.69
2_6	82.69^{a}± 21.16	204.96^{a}± 26.34	10.44^{b}± 18.24	68.87^{a}± 16.78
mock	0.00^{f}± 0	0.29^{d}± 0.25	0.00^{b}± 0	10.18^{d}± 12.7
PH-1	70.88^{a}± 29.6	162.65^{a}± 51.41	22.40^{a}± 21.98	69.64^{a}± 13.85

Table 3.2 Summary table for overall results of wild type and MAT deletion strains.
Letters indicate the difference is significant at α= 0.05, P value=<0.001 (significant differences from the wild type are highlighted for the *mat1-1-1* and *mat1-2-1*).

Strain Class	Severity Wheaton	Severity Alsen	Mycotoxin on Wheaton (ppm)	Severity Maize Stalk
PH-1 (WT)	$70.9^{a} \pm 29.6$	$22.40^{a} \pm 21.9$	$162.6^{a} \pm 51.4$	$69.6^{a} \pm 51.4$
mat1-1-1	$63.3^{a} \pm 30.5$	$13.77^{b} \pm 18.8$	$137.2^{a} \pm 55.1$	$54.30^{b} \pm 22.9$
mat1-2-1	$38.0^{b} \pm 33.0$	$8.78^{c} \pm 13.9$	$76.0^{b} \pm 68.4$	$49.8^{b} \pm 23.4$
MAT1	$64.9^{a} \pm 32.8$	$19.52^{a} \pm 24.6$	$151.5^{a} \pm 67.3$	$70.6^{a} \pm 15.8$

Table 3.3 Correlation of Disease Severity of strains on different hosts. PH-1 (yellow), mat1-1-1 (green), and mat1-2-1 (orange). 95 % confidence interval.

Correlation Disease Severity % of strains on different hosts

PH-1	Alsen	Wheaton	Golden Jubilee
Alsen	1	0.07197845	0.1498363
Wheaton	0.07197845	1	-0.5153479
Golden Jubilee	0.1498363	-0.5153479	1

mat1-1-1	Alsen	Wheaton	Golden Jubilee
Alsen	1	-0.262638	0.41889
Wheaton	-0.262638	1	-0.5082946
Golden Jubilee	0.41889	-0.5082946	1

mat1-2-1	Alsen	Wheaton	Golden Jubilee
Alsen	1	0.3643069	0.2563893
Wheaton	0.3643069	1	-0.1435806
Golden Jubilee	0.2563893	-0.1435806	1

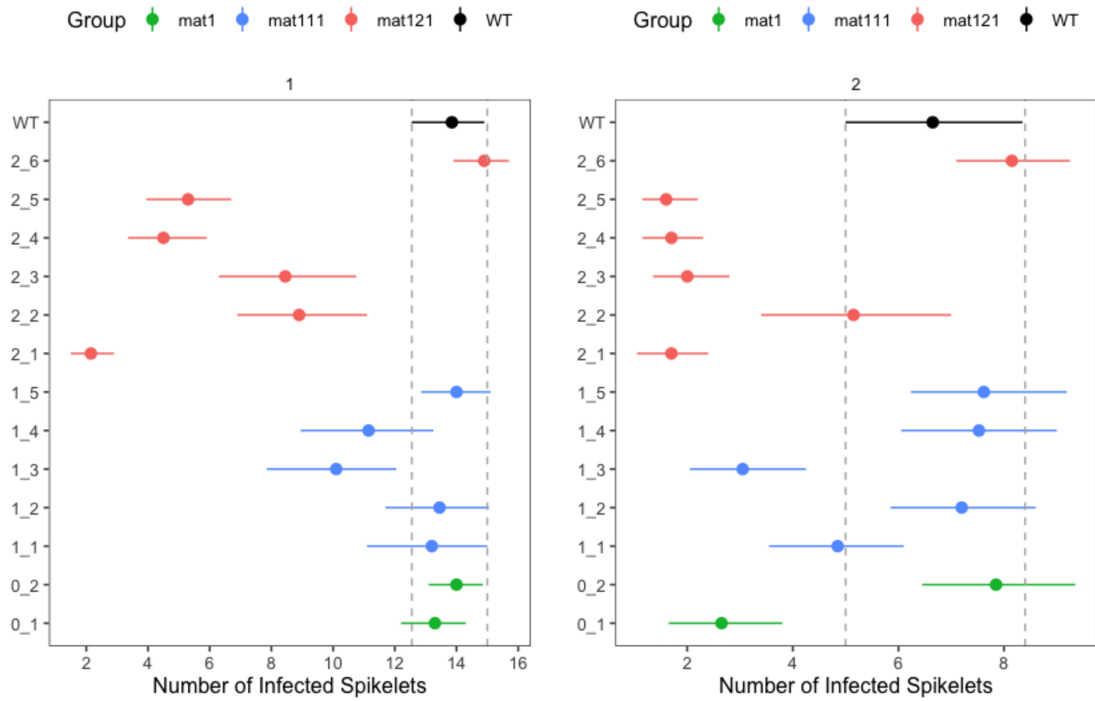


Figure 3.1 Average number of infected spikelets [95% confidence interval (CI)] on Wheaton of wild type and MAT deletion strains *of Fusarium graminearum* for two experiments.
Points represent mean number of infected spikelets for each strain. Dashed lines correspond to PH-1 CI. Strains that overlap with PH-1, behave similarly to PH-1.

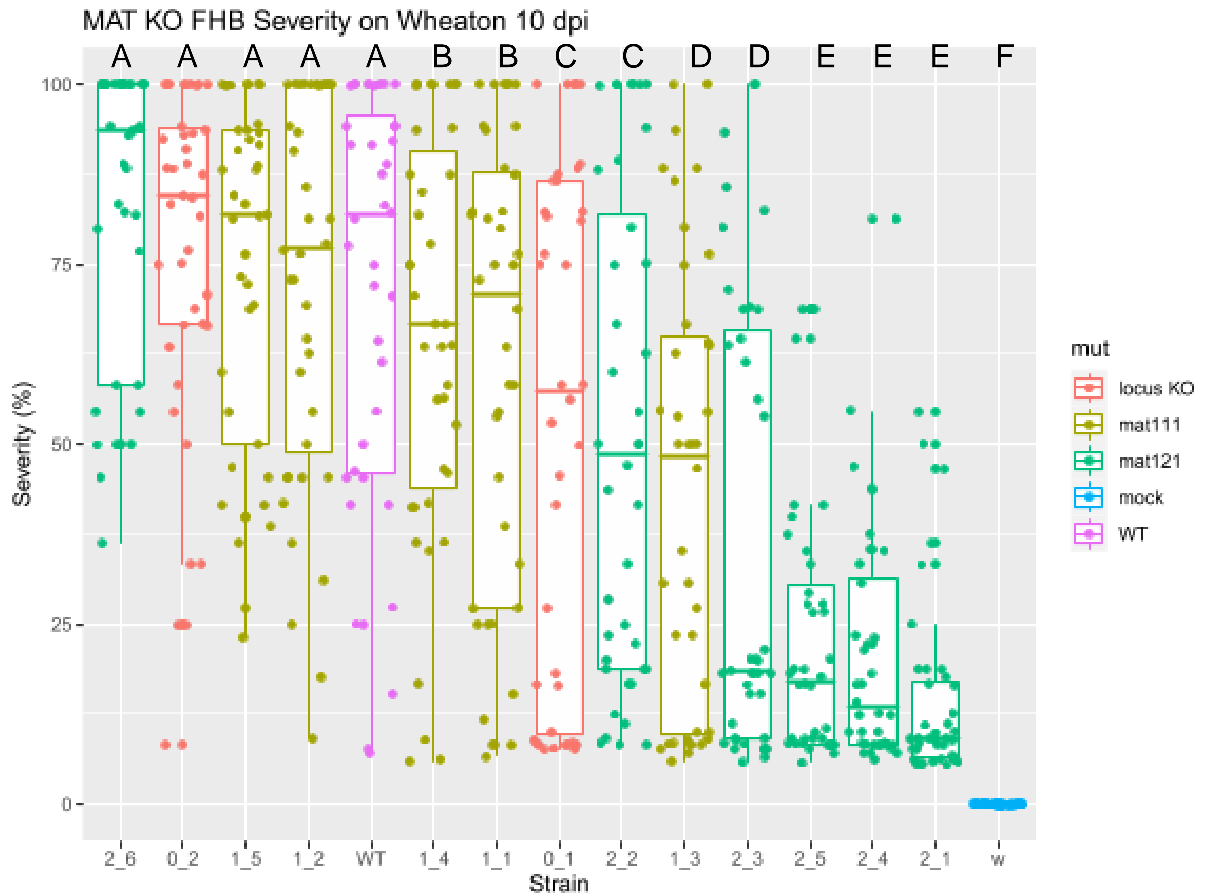


Figure 3.2 Mean FHB Severity on susceptible wheat "Wheaton" at and 10 days post inoculation of *mat1-1-1* and *mat1-2-1* deletion strains.
Each dot shows a single data point. The horizontal bars inside the boxes indicate the mean value of that treatment. The letters on top of the bars represent the significant groupings determined by Scott Knott (P <0.001, α=0.05). These data were collected by Dr. Franklin Machado and Dr. Aline Viera De Barros and used with permission.

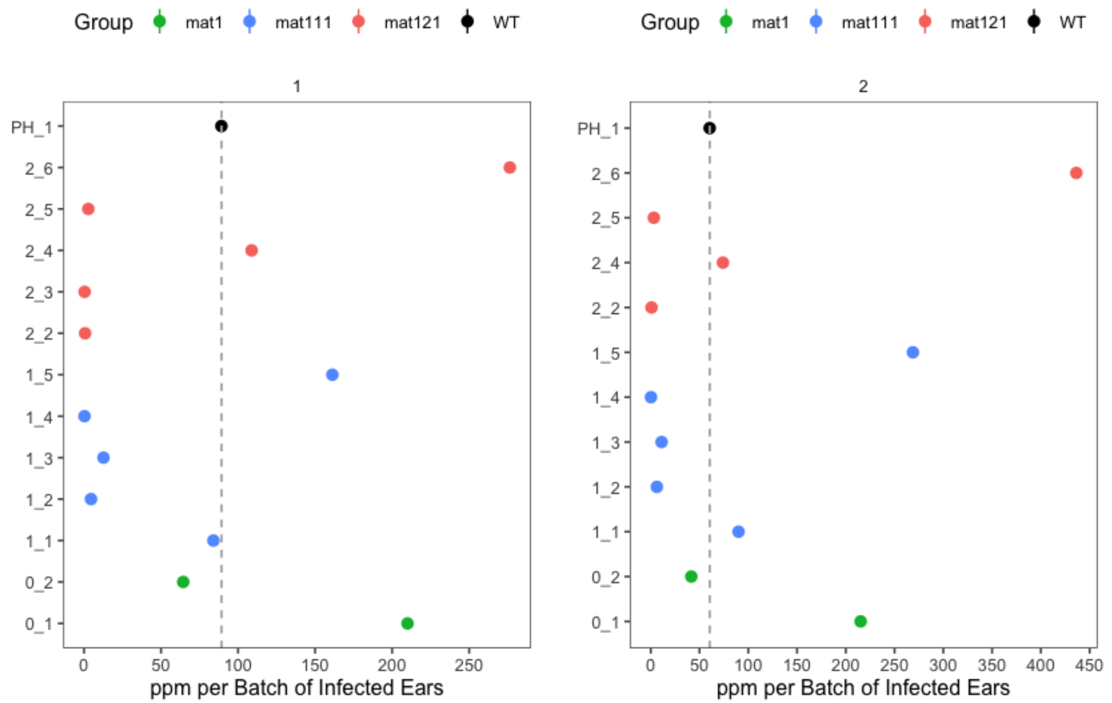


Figure 3.3 Average amount (ppm) of DON on infected wheat head batches [95% confidence interval (CI)] of wild type and MAT deletion strains *of Fusarium graminearum* for two experiments.

Points represent mean amount (ppm) of DON on infected Wheaton wheat heads for each strain. Dashed lines correspond to PH-1 CI. Strains that overlap with PH-1, behave similarly to PH-1.

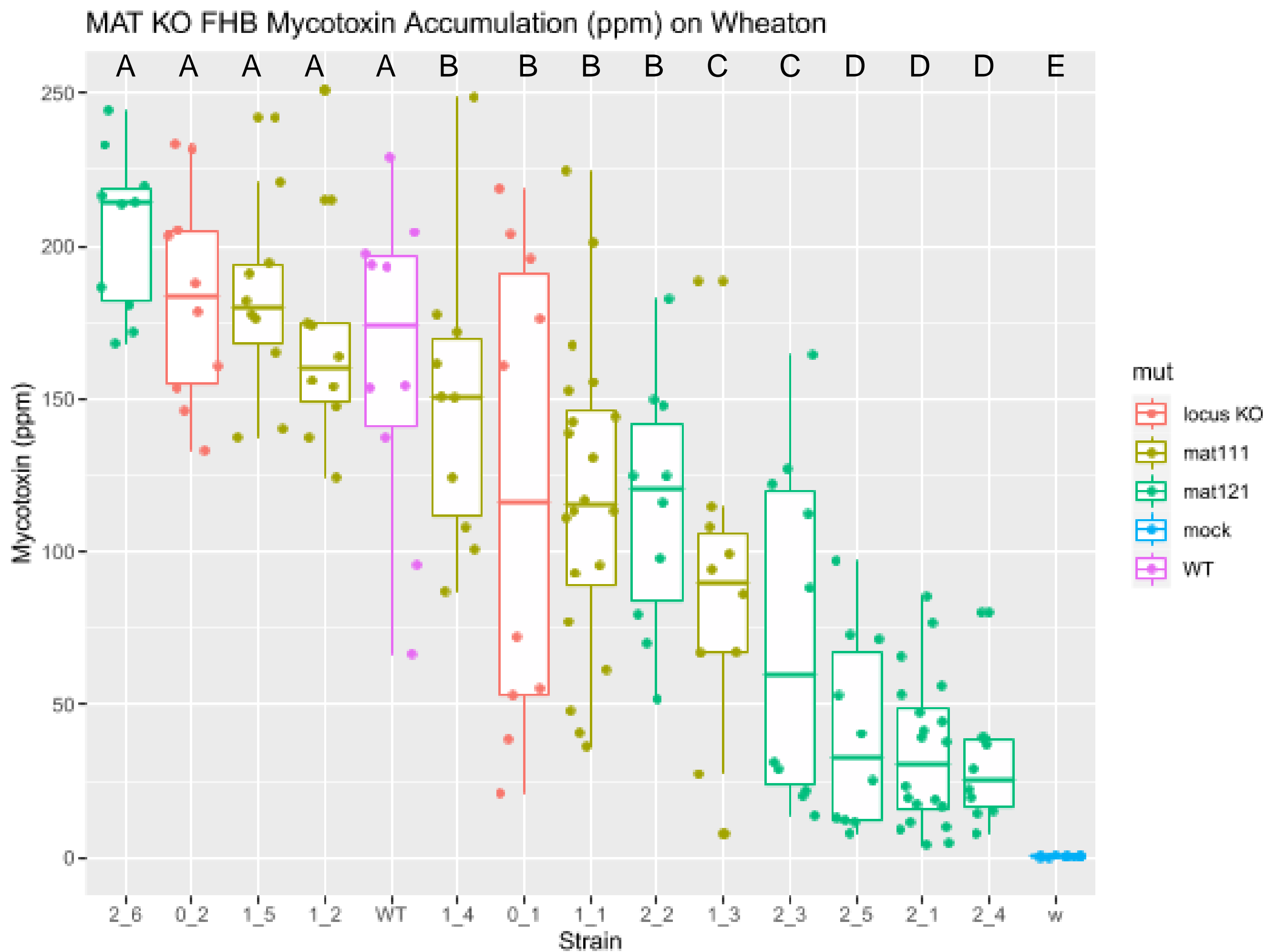


Figure 3.4 Mycotoxin Accumulation on "Wheaton" 10 days posts inoculation of *mat1-1-1* and *mat1-2-1* deletion strains.
Each dot shows a single data point. The horizontal bars inside the boxes indicate the mean value of that treatment. The letters on top of the bars represent the significant groupings determined by Scott Knott (P <0.001, α=0.05). These data were collected by Dr. Franklin Machado and Dr. Aline Viera De Barros and used with permission.

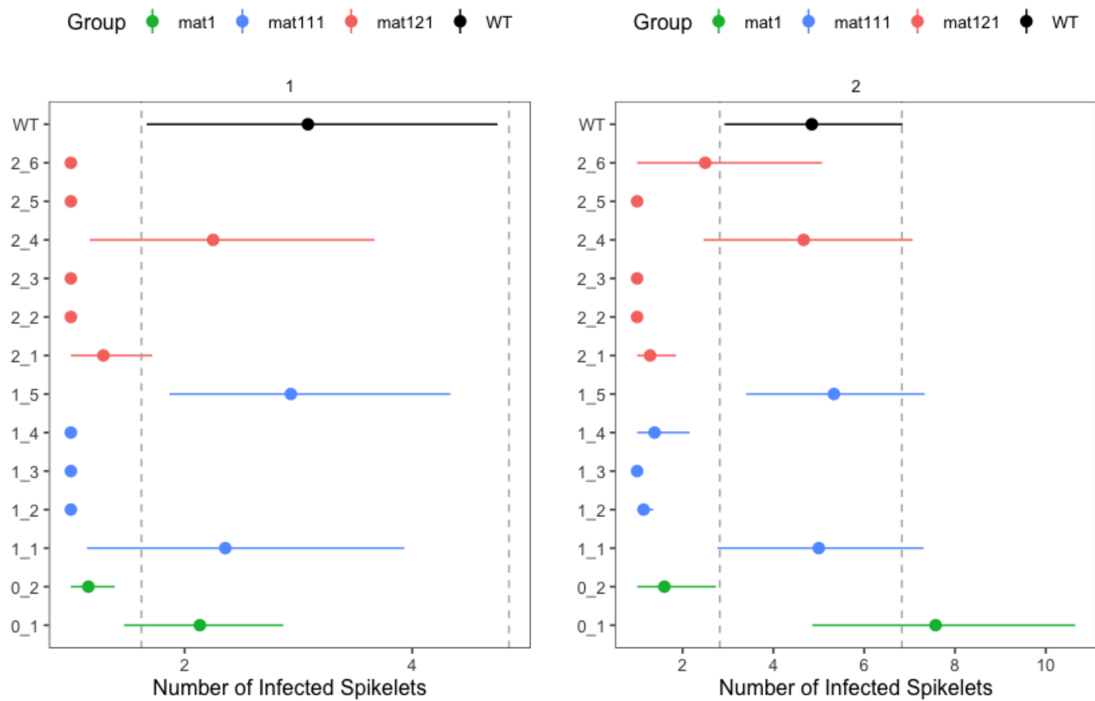


Figure 3.5 Average number of infected spikelets [95% confidence interval (CI)] on Alsen of wild type and MAT deletion strains *of Fusarium graminearum* for two experiments.
Points represent mean number of infected spikelets for each strain. Dashed lines correspond to PH-1 CI. Strains that overlap with PH-1, behave similarly to PH-1.

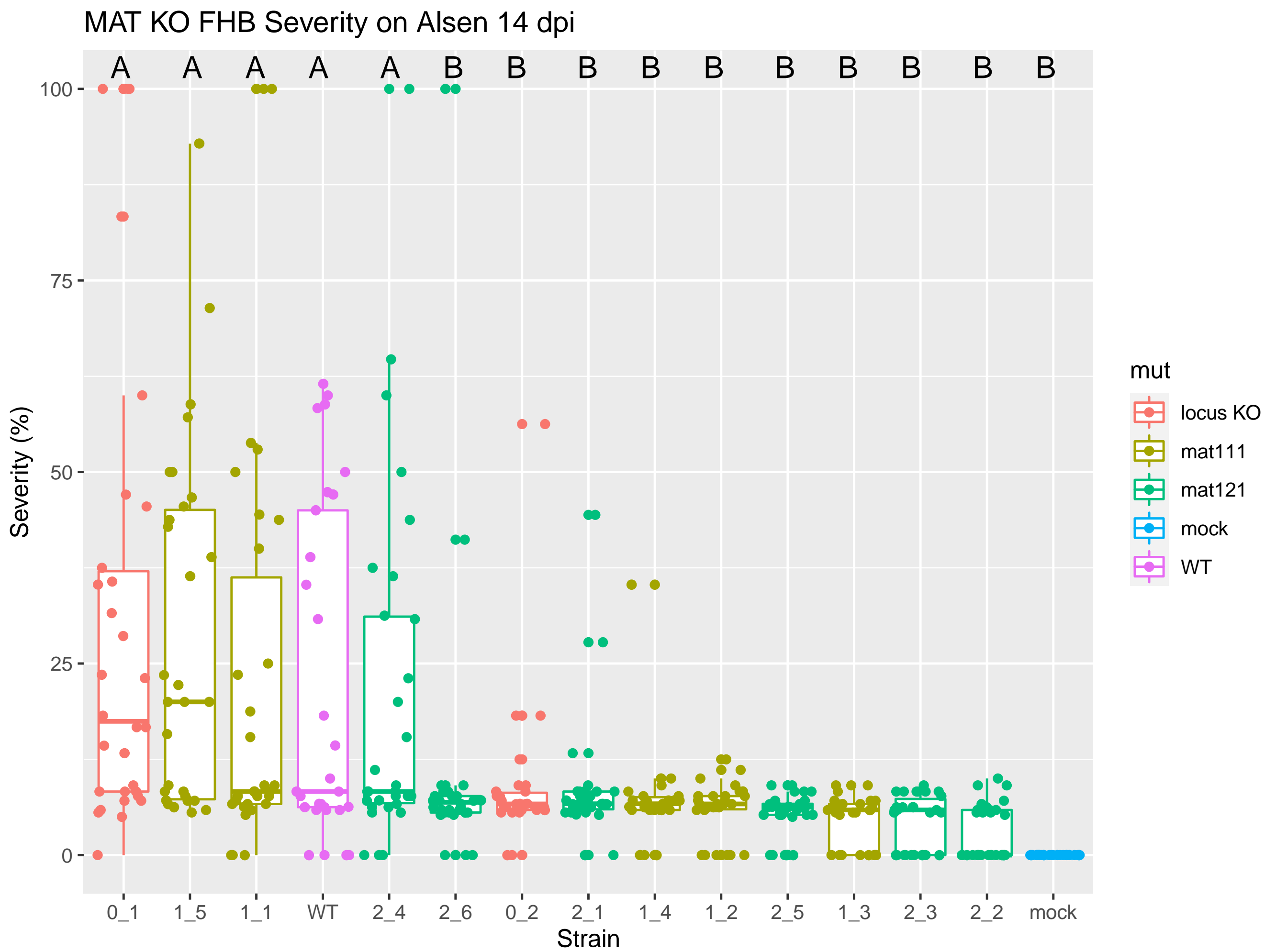


Figure 3.6 Mean FHB Severity on partially resistant wheat "Alsen" at 14 days post inoculation of *mat1-1-1* and *mat1-2-1* deletion strains.
Each dot shows a single data point. The horizontal bars inside the boxes indicate the mean value of that treatment. The letters on top of the bars represent the significant groupings determined by Scott Knott ($P < 0.001$, $\alpha = 0.05$).

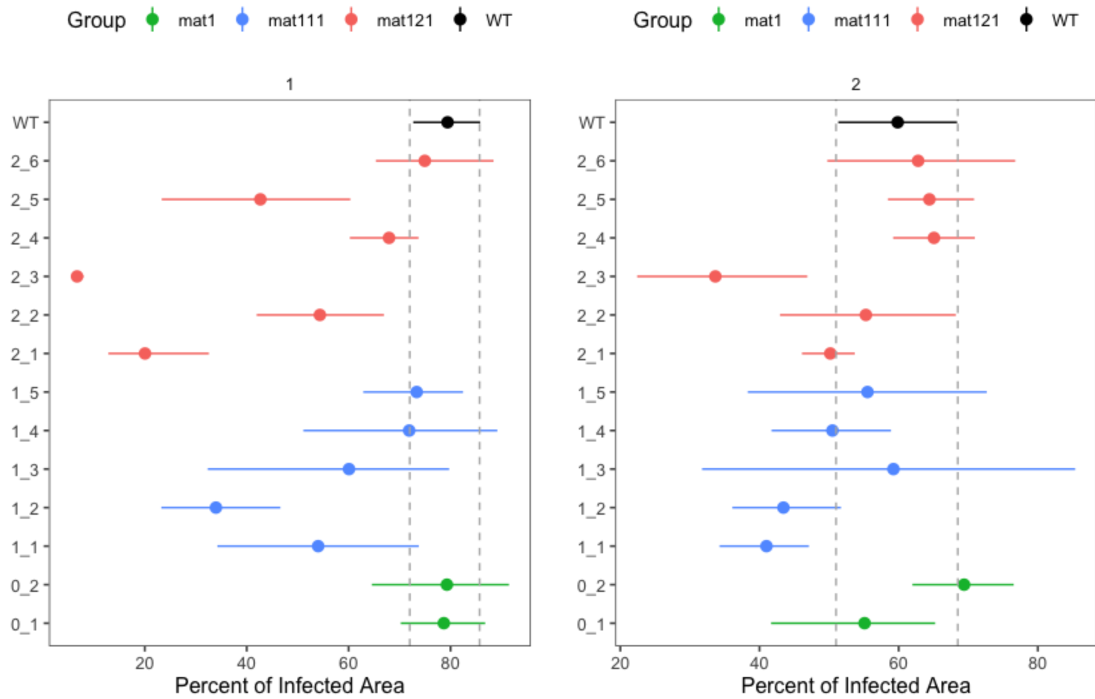


Figure 3.7 Average infected area [95% confidence interval (CI)] on Golden Jubilee of wild type and MAT deletion strains *of Fusarium graminearum* for two experiments.
Points represent mean infected area for each strain. Dashed lines correspond to PH-1 CI. Strains that overlap with PH-1, behave similarly to PH-1.

MAT KO GSR Severity on Golden Jubilee 14 dpi

A A A A A A A A B B B C C D D

Severity (%)

100 75 50 25 0

0_2 WT 2_6 0_1 2_4 1_5 1_4 1_3 2_2 2_5 1_1 1_2 2_1 2_3 mock

Strain

mut: locus KO, mat111, mat121, mock, WT

Figure 3.8 Mean Percent of Diseased internodal area 14 days post inoculation of wild type and *mat1-1-1* and *mat1-2-1* deletion strains.
Each dot shows a single data point. The horizontal bars inside the boxes indicate the mean value of that treatment. The letters above the bars represent the significant groupings determined by Scott Knott (P <0.001, α=0.05). The figure below shows representative symptoms produced by each strain (presented in the same order as in the graph, left to right).

CHAPTER 4. MATINGS AND ANALYSIS OF MARKER SEGREGATION AMONG Δ*MAT1-1-1*, Δ*MAT1-2-1*, AND WILD TYPE STRAINS.

4.1 Introduction

Although *F. graminearum* is homothallic and primarily undergoes self-fertilization, the heterogeneity of the population in the field suggests that outcrossing is frequent (Robert L Bowden & Leslie, 1992; A. C. Kelly et al., 2015; Walker, Leath, Hagler Jr, & Murphy, 2001; Zeller, Bowden, & Leslie, 2004). Direct evidence for recombination has been reported from populations of *F. graminearum* in North America and Europe (A. C. Kelly & Ward, 2018; Talas, 2016; F. Talas & B. A. McDonald, 2015). There is also evidence for less frequent recombination among different phylogenetic species within the FSASC that cause FHB (Boutigny et al., 2011; Leslie, Zeller, & Summerell, 2001; O'Donnell et al., 2000). A better understanding of population diversity and how populations can evolve through recombination could improve risk assessment for FHB. An ability to conduct genetic analyses of genotype-phenotype associations through controlled crosses would help us to increase our understanding.

Outcrosses of *F. graminearum* in the laboratory occur at frequencies of up to 35%, so genetic analysis via controlled crosses is a possibility (R. L. Bowden & Leslie, 1998). However, identification of the minority of perithecia that result from heterothallic outcrossing versus homothallic inbreeding requires the incorporation of genetic markers. For most of the previous studies of heterothallic mating in *F. graminearum*, each parent was marked with a different complementary unlinked nitrate utilization (NIT) marker (*NIT1* and *NITM*). Only recombinant strains can grow on nitrate medium, thus facilitating identification of crossed perithecia. In a cross of NIT-marked strains of *F. graminearum*, the NIT markers and vegetative incompatibility markers segregated as expected, confirming the potential of heterothallic matings for genetic analysis (R. L. Bowden & Leslie, 1998). The importance of outcrossing and its contribution in the field has been observed as well by crossing NIT-marked strains of *F. graminearum*, and inoculating wheat in

the field with the progeny population at different locations (Voss et al., 2010). Aggressiveness of the progeny varied across a normal distribution. Analysis of the segregating populations across environments showed variation in aggressiveness and DON were strongly influenced by pathogen genetics, with heritability ratings of 0.5-0.7 (Voss et al., 2010). Transgressive strains that are more aggressive than either parent can result from crosses (Voss et al., 2010). (Cumagun & Miedaner, 2004).

Controlled crosses among phylospecies within the FSASC are also possible (R. L. Bowden & Leslie, 1998). Although there is lower fertility between phylospecies versus within them (R. L. Bowden & Leslie, 1998), it is nonetheless possible to do a genetic analysis with crosses among these strains (Jurgenson et al., 2002). In a cross of NIT-marked strains of *F. graminearum* producing DON, and another phylospecies, *F. asiaticum*, which produces nivalenol (NIV), the *TRI5* gene responsible for controlling mycotoxin type segregated in the expected 1:1 ratio. However crossover distribution across linkage groups was non-random (Leslie et al., 2001) and the progeny carrying parental linkage groups were over-represented (Jurgenson et al., 2002).

Incorporation of NIT markers needs to occur in both parents, and multiple cirrhi need to be picked and individually screened to identify heterothallic perithecia. Bec (2011) incorporated a green fluorescent protein (GFP) marker into a tester parent and was able to screen quickly for heterothallic matings by looking for green fluorescent cirrhi among the majority of dark cirrhi from homothallic perithecia produced by the unmarked parent as a female. Analysis of segregation patterns of molecular markers among these strains confirmed that they segregated in a 1:1 expected ratio. This method required less effort, and increased capability to screen multiple strains as the female parent.

Presence of the GFP transgene complicates utilization of these strains for field studies. Deletions created by new gene editing technologies e.g. CRISPR could be a useful approach for eventual production of strains that could be evaluated in the field. MAT deletion strains might be well-suited for the purpose, because they have already been demonstrated to undergo heterothallic matings with compatible

deletion mutants or wild type strains, and to produce expected marker segregation ratios (J. Lee et al., 2003). They would also be unable to self, which might decrease risk of escape in a field setting. In the previous two chapters I evaluated the morphological and pathogenicity phenotypes of a group of MAT deletion strains. I observed that, although all lost self-fertility and were capable of cross-fertility, individual deletion transformants displayed a range of quantitative phenotypes. Because of this, I wanted to confirm the ability of the strains to undergo normal heterothallic matings that produced progeny with expected segregation patterns for molecular and phenotypic markers. In this third chapter of my thesis, I present the results of this investigation.

4.2 Materials and methods

4.2.1 Fungal Strains and Culture.

Fungal strains used in this chapter are summarized in (**Table 4.1**). All strains were routinely grown at 23°C with constant light (Sylvania F032/741/ECO). Strains were single-spored and stored on silica gel at -20°C or -80°C (Tuite, 1969, after Perkins, 1962). Strains were started on PDA for 5 days, before collecting colonies with sterile toothpicks or cutting plugs to subculture on sporulation inducing media. Production of asexual spores (macroconidia) was done on mungbean agar (MBA) (40 g mungbean and 10 g Bacto® Agar per L) at 23°C for 7-10 days. Mungbean agar was prepared by boiling 40 g of mungbeans in 1 L of water for about 23 min or until the beans began to split. Beans were filtered out by using a double layer of cheesecloth, the liquid was measured, and water was added to 1 L. Ten grams of Bacto® Agar were added before autoclaving. To harvest spores, 2 mL of sterile water was applied to the surface of the Petri plate and then spores were released by rubbing with a sterile plastic micro-pestle. Asexual spore suspensions were filtered through a double layer of sterile cheesecloth to remove mycelia and collected in a sterile 50 mL Falcon tube. Spores were counted by using a

hemocytometer. Spores were centrifuged at 3330 x g, then washed once in sterile water and resuspended at a concentration of 1 x 10^5 per mL.

4.2.2 Crossing Procedure and Collection of Random Progeny.

Crosses were made on carrot agar media (CAM) (Klittich & Leslie, 1988). CAM was prepared by peeling and chopping 400 g of carrots into 1-inch pieces and autoclaving in 1 L of water. After cooling, the carrots were processed to a slurry in a food processor. The slurry was measured, and water added to 1 L. Fifteen g of Bacto® agar was added before autoclaving. Strains were crossed by using a modification of the method of (R. L. Bowden, and Leslie, J. F., 1999). A 10 µL drop of spore suspension (1 X 10^5 per mL) of each mate was placed on each side of a 60 mm Petri Plate containing CAM. After four days of incubation at 23°C with constant fluorescent light, perithecial production was induced by applying 1 mL of sterile 2.5% Tween 60 to the surface of each plate, and gently rubbing with a sterile glass rod to flatten the culture and remove excess mycelia. Rubbing the surface ensured distribution of spores of both strains across the entire plate. Following induction, the plates were incubated at 23°C with constant fluorescent light until perithecial maturation. Two-three weeks after induction, the mature perithecia usually extruded cirrhi that each contained several thousand ascospores. A sterile glass needle was used to pick up individual cirrhi, which were then dispersed in 200 µL water and spread out on 2% water agar. After 10-14 hours of growth, isolates arising from single ascospores were observed under a microscope and transferred to individual 60 mm Petri plates. Twenty-four single ascospores were collected from each perithecium, and four perithecia were collected per cross.

4.2.3 DNA extraction.

Five or eight mL of YEPD medium (20 g dextrose, 20 g bacto-peptone, 10 g yeast extract per L) was inoculated with a fragment taken with a sterile yellow pipette tip from the edge of an actively growing colony. Cultures were incubated at

25°C for 5-7 days at 250 rpm. Recovered mycelia were flash-frozen in liquid nitrogen, lyophilized, and pulverized in individual 2 ml Eppendorf tubes by using a mini-pestle, or in 96-deep well plates by using a 2000 GENO Grinder (Spex Cretiprep) (500 strokes/sec for 30 sec). Two hundred µl of warm lysis buffer (0.5 M NaCl, 1% SDS, 10 mM Tris HCl, Ph7.5, 10 mM EDTA) was added per approximately 50 mg fungal tissue, and samples were incubated for 30 min, gently shaking once during the incubation. After incubation two hundred µl of PCI (25 parts phenol, 24 parts chloroform, 1-part iso-amyl-alcohol), mixed by gently shaking two times, then incubated at 65°C for an additional 30 min. The contents were mixed once again during incubation. The samples on plates were centrifuged in a tabletop centrifuge for 20 min at maximum speed to separate the phases. The aqueous phase was then transferred to new tubes or a new 2 mL 96 well plate and then DNA was precipitated from the aqueous phase by using 1 volume of isopropanol. The resulting pellet was washed twice with 70% ethanol and then resuspended in 50 µl of TE, pH 7.9, or sterile water with 2 µl of a 5-mg/ml concentration of RNase A, at 65°C for 1 h.

4.2.4 Progeny Phenotyping.

Cultures obtained from single ascospores were subcultured on hygromycin B amended PDA plates (50 µg/mL Hygromycin B). The strains were cultured from 2-4 days at 23°C with constant light (Sylvania F032/741/ECO). Colonies were evaluated for hygromycin resistance, and for colony morphology on unamended PDA. Progeny strains were also cultured on CAM for 7 days, or until the mycelium reached the edges of the plates, and then the mycelia were flattened to induce perithecia, as described above. After 14 days the strains were observed under the dissecting microscope, and individual perithecia were crushed and observed under the compound microscope, to determine fertility. A visual flow chart of these procedures is provided in (**Figure 4.1**).

4.2.5 Progeny Genotyping.

Segregation of several molecular markers was evaluated as appropriate for each cross. *MAT1-1-1* and *MAT1-2-1* specific markers were identified in a multiplex PCR analysis as described in Chapter 2. Cleaved Amplified Polymorphic Sequences (CAPS) markers were utilized for analysis of the cross between PH-1 and Gz3639 as described by (Bec, 2011; Bec et al., 2015). Chemotyping using PCR was done by using the TRI3 multiplex assay described by (Ward et al. 2008) to identify 15ADON, 3ADON, or NIV chemotypes. Each PCR reaction consisted of a 20 µl total reaction volume including 2 µl of 10X PCR buffer, 1.6 µl of 25 mM MgCl2, 2 µl of 10 mM dNTP mix, 1 µl of each primer (10 nM) (both MAT primer sets were used for the MAT multiplex) and 1 µl of template DNA (20-50 ng/l),10.7 µl of water and 0.7 µl of Taq polymerase. A strain of DH5a containing the pTAQ gene was provided by Dr. Pradeep Kachroo, and preparation of the Taq polymerase enzyme was as described in (Desai & Pfaffle, 1995). All primers are listed in (**Table 4.2**)**.**

4.2.6 FHB pathogenicity assay.

The susceptible HRSW variety Wheaton was used. Wheat seeds were planted in a mixture of topsoil (Maury silt loam) and PromixBC grown substrate (3:2) in plastic planting cones at a rate of three seeds per Cone-tainer ™ (Steuwe and Sons, Inc.). The seeds were lightly covered with a moist layer of soil mixture. Seeds were germinated in the greenhouse at ambient temperature (25°C-28°C). After germination, seedlings were thinned to one per pot. Wheat plants were grown in a greenhouse with a 14/10 photoperiod, provided by "Hortilux" LU430S/HTL/EN high pressure sodium lights, and ambient temperatures between 25°C-28°C. Plants were fertilized with 150 ppm of N:P:K (20:10:20) fertilizer formulation twice a week beginning two weeks after planting, with the last fertilization at heading. Flowering typically occurred after 3-4 weeks. Spores used for FHB assays were collected from mungbean agar and counted by using a hemocytometer. For use as

inoculum, spores were centrifuged at 3330 x g, then washed once in sterile water and resuspended at a concentration of 1 x 10^5 per mL. Once prepared, the spore suspension was stored at 4°C until used. The same stock was used for no more than 7 days. At early- to mid- anthesis, a single centrally positioned floret on the primary flowering stem of each plant was inoculated with 10 µL of the spore suspension as described by (Miedaner, Moldovan, & Ittu, 2003) and covered with a small plastic bag for 24 hours to increase humidity. Symptom severity was recorded at seven, ten- and 14-days post-inoculation, as the number of symptomatic spikelets over the total number of spikelets in a head (percentage). Each treatment had fifteen replicates. The experiment was done twice.

4.2.7 Data Analysis

All experiments were conducted as a completely randomized design. Data were visualized and analyzed by 95 % Confidence Intervals (CI) and by Scott Knott. CI was done by using 'mean_cl_boot' from the 'Hmisc' package, which implements basic nonparametric bootstrapping to obtain confidence limits for a population of means without assuming a normal distribution (Harrell Jr & Harrell Jr, 2019). CI was performed for multiple experimental replications. If confidence limits of mutants overlapped with the wild type PH-1, these were considered similar. For the Scott Knott, data from multiple replicated experiments were combined. The Scott Knott was used to group the isolates according to the means of measurements and counts (Jelihovschi et al., 2014).The overall means of measurements (% of disease, number of infected spikelets) of the strain groups were also compared by using the Tukey test, with $\alpha = 0.05$, after performing an analysis of variance. All analyses were run in R (R Core Team 2019). A chi-square calculator was used to determine p-values (GraphPad by Dotmatics).

4.3 Results

4.3.1 Segregation of phenotypic traits.

Segregation patterns of several phenotypic traits among groups of 96 random progeny collected from various crosses were analyzed. Summaries of all phenotyping results are presented in **Table 4.3 – 4.8.**

Hygromycin sensitivity was assayed by replicating the colonies onto media containing levels of the antibiotic inhibitory to the wild type. Resistance to hygromycin segregated in a 1:1 ratio among progeny recovered from crosses between MAT deletions and hygromycin-sensitive wild type strains PH-1 and *F. meridionale* (**Figure 4.2 and 4.4**). Nearly all the hygromycin resistant progeny from these crosses were also self-sterile, as expected. One progeny strain was resistant to hygromycin and self-fertile. This may be a recombinant, but it could also be a mixed strain, so this needs to be verified by single sporing the strain again and ensuring that it retains self-fertility. Progeny strains produced from crosses between complementary heterothallic MAT deletion strains were all resistant to hygromycin since the deletion transformants all included HygB as a selectable marker. Mycelial morphology of progeny strains was also evaluated (**Figures 4.2 - 4.6**). In a cross involving the *mat1-1-1* deletion strain 1_2 and the *mat1-2-1* deletion strain 2_3, which has the velvet phenotype, progeny did not segregate 1:1 for velvet, and velvet progeny were recovered from only one of the four perithecia (**Figure 4.3**). In a cross between strain 2_3 and another *mat1-1-1* deletion strain, 1_2, the velvet trait also did not segregate 1:1, and the velvet progeny were only present in two perithecia (data not shown). All the velvet progeny strains were female-sterile, unable to produce protoperithecia or perithecia as a female parent, like strain 2_3. None of the other crosses involved velvet parents, and none of them produced progeny with the velvet phenotype. Progeny from a cross between a *mat1-1-1* deletion and the GFP-labeled Gz3639 strain were all resistant to hygromycin as expected, because the GFP vector also includes the HygB cassette (**Figure 4.5**). Expression of GFP did not segregate 1:1 among progeny of a cross

between a *mat1-1-1* deletion strain and the GFP-labeled strain Gz3639 (**Figure 4.6**). All parental controls behaved as expected in all hygromycin assays.

4.3.2 Molecular probes and segregation analysis.

Analysis of molecular markers showed that they mostly segregated with expected patterns among the progeny of all the crosses. Summaries of all genotyping results are presented in **Table 4.3 – 4.8.**

For crosses between wild type and *mat1-1-1* deletion strains, there was a 1:1 segregation pattern of strains with both *MAT1-1-1* and *MAT1-2-1* (wild type, ++) and strains with only *MAT1-2-1* (*mat1-1-1* deletion, -+) (**Table 4.3** and **Table 4.4**). For crosses between heterothallic MAT strains, the *MAT1-1-1* and *MAT1-2-1* markers segregated 1:1 (**Table 4.5** and **Table 4.6**), and there were no strains that had both or neither. This was expected because these two genes are closely linked. Even though the velvet phenotype was only observed among *mat1-2-1* deletion strains, it was not linked to the MAT1 locus, as the velvet progeny from the cross of *mat1-2-1* deletion strain 2_3 segregated 1:1 for the two idiomorphs.

Segregation of a CAPS marker (Bec et al., 2015) was evaluated in a cross between a *mat1-1-1* deletion strain in the PH-1 background and the GFP-labeled strain of Gz3639. The CAPS marker and the MAT genes segregated in a 1:1:1:1 ratio, indicating that these two loci are unlinked (**Table 4.7**) (**Figure 4.9**).

Segregation patterns were also evaluated among progeny of a cross between a *mat1-1-1* deletion strain of *F. graminearum* and a wild type *F. meridionale* strain. *Fusarium meridionale* is another species in the FSASC that also causes FHB (Del Ponte et al., 2022). *F. graminearum* produces DON while *F. meridionale* produces NIV, and these two chemotypes can be differentiated based on PCR amplification of the TRI3 gene (Ward et al. 2008). The TRI3 alleles segregated in a 1:1 ratio as expected. Hygromycin sensitivity, self-fertility, and MAT gene markers also segregated 1:1 and were mostly linked to each other, as expected (**Table 4.8**). The MAT locus was unlinked to the TRI3 locus. There were three strains that didn't amplify with the MAT markers. There were three other strains with anomalous

patterns that might be either recombinants or mixed cultures. These will need to be further investigated.

4.3.3 FHB pathogenicity assay.

The aggressiveness of twenty progeny from a cross between two complementary MAT deletion strains (*mat1-1-1* 1_2 x *mat 1-2-1* 2_3) was evaluated on Wheaton HRSW. These deletion strains were chosen because they showed moderate and low aggressiveness on Wheaton, respectively, in the experiments reported in Chapter 3 of this thesis. My goal was to determine whether the level of aggressiveness was linked to the type of deletion (*mat1-1-1* versus *mat1-2-1*). Ten "flat" progeny strains representing each deletion type (*mat1-1-1* and *mat1-2-1*), as determined by the multiplex PCR, were randomly selected. With only one exception, all the velvet progeny strains from the cross produced extremely low numbers of conidia, and so they were not used for plant inoculations. Ten *mat1-1-1* deletion progeny strains, ten *mat1-2-1* deletion progeny strains, the respective parental strains, and the PH-1 progenitor wild type strain, along with a water control, were included in this analysis. Confidence intervals showed that none of the strains was like PH-1 in either the first or second experiment (**Figure 4.10**). I obtained three significantly different groups based on Scott Knott analysis (**Figure 4.11**). All progeny and the 1_1 parental strain belonged to one group, which was statistically less aggressive than the PH-1 wild type. The parental strain 2_3, with the velvet phenotype, was in the third group together with the water control. There was no overall relationship between mating deletion and aggressiveness (**Figure 4.12**). Summaries of the data for individual strains are included in (**Table 4.9**), and overall results for each class are included in (**Table 4.1**).

4.3.4 Stability of Velvet phenotype

I collected spikelets from Wheaton plants that were inoculated with the 2_3 *mat1-2-1* deletion strain to test the stability of the velvet phenotype during host colonization. I also tested one of the velvet progeny strains (4C59) from the cross of *Δmat1-1-1* strain 1_2 and *Δmat1-2-1* strain 2_3. This was the only progeny strain that produced enough spores for inoculation. Fourteen days after inoculation, strains reisolated from the tissues inoculated with strain 2_3 retained the velvet aerial mycelium in culture (**Figure 4.13**). The 4C59 progeny strain grew from the tissue with a normal appearance initially, but later reverted to velvet at the edges of the colony (**Figure 4.13**). Subculturing from both normal and velvet portions of the colony resulted in stable flat or velvet phenotypes (**Figure 4.14**). Somewhat surprisingly, the wild type PH-1 control showed a velvet phenotype when it first grew from the plant tissue, but then it produced normal sectors after a few days (**Figure 4.13**).

4.4 Discussion

The goal of my project was to identify and characterize suitable heterothallic tester strains that can be used to make controlled crosses with wild type strains for analysis of pathogenicity, toxigenicity, and fitness. Results from this chapter demonstrate that *mat1-1-1* deletion strains outcross successfully with *mat1-2-1* deletion strains of the same PH-1 background, as well as with other wild type strains of *F. graminearum* and another phylospecies, *F. meridionale*. All the crosses produced sufficient progeny for analysis, and all showed expected Mendelian patterns of marker segregation and recombination for several morphological markers, and all molecular markers. The multiplex assay for MAT genes was very helpful for minimizing necessary steps: it would be helpful to expand the multiplex to include the chemotyping markers and other markers in the future. A small number of strains with unexpected segregation patterns were

observed; these could be due to cross contamination, or possibly recombination. These strains need to be single-spored again and reanalyzed.

I incorporated a strain expressing GFP in one of my crosses. The fluorescence was easy to screen among the progeny, but it did not segregate in a 1:1 ratio. Bec observed a similar phenomenon in her crosses with this strain (Bec 2011). One possibility is that the transgene is unstable during meiosis, but the transformation vector contained both the GFP and HygB genes, and since half the progeny strains are still hygromycin resistant, it seems more likely that the GFP is being silenced in some way. Analysis by PCR to determine if the GFP gene is still present would be informative. Additional fluorescent strains could also be tested since others have found that the GFP trait segregates normally in their crosses (H.-K. Kim et al., 2012)

The velvet mycelium also did not segregate as a single locus, and the phenotype was not linked to the *mat1-2-1* deletion, even though all the original velvet strains were in a *mat1-2-1* background. All the velvet progeny strains were completely female sterile and produced no perithecia or protoperithecia, so the velvet morphology and loss of female fertility appear to be linked. In the cross between 1_2 and 2_3, velvet strains were only observed from progeny derived from one perithecia. In the cross between 1_1 and 2_3, only two of the four perithecia contained velvet progeny. It is not clear why all perithecia don't contain velvet progeny, but it suggests that the trait is not controlled by nuclear genes and may be epigenetic.

I followed up with a theory that the velvet strains could regain their normal phenotype by inoculating and reisolating them from the host organism, which has been noted for degraded fungal plant pathogens by others (Hajek et al., 1990; Hartmann & Wasti, 1974; Hayden, Bidochka, & Khachatourians, 1992; Kawakami, 1960; Lord & Roberts, 1986; Prenerová, 1994; Shah et al., 2005; Steinkraus, Geden, & Rutz, 1991; Wasti & Hartmann, 1975). However, the 2_3 velvet strain did not regain a normal phenotype when recovered from inoculated tissue. The *mat1-2-1* deletion progeny strain 4C59, originally velvet, initially grew out with a wild type appearance, but then reverted to velvet and the velvet parts of the colony

were stable. Surprisingly, from one of the recovery trails I performed, the wild type also showed signs of sectoring to a velvet phenotype. This is additional evidence that the PH-1 strain is prone to this type of degradation.

The connection between velvet and *mat1-2-1*, if any, remains unclear: all the strains were produced and stored at the same time, and they were all originally flat. Only the *mat1-2-1* strains (4 out of 6) now show this phenotype. One possibility is that there is something different about the batch of protoplasts or some other factor related to the transformation experiment that produced the *mat1-2-1* deletions, as opposed to the other deletion strains. Another possibility is that the deletion of *mat1-2-1* somehow promotes instability, perhaps by altering patterns of transposon movement and integration, or stress response. This question needs further study.

The results of the FHB pathogenicity assay clearly demonstrate that aggressiveness is not linked to the MAT genes. The reduction in aggressiveness of the 2_3 strain and most of the other *mat1-2-1* deletion strains must be due to other factors, and not to the deletion itself. It is possible that it is related to the velvet phenotype, but aggressiveness assays remain to be done with the velvet progeny strains. Only one of them produced enough spores for inoculations so it will be necessary to come up with a different type of inoculum, possibly mycelial fragments. The literature reports that crosses usually produce transgressive strains (Bec, 2011; Voss et al., 2010). However, my results showed that all the progeny strains were less aggressive than the PH-1 wild type, and equal in aggressiveness to the *mat1-1-1* deletion parent (**Table 4.10** and **Figure 4.12**). One possibility is that I didn't look at enough strains to identify transgressives, which are likely to be relatively rare. It may also be due to inbreeding. The two parental strains are both from the same PH-1 background, so they may not be divergent enough to produce transgressive recombinant strains.

I have identified several *mat1-1-1* deletion strains that have high female fertility, that are morphologically and pathogenically like the wild type, and that can produce progeny with normal segregation patterns. These strains should be suitable as test maters for future genetic analyses.

Table 4.1 List of strains used in this study.
Strain labels that begin with 1 are *mat1-1-1* deletion strains, while strains that begin with 2 are *mat1-2-1* deletion strains.

Strains					
Code	Name (Bec, 2011)	Genotype	Transformation Protocol	Colony type	Hygromycin B Resistance
1_1	mat111 sm1	*mat1-1-1/MAT1-2-1*	split-marker	Flat	R
1_2	mat111 sm5	*mat1-1-1/MAT1-2-1*	split-marker	Flat	R
2_3	mat121 sm16	*MAT1-1-1/mat1-2-1*	split-marker	Velvet	R
PH-1		*MAT1-1-1/MAT1-2-1*	none	Flat	S
Gz3639 GFP		*MAT1-1-1/MAT1-2-1*	ectopic	Flat	R
Fusarium meridionale 004		*MAT1-1/MAT1-2*	none	Flat	S

Table 4.2 List of primers used in this study.

Gene of Interest	Amplicon	Primer Sequence 5' - 3'	Reference
MAT1-1-1	MAT1-1-1 internal probe forward for MAT multiplex	TCGAGGAAACTCTTGCCTTA	This study
	MAT1-1-1 internal probe reverse for MAT multiplex	CGAGGACCATGTTACCAAAG	This study
MAT1-2-1	MAT1-2-1 internal probe forward for MAT multiplex	CAGGGTTGAGTTCGGAAAGC	This study
	MAT1-2-1 internal probe reverse for MAT multiplex	TCCAGCATCGTCCAAGAACT	This study
CAPSEcoRI	CAPSEcoRI internal probe forward	GGTTCGGTGAGTCTTTAAGCCCC	Bec et al., 2015
	CAPSEcoRI internal probe reverse	CGGCTTGAGGGTTTTCGAGC	Bec et al., 2015
Fg16	TRI3 chemotyping forward	CTCCGGATATGTTGCGTC AA	(Carter et al., 2000; Zhang et al., 2007)
	TRI3 chemotypinh reverse	GGTAGGTATCCGACATGG CAA	(Carter et al., 2000; Zhang et al., 2007)

Table 4.3 Characterization of progeny sets deriving from *mat1-1-1* (1_1) x PH-1. Highlighted are the parental characteristics. Phenotypic characters included Hygromycin B sensitivity (HygR or HygS), type of mycelium (Flat vs Velvet), and fertility (self-fertile or self-sterile). Genotypes were determined by using PCR MAT Multiplex to detect presence of *MAT1-1-1* and *MAT1-2-1*. Genotypes were: both genes; *MAT1-1-1* only; *MAT1-2-1* only; or neither gene [++, +-, -+, --]. Chemotype primers were used to identify TRI3 allele (DON or NIV), and CAPs markers were used to parental background (PH-1) or Gz3639 GFP (Gz). Segregation ratios for the different characteristics are shown, with P values from the Chi-Square analysis.

***mat1-1-1* (1_1) x PH-1**

Type	Number of progeny
Phenotypic (N=96)	
HygR (1_1)	46
HysS (PH-1)	50
Genotypic (N=40)	
HygR Flat Fertile Δmat1-1-1	1
HygR Flat Fertile MAT	1
HygR Flat Sterile Δmat1-1-1	18
HygS Flat Fertile MAT	19
HygS Flat Sterile Δmat1-1-1	1

Marker Segregation	**Segregation**	**Chi Square**
HygS : HygR	46:50	P=0.6831
MAT : Δmat1-1-1	20:20	P=1
HygR Sterile : HygR Fertile : HygS Sterile : HygS Fertile	18:2:1:19	P=0.0001
HygR Δmat1-1-1 : HygR MAT : HygS Δmat1-1-1 : HygS MAT	19:1:1:19	P=0.0001

Table 4.4 Characterization of progeny sets deriving from *mat1-1-1* (1_2) x PH-1.
Highlighted are the parental characteristics. Subsets are considered any progeny with more than Hygromycin B sensitivity (HygR or HygS) and type of mycelium (Flat vs Velvet). Genotypes were determined using PCR MAT Multiplex [MAT (both genes) or Δmat1-1-1 or Δmat2-1-1], chemotypes Fg16 (DON or NIV), and CAPs markers determined parental background (PH-1) or Gz3639 GFP (Gz). Segregation ratios for the different characteristics are shown, with P values from the Chi-Square analysis.

***mat1-1-1* (1_2) x PH-1**

TYPE	NUMBER OF PROGENY
Phenotypic (N=96)	
HygR (1_2)	46
HygS (PH-1)	50
Genotypic (N=20)	
HygR Flat Fertile Δmat1-1-1	1
HygR Flat Sterile Δmat1-1-1	9
HygS Flat Fertile MAT	10

Marker Segregation	**Segregation**	**Chi Square**
HygS : HygR	50:46	P=0.6831
MAT : Δmat1-1-1	10:9	P=0.8185
HygR Sterile : HygR Fertile : HygS Sterile : HygS Fertile	9:1:0:10	P=0.0009
HygR Δmat1-1-1 : HygR MAT : HygS Δmat1-1-1 : HygS MAT	10:0:0:10	P=0.0002

Table 4.5 Characterization of progeny sets deriving from *mat1-1-1* (1_1) x *mat1-2-1* (2_3). Highlighted are the parental characteristics. Subsets are considered any progeny with more than Hygromycin B sensitivity (HygR or HygS) and type of mycelium (Flat vs Velvet). Genotypes were determined using PCR MAT Multiplex [MAT (both genes) or Δmat1-1-1 or Δmat2-1-1], chemotypes Fg16 (DON or NIV), and CAPs markers determined parental background (PH-1) or Gz3639 GFP (Gz). Segregation ratios for the different characteristics are shown, with P values from the Chi-Square analysis.

mat1-1-1* (1_1) x *mat1-2-1 (2_3)

TYPE	NUMBER OF PROGENY
Phenotypic (N=95)	
HygR Flat (1_1)	82
HygR Velvet (2_3)	13
Genotypic (N=27)	
HygR Flat Fertile MAT	1
HygR Flat Sterile Δmat1-1-1	5
HygR Flat Sterile Δmat1-2-1	8
HygR Velvet Sterile Δmat1-1-1	9
HygR Velvet Sterile Δmat1--1	4

Marker Segregation	**Segregation**	**Chi Square**
HygR Δmat1-1-1 : HygR Δmat1-2-1	14:12	P=0.6949
HygR Flat : HygR Velvet	82:13	P=0.0001
HygR Flat Δmat1-1-1 : HygR Velvet Δmat1-2-1 : HygR Flat Δmat1-2-1 : HygR Velvet Δmat1-1-1	5:4:8:9	P=0.4548

Table 4.6 Characterization of progeny sets deriving from *mat1-1-1* (1_2) x *mat1-2-1* (2_3). Parental types are highlighted. Subsets are considered any progeny with more than Hygromycin B sensitivity (HygR or HygS) and type of mycelium (Flat vs Velvet). Genotypes were determined using PCR MAT Multiplex [MAT (both genes) or Δmat1-1-1 or Δmat2-1-1], chemotypes Fg16 (DON or NIV), and CAPs markers determined parental background (PH-1) or Gz3639 GFP (Gz). Segregation ratios for the different characteristics are shown, and Chi Square values are presented for expected 1:1 or 1:1:1:1 ratios.

mat1-1-1* (1_2) x *mat1-2-1 (2_3)

Type	Number of progeny
Phenotypic (N=96)	
HygR Flat (1_1)	87
HygR Velvet (2_3)	9
Genotypic (N=96)	
HygR Flat Fertile MAT	1
HygR Flat Sterile Δmat1-1-1	45
HygR Flat Sterile Δmat1-2-1	41
HygR Velvet Nothing Δmat1-1-1	3
HygR Velvet Nothing Δmat1-2-1	6

Marker Segregation	**Segregation**	**Chi Square**
HygR Δmat1-1-1 : HygR Δmat1-2-1	48:47	P=0.9183
HygR Flat : HygR Velvet	87:9	P=0.0001
HygR Flat Δmat1-1-1 : HygR Velvet Δmat1-2-1 : HygR Flat Δmat1-2-1 : HygR Velvet Δmat1-1-1	45:6:41:3	P=0.0001

Table 4.7 Characterization of progeny sets deriving from *mat1-1-1* (1_1) x Gz3639 GFP.
Parental types are highlighted. Subsets are considered any progeny with more than Hygromycin B sensitivity (HygR or HygS) and type of mycelium (Flat vs Velvet). Genotypes were determined using PCR MAT Multiplex [MAT (both genes) or Δmat1-1-1 or Δmat2-1-1], chemotypes Fg16 (DON or NIV), and CAPs markers determined parental background (PH-1) or Gz3639 GFP (Gz). Segregation ratios for the different characteristics are shown, and Chi Square values are presented for expected 1:1 or 1:1:1:1 ratios.

mat1-1-1* (1_1) x *Gz3639 GFP

Type	Number of progeny
Phenotypic (N=96)	
HygR nonGFP (1_1)	86
HygR GFP (Gz3639 GFP)	10
Genotypic (N=39)	
HygR Flat Fertile MAT Gz	11
HygR Flat Fertile MAT PH-1	10
HygR Flat Sterile Δmat1-1-1 Gz	7
HygR Flat Sterile Δmat1-1-1 PH-1	10
HygR Flat Sterile MAT PH-1	1

Marker Segregation	**Segregation**	**Chi Square**
HygR nonGFP vs HygR GFP	86:10	0.0001
HygR Δmat1-1-1 vs HygR wild type	17:22	0.4233
HygR Δmat1-1-1 PH-1, HygR wild type Gz, HygR Δmat1-1-1 Gz, HygR wild type PH-1	10:11:7:10	0.814

Table 4.8 Characterization of progeny sets deriving from *mat1-1-1* (1_1) x *Fusarium meridionale* 004. Parental types are highlighted. Subsets are considered any progeny with more than Hygromycin B sensitivity (HygR or HygS) and type of mycelium (Flat vs Velvet). Genotypes were determined using PCR MAT Multiplex [MAT (both genes) or Δmat1-1-1 or Δmat2-1-1], chemotypes Fg16 (DON or NIV), and CAPs markers determined parental background (PH-1) or Gz3639 GFP (Gz). Segregation ratios for the different characteristics are shown, and Chi Square values are presented for expected 1:1 or 1:1:1:1 ratios.

***mat1-1-1* (1_1) x *Fusarium meridionale* 004 (wild type)**

Type	Number of progeny
Phenotypic (N=96)	
HygR Flat (1_1)	52
HygS Flat (Fm)	44
Genotypic (N=40)	
HygR Flat Fertile MAT NIV	1
HygR Flat Sterile Δmat1-1-1 15ADON	11
HygR Flat Sterile Δmat1-1-1 NIV	8
HygS Flat Fertile Δmat1-1-1 NIV	1
HygS Flat Fertile MAT 15ADON	3
HygS Flat Fertile MAT NIV	12
HygS Flat Sterile MAT 15ADON	1
HygS Flat Fertile 0 0 15ADON	1
HygS Flat Fertile 0 0 NIV	1
HygS Flat Fertile 0 0 15ADON	1

Marker Segregation	**Segregation**	**Chi Square**
HygS : HygR	52:44:00	P=0.4142

MAT : Δmat1-1-1	17:20	P=0.6219
HygR Sterile : HygR Fertile : HygS Sterile : HygS Fertile	19:1:2:18	P=0.0001
HygR Δmat1-1-1 : HygR MAT : HygS Δmat1-1-1 : HygSMAT	19:1:1:16	P=0.0001
DON Δmat1-1-1 : DON:MAT : NIV Δmat1-1-1 : NIV MAT	11:4:9:13	P=0.1841

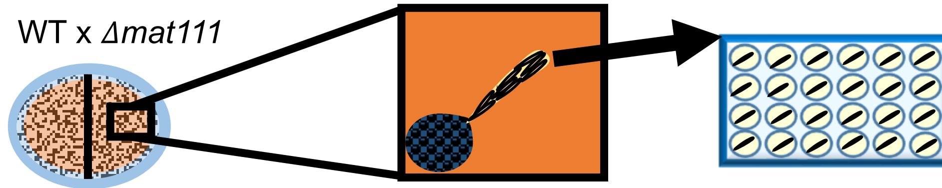
Table 4.9 Summary table for results of MAT progeny deletion strain FHB Severity (%). The groupings were determined using Scott Knott. Letters indicate the difference is significant at α= 0.05, with P values = <0.001.

	FHB on Wheaton
Strain	Mean Severity (%)
1_2	16.27^{b}± 13.88
2_3	1.73^{c}± 3.69
4A13	19.88^{b}± 14.35
4A14	18.45^{b}± 12.61
4A16	16.16^{b}± 19.07
4A7	18.22^{b}± 13.34
4B26	13.80^{b}± 12.13
4B28	13.45^{b}± 9.18
4B37	13.27^{b}± 16.36
4B38	14.64^{b}± 11.18
4B40	16.51^{b}± 11
4B45	19.70^{b}± 15.68
4B47	14.53^{b}± 11.66
4B48	17.75^{b}± 15.23
4C56	18.21^{b}± 14.08
4C59	15.86^{b}± 17.67
4C64	13.54^{b}± 11.13
4C70	14.08^{b}± 10.52
4C71	14.00^{b}± 9.23
4D77	13.57^{b}± 13.01
4D81	15.69^{b}± 11.52
4D89	13.95^{b}± 15.11
4D93	15.34^{b}± 11.77
PH-1	51.99^{a}± 28.68
w	0.00^{c}± 0

Table 4.10 Summary table for results of MAT progeny deletion strains combined FHB Severity (%). The groupings were determined using Tukey Test. Letters indicate the difference is significant at $\alpha = 0.05$, with P values = < 0.001.

	FHB on Wheaton
Strain	Mean Severity (%)
PH-1	51.99[a]± 28.68
mat111	16.39[b]± 13.68
mat121	14.02[b]± 13.13
mock	0[c]± 0

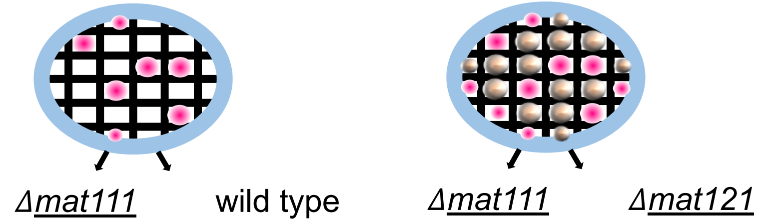
Crossing



Cirrhi from four perithecia from the *mat1-1-1* deletion side of the plate are collected and 24 ascospores from each, 96 total, are isolated, cultured, and single-spored.

Progeny strains are tested for segregating traits:

I. Hyg B resistance marker & II. Colony Characteristics



III. Female Fertility

Figure 4.1 Schematic of phenotypic segregation pattern analysis if heterothallic *Fusarium graminearum*

1_1 x PH-1

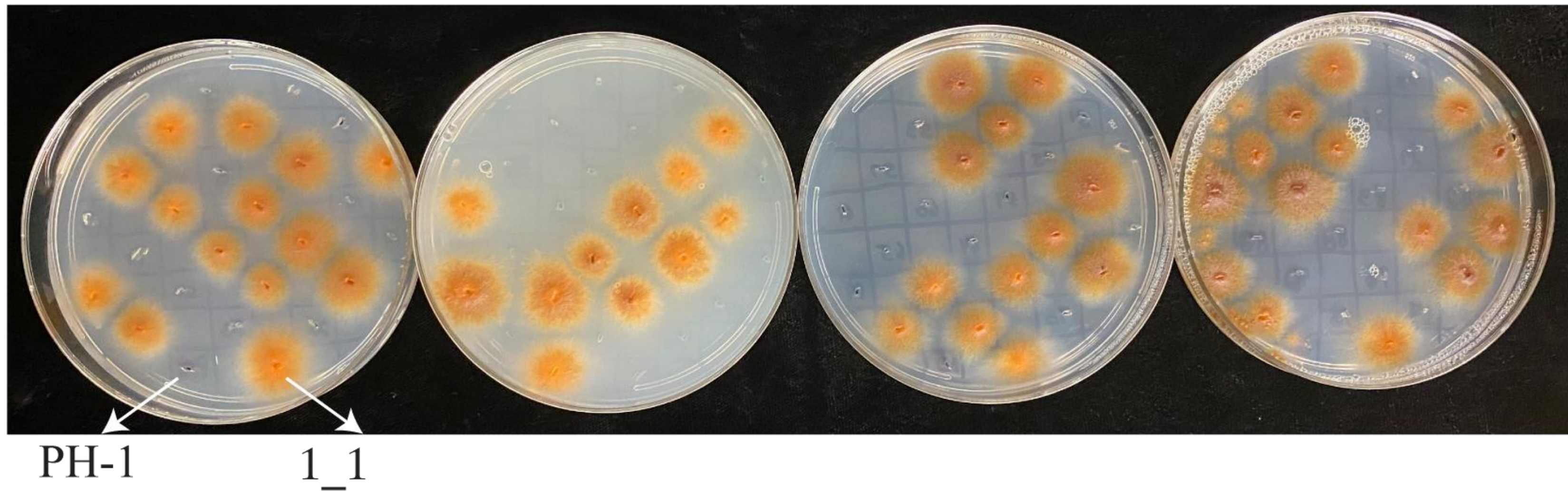
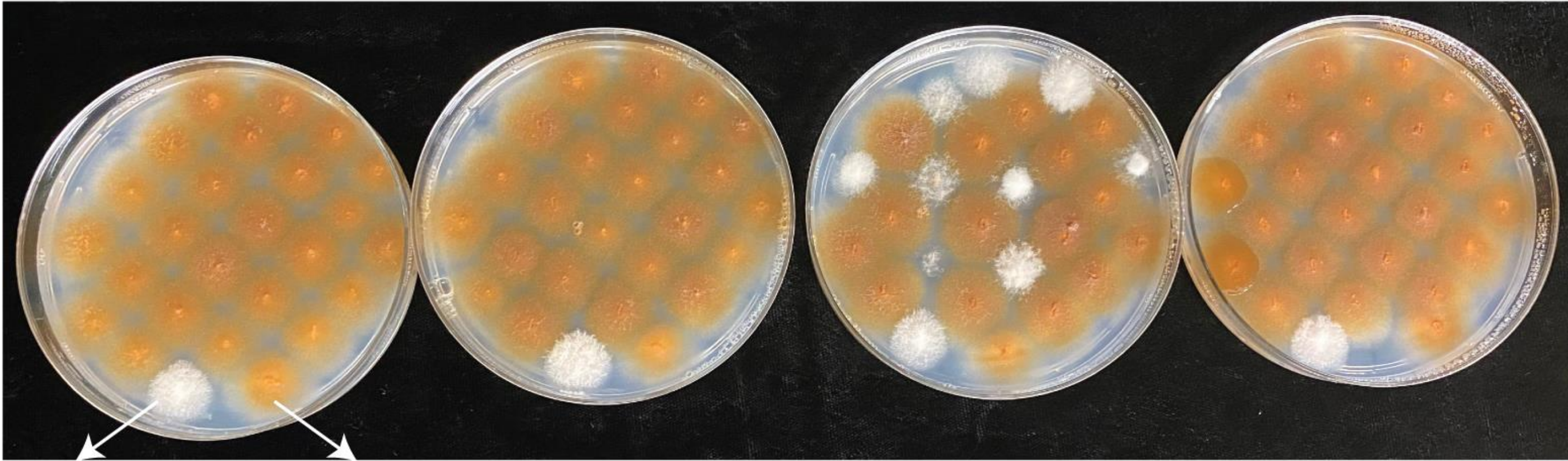


Figure 4.2: Progeny and parental strains (1_1 x PH-1) inoculated on PDA + 50 mg/mL Hygromycin B, 4 days after inoculation.

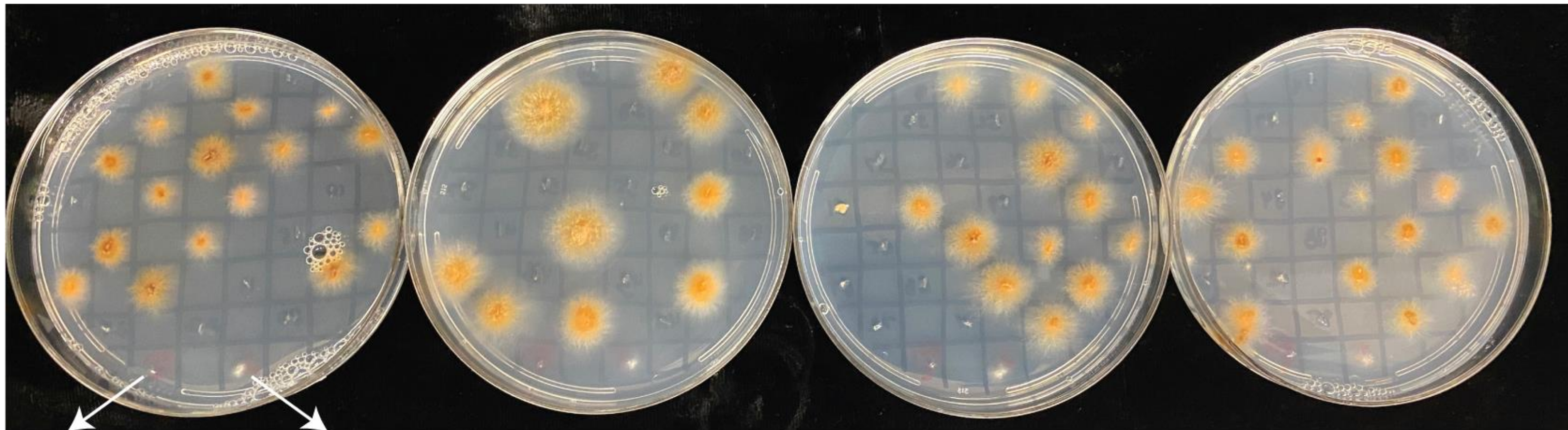
1_2 x 2_3



2_3 1_2

Figure 4.3 Progeny and parental strains (1_2 x 2_3) inoculated on PDA + 50 mg/mL Hygromycin B, 4 days after inoculation.

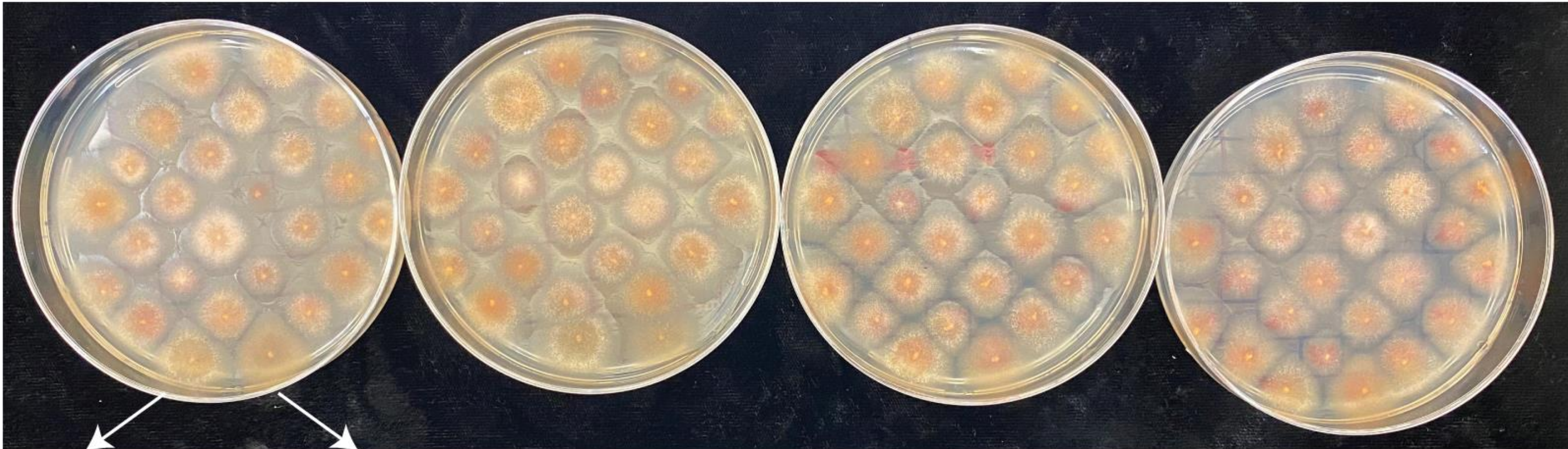
1_1 x *Fusarium meridionale* (Fm004)



Fm004 1_1

Figure 4.4: Progeny and parental strains (1_1 x Fm004) inoculated on PDA + 50 mg/mL Hygromycin B 4 days after inoculation.

1_1 x Gz3639 GFP



Gz3639 GFP 1_1

Figure 4.5 Progeny and parental strains (1_1 x Gz3639 GFP) inoculated on PDA + 50 mg/mL Hygromycin B, 4 days after inoculation.

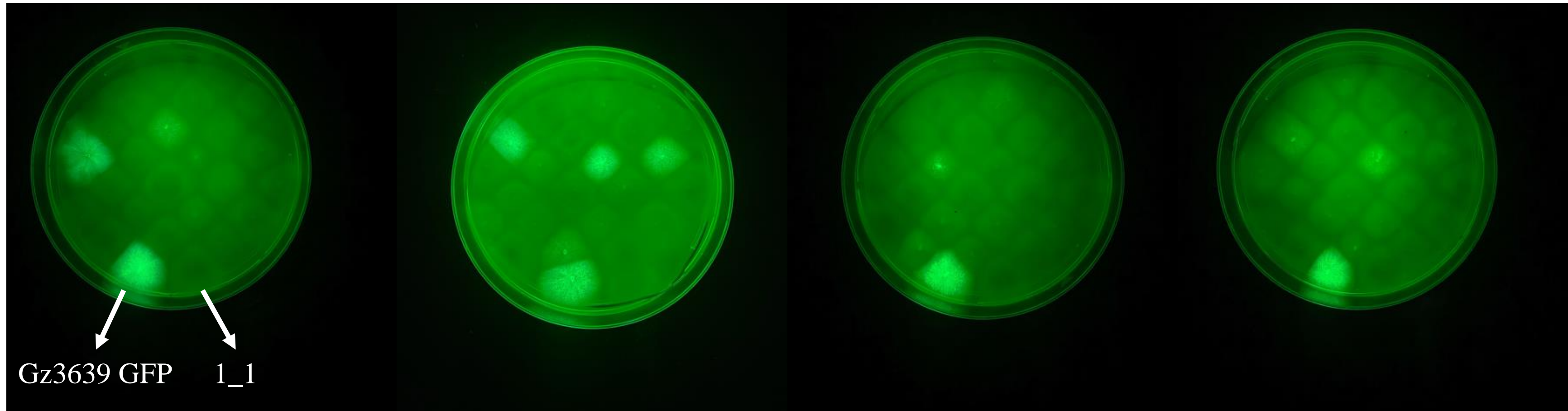


Figure 4.6 Progeny and parental strains inoculated (1_1 x Gz3639 GFP) on PDA + 50 mg/mL Hygromycin B 4 days after inoculation imaged under a light that reveals GFP.

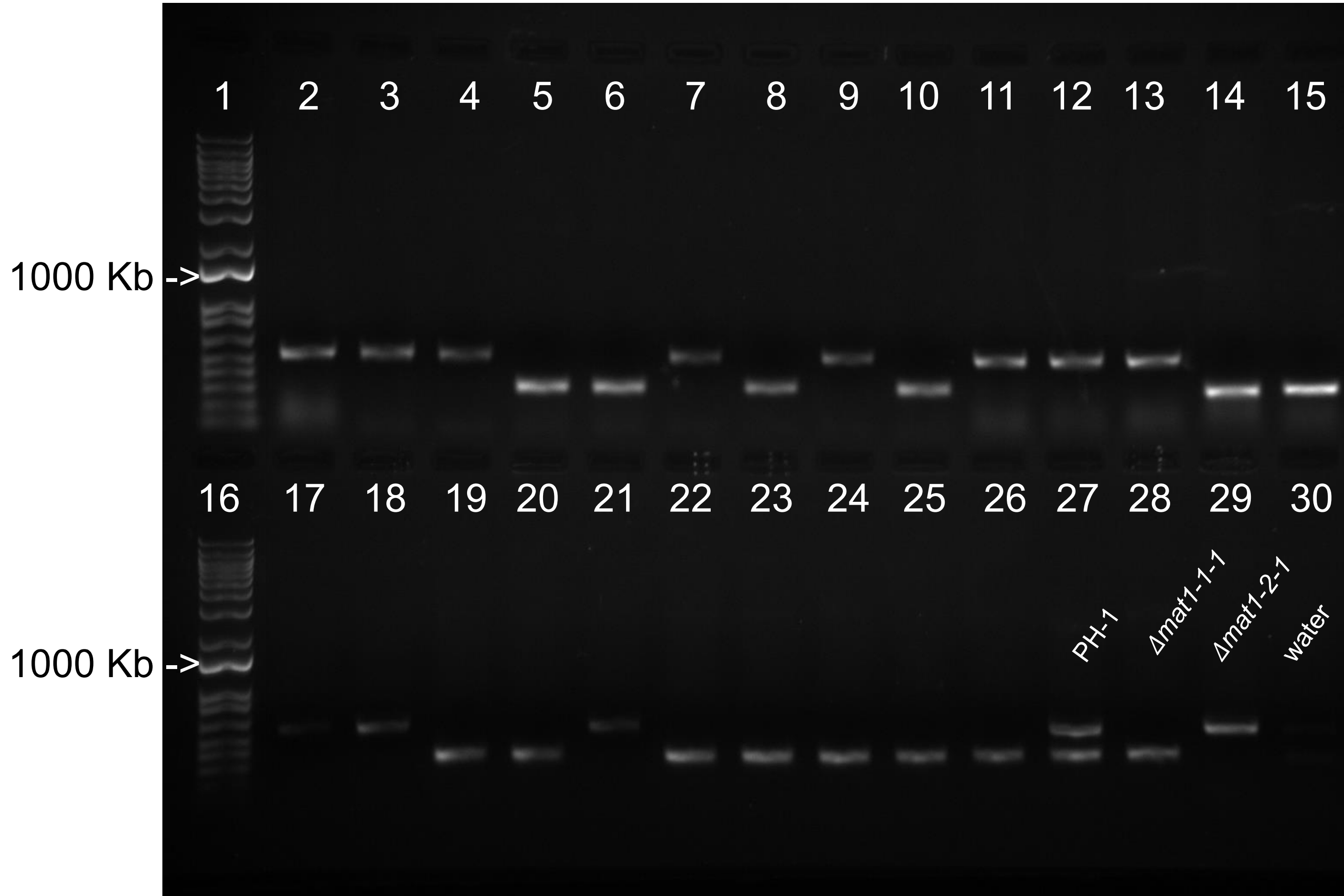


Figure 4.7 Amplification of *MAT1-1-1* and *MAT1-2-1* gene sequence of parental strains, progeny strains, and positive and negative controls.
Lanes 1 and 16 = 1 Kb ladder, Lane 2 to15 and 17 to 26 = progeny from a cross between 1_2 x 2_3, Lane 27 = PH-1, Lane 28 = *mat1-1-1* deletion strain, Lane 29 = *mat1-2-1* deletion strain, Lane 30 = water PCR control.

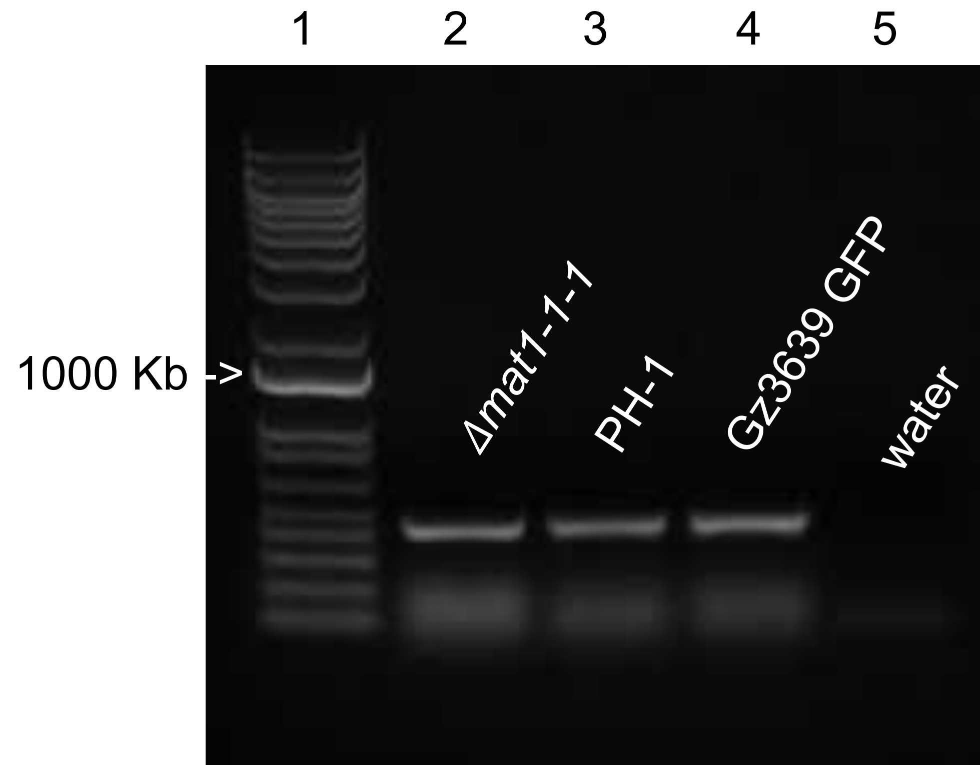


Figure 4.8 Amplification of CAPSEcoR1 sequence of positive and negative controls.
Lane 1 = 1 Kb ladder, Lane 2 = *MAT1-1-1* deletion strain (PH-1) , Lane 3 = Gz3639 GFP, Lane 5 = water.

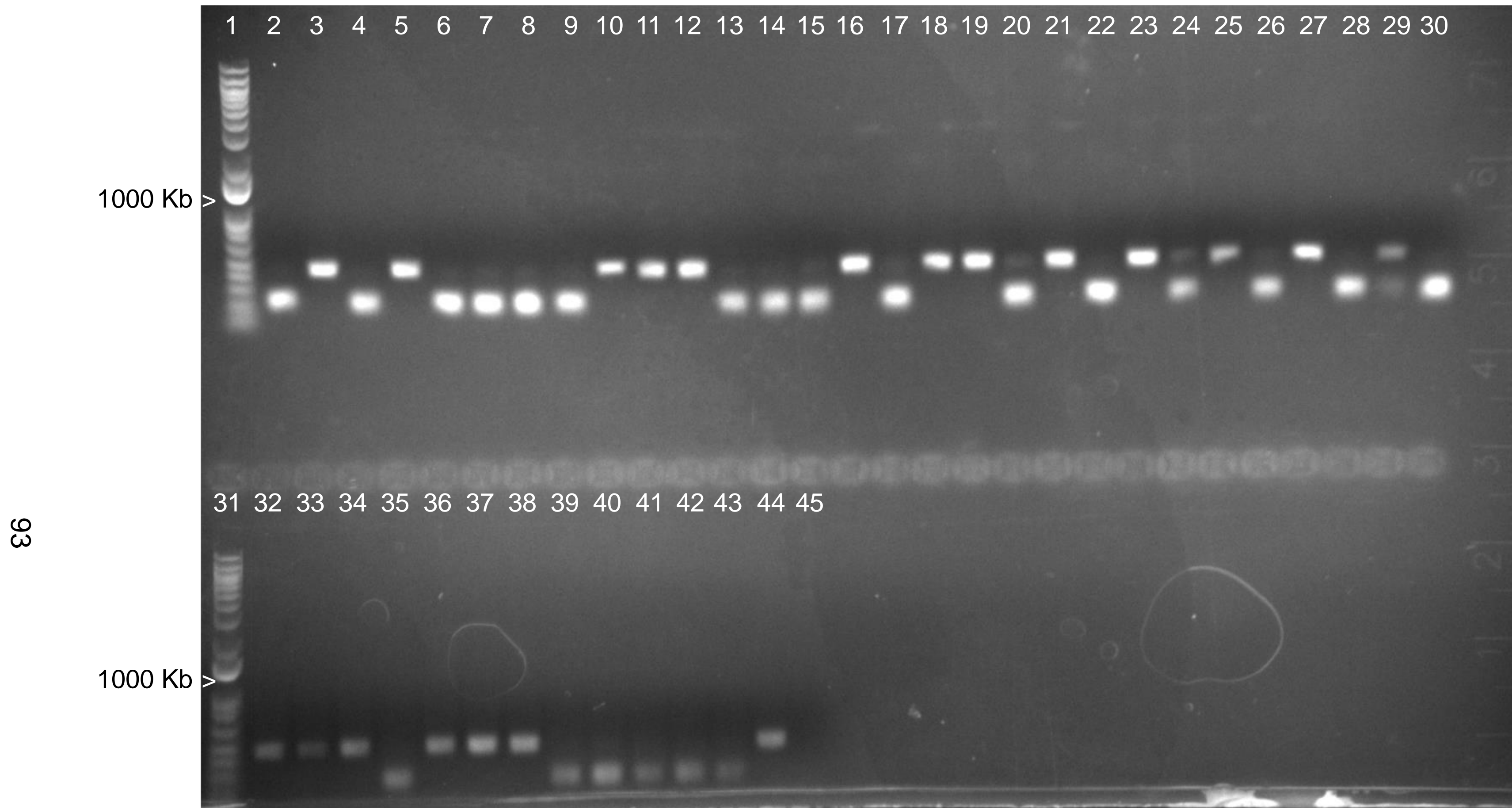


Figure 4.9 Segregation of CAPS EcoR1 marker among progeny of *MAT1-1-1* deletion strain (PH-1) x Gz3639 GFP cross derived from four randomly selected perithecia.
CAPs segregated in a 1:1 ratio. Lanes 2 – 41 are randomly collected progeny, Lane 42 = *mat1-1-1* deletion strain (PH-1 background), Lane 43 = PH-1, Lane 44 = Gz3639 GFP, Lane 45 = water PCR control.

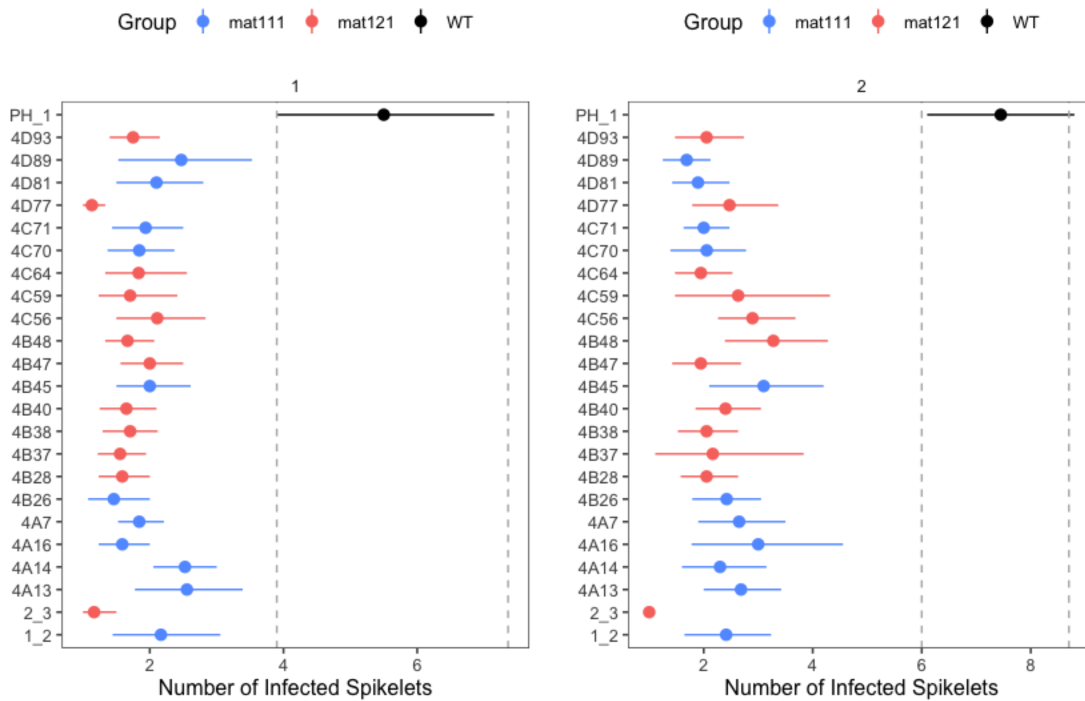


Figure 4.10 Average number of infected spikelets [95% confidence interval (CI)] on Wheaton of wild type, MAT deletion parental strain and progeny *of Fusarium graminearum* for two experiments.
Points represent mean number of infected spikelets for each strain. Dashed lines correspond to PH-1 CI. Strains that overlap with PH-1, behave similarly to PH-1.

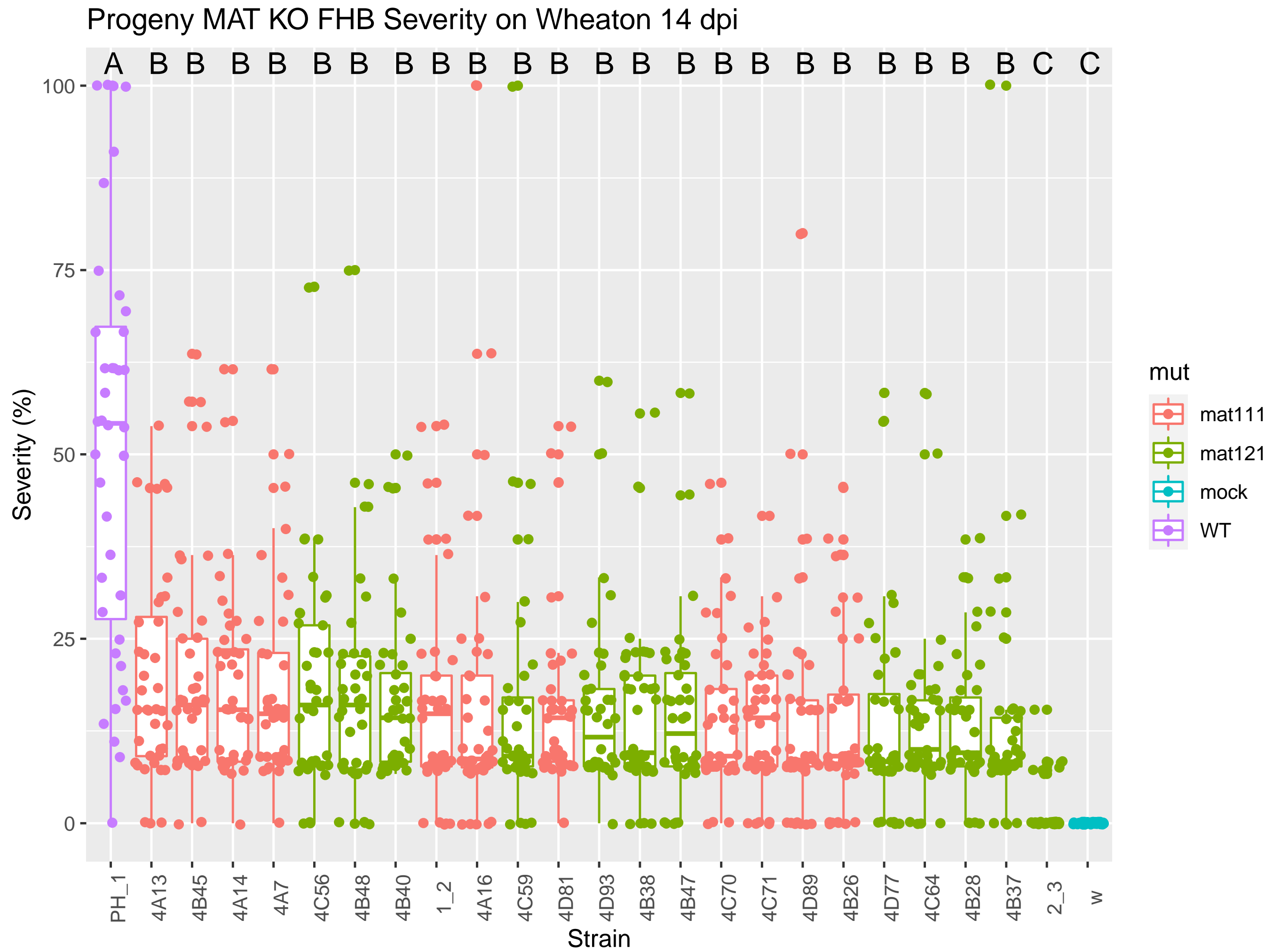


Figure 4.11 FHB caused by progeny between 1_2 *(MAT1-1-1* deletion strain*)* x 2_3 (*MAT1-2-1* deletion strain).
Significantly different groups (letters) were determined using Scott Knott tests to determine PH-1 (A), 4A13-4B37 (B), and 2_3 - w (C). Each dot shows a single data point. The horizontal bars inside the boxes indicate the mean value of that treatment. The letters on top of the bars represent the significant groupings determined by Scott Knott ($P < 0.001$, $\alpha=0.05$).

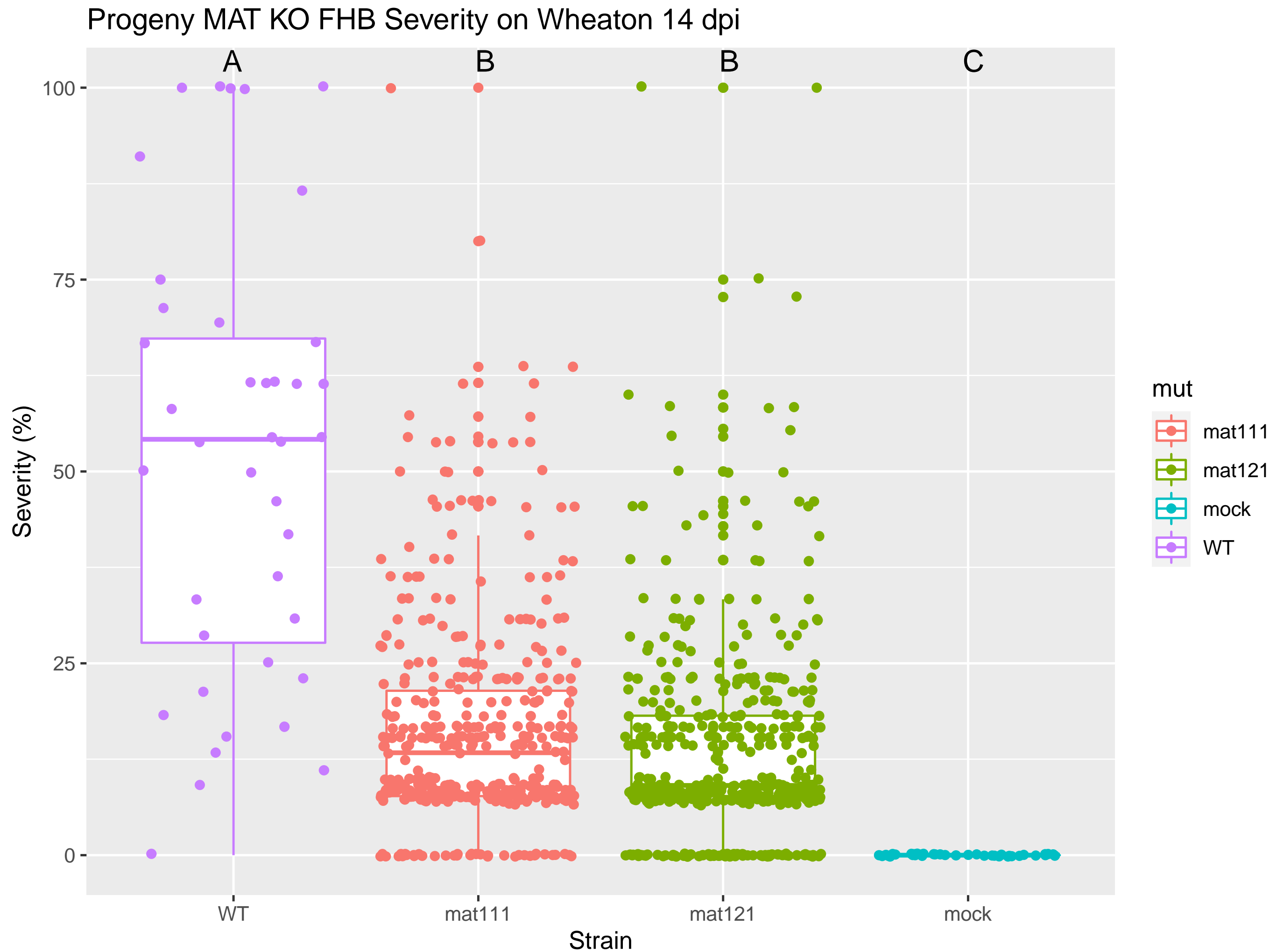


Figure 4.12 Wild type, MAT deletion strains and mock (water) Severity (%) on Wheaton.

This figure shows the significant differences between the wild type PH-1 from all the *mat1-1-1* and *mat1-2-1* deletion progeny strains and the mock inoculated negative control. The progeny strains were grouped based on their genotype, determined with the multiplex MAT PCR test. Statistical comparisons were done using an ordinary one-way ANOVA using Tukey's post-hoc test to correct for multiple comparisons. Each dot shows a single data point. The horizontal bars inside the boxes indicate the mean value of that treatment. The letters on top of the bars represent the significant groupings determined by Scott Knott ($P < 0.001$, $\alpha = 0.05$).

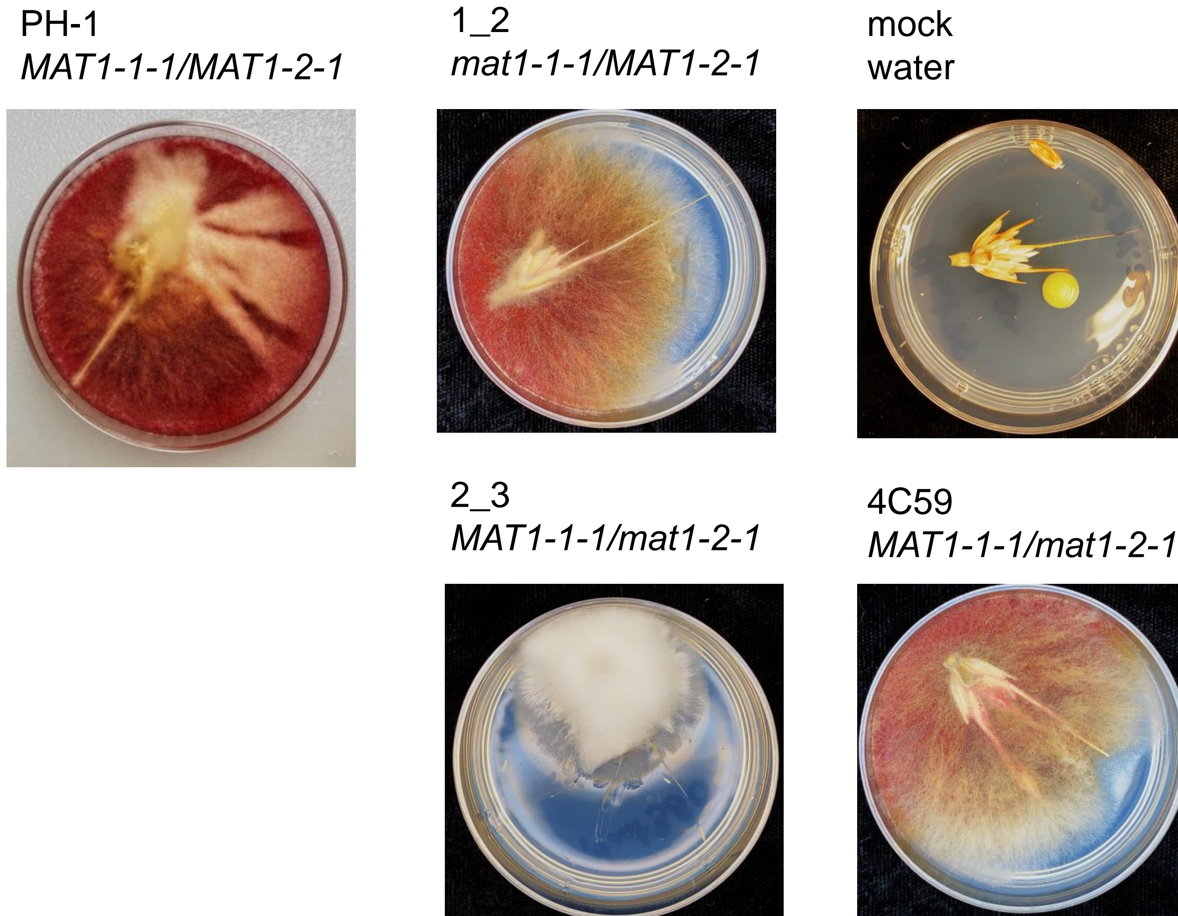


Figure 4.13 Strains from wild type (PH-1), MAT deletion strains (1_2 and 2_3), and velvet progeny strain (4C59) re-isolated from wheat head spikelets 14 days after inoculation.
Below the labels are the genotypes of the strains. PH-1 wild type exhibited sectoring of velvet aerial mycelium. The flat versus velvet phenotypes of the deletion strains were stable. The velvet mat1-2-1 deletion progeny strain 4C59, started to grow like the wild type, but then became more velvet over time.

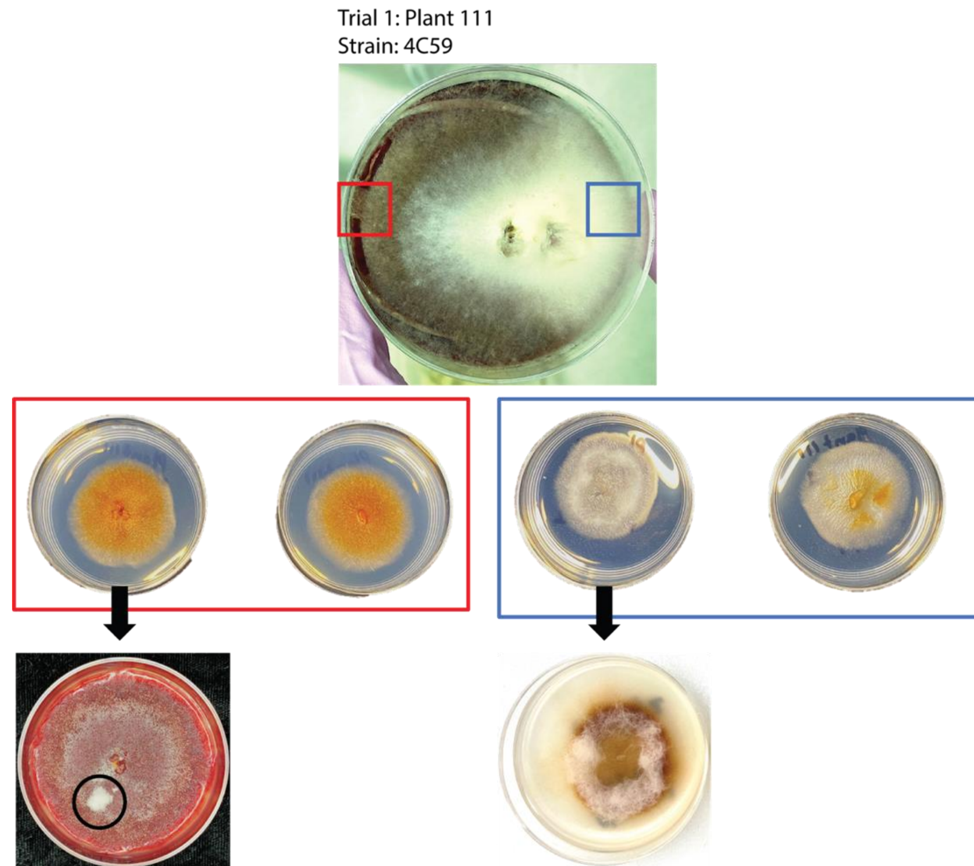


Figure 4.14 Progeny strain 4C59 recovered from wheat head spikelet showing two different types of mycelia, velvet and flat.
The red box corresponds to subcultures from the flat side and the blue box to the velvet side. The black arrows show subcultures from those plates after 7 days, with the flat showing a small velvet region (circled).

CHAPTER 5. EFFECT OF MAT1, *MAT1-1-1*, AND *MAT1-2-1* DELETIONS ON FUNGAL GENE EXPRESSION IN WHEATON WHEAT HEADS.

5.1 Introduction

Several previous studies have evaluated the transcriptome of *F. graminearum* during infection of wheat heads (Neil A Brown, Antoniw, & Hammond-Kosack, 2012; Neil Andrew Brown & Hammond-Kosack, 2015) (reviewed by (Kazan & Gardiner, 2018). These data have revealed potential mechanisms of disease resistance on the host side, including activation of genes encoding proteins that detoxify DON or suppress other pathogen virulence factors (Gottwald, Samans, Lück, & Friedt, 2012). Resistance is also associated with earlier and stronger induction of defense-related genes (Bernardo et al., 2007). Potential fungal pathogenicity genes induced during infection include the TRI genes that are involved in production of DON, and other genes that are associated with nutrient access and suppression of host defense. *In planta* comparisons of symptomatic and asymptomatic inoculated wheat flower tissues revealed that the TRI genes were induced before symptoms develop, in association with high production of DON (Neil A Brown, Evans, Mead, & Hammond-Kosack, 2017). In addition, amino acid and polyamine transporters were induced along with CAZymes that are involved in plant cell wall degradation and aid the fungus to enter and move within the plant cells (Neil A Brown et al., 2012; Neil Andrew Brown & Hammond-Kosack, 2015).

Transcriptomics studies of *F. graminearum* MAT deletion strains have also been done, but only *in vitro* (H.-K. Kim et al., 2015). The MAT gene products are transcription factors that control multiple other genes involved in necessary events in sexual development before cellular fusion, and during the formation of fertile perithecia and ascospores (H.-K. Kim et al., 2012; H.-K. Kim et al., 2015; J. Lee, Leslie, & Bowden, 2008). For example, genes encoding pheromones and pheromone receptors, which facilitate plasmogamy in protoperithecia during early sexual development and are essential for fertility, are downregulated in MAT deletion strains *in vitro* (H.-K. Kim et al., 2012; Zheng et al., 2013). However, transcriptomic studies have revealed that the MAT genes also have direct or indirect effects on many more genes, with a wide range of functions beyond mating. In

microarray assays, a majority (59%) of expressed genes were down-regulated in a *mat1-2-1* deletion strain of *F. graminearum in vitro,* compared to the wild type (S.-H. Lee, Lee, Choi, Lee, & Yun, 2006). In addition to genes directly involved in mating, these also included stress response, metabolism, and development genes, all of which could influence pathogenicity or toxigenicity. As another example, genes involved in production of the mycotoxin ZEA were repressed by the MAT genes during perithecium induction (Son et al., 2011).

Not only are the MAT genes master regulators of multiple other genes, but they in turn are controlled by other developmental regulators, thus occupying a central position in overall developmental control. For example, strains deleted for *FgVelB*, a developmental regulator that affects chromatin structure and controls asexual and sexual sporulation and secondary metabolism, showed down regulation of the MAT genes and reduced perithecial formation as well as pathogenicity (Jiang, Liu, Yin, & Ma, 2011; J. Lee, Myong, et al., 2012; Merhej et al., 2012). The mycotoxin genes *TRI5* and *TRI6* were also down regulated, in contrast with the wild type where *TRI6* was induced during sexual development. Reduced pathogenicity also resulted from a deletion of the gene *Gpmk1*, a MAP kinase that belongs to a signal transduction pathway that is involved in multiple environmental responses (Jenczmionka, Maier, Lösch, & Schäfer, 2003). The mutation was also reduced in the production of mating-type specific pheromones and in perithecial formation.

In the earlier chapters of this thesis, I explored effects on morphology, fertility, and pathogenicity of MAT deletions, and showed that the deletion strains could be used for heterothallic matings and marker segregation analysis. The individual deletion strains had a range of quantitative phenotypes related to female fertility, interfertility, pathogenicity, and toxigenicity. These changes did not appear to be directly related to the specific MAT gene deletions, so in this final chapter I set out to see if the deletion strains were associated with other significant changes in the transcriptome *in planta*.

5.2 Materials and methods

5.2.1 Fungal strains and growth conditions.

Strain PH-1 was obtained from Dr. Frances Trail. MAT gene deletion strains were constructed earlier (Bec et al., 2021). All fungal strains were routinely grown at 23°C with constant light (Sylvania F032/741/ECO). Gene deletion strains were single-spored and stored on silica gel at -20°C or -80°C (Tuite, 1969, after Perkins, 1962). Strains were never subcultured more than once. Strains were started on PDA for 5 days before collecting colonies with sterile toothpicks and subculturing on sporulation inducing media. Production of asexual spores was done on mungbean agar (MBA) (40 g mungbean and 10 g Bacto Agar per L) at 23°C. Mungbean agar was prepared by boiling 40 g of mungbeans in 1 L of water for about 23 min or until the beans began to split. Beans were filtered out using a double layer of cheesecloth, the liquid was measured, and water was added to 1 L. Ten grams of Bacto Agar were added before autoclaving. Once cultures had grown for 7-10 days, 2 mL of sterile water was applied to the surface of the Petri plate and the spores were released by rubbing with a sterile plastic micro-pestle. Spore suspensions were filtered through a double layer of cheesecloth to remove mycelia, and collected in a sterile 50 mL Falcon tube. Spores were counted by using a hemocytometer. For use as inoculum, spores were centrifuged at 3330 x g, then washed once in sterile water and resuspended to adjust to 1 x 10^5 per mL.

5.2.2 Fusarium Head Blight Pathogenicity Assay.

The susceptible HRSW variety Wheaton were used for this study. Wheat seeds were planted in a mixture of topsoil (Maury silt loam) and PromixBC grown substrate (3:2) in plastic planting cones at a rate of three seeds per Cone-tainer™ (Steuwe and Sons, Inc.). The seeds were lightly covered with a moist layer of soil mixture. Seeds were germinated in the greenhouse at ambient temperature of 25°C-28°C. After germination, seedlings were thinned to one per cone. Wheat plants were grown in a greenhouse with a 14/10 photoperiod, provided by "Hortilux" LU430S/HTL/EN high pressure sodium lights, and

ambient temperatures between 25-28°C. Plants were fertilized with 150 ppm of N:P:K (20:10:20) fertilizer formulation twice a week, beginning two weeks after transplanting with last fertilization at heading. Flowering typically occurred after 3-4 weeks. At early- to mid- anthesis, a single centrally positioned floret on the primary flowering stem of each plant was inoculated with 10 µL of a 1 x 10^5 per mL spore suspension as described by (Miedaner et al. 2003) and covered with a small plastic bag for 24 hours to increase humidity.

5.2.3 Experimental Design

One *mat1-1-1* deletion strain, two *mat1-2-1* deletion strains, one MAT1 whole locus deletion strain, and the wild type PH-1, were used in this study (**Table 5.1**). Fifteen wheat heads were inoculated for each time point and for each treatment. The experiment used a completely randomized design. Treatments were applied over the course of several days from the same batch of spore suspension that was stored at 4°C. Treatments were randomized and applied to the spikelets on each plant as soon as they entered anthesis.

5.2.4 Preparation of RNA.

Following inoculation, RNA was isolated from the inoculated spikelets at 2 dai and at 4 dai. The total RNA extraction protocol was modified from one previously published by (O'Connell et al., 2012). Time points were chosen based on published studies in which *F. graminearum* moved from the inoculated spikelet into the rachis between 2 and 4 dai. (Neil A Brown et al., 2012; Jansen et al., 2005; Mary Wanjiru, Zhensheng, & Buchenauer, 2002; Miller, Chabot, Ouellet, Harris, & Fedak, 2004). Single inoculated spikelets were collected 2 dai, and inoculated spikelets and subtending rachis were collected at 4 dai. All tissues were flash frozen in liquid N_2 immediately after collection.

Five randomly selected frozen spikelets, or spikelets plus rachis, were pulverized together to a fine powder in a sterile mortar and pestle and transferred to a 1.5 mL Eppendorf tube. One mL of Trizol reagent and 200 µL of chloroform were added to the tube and mixed vigorously by hand and then incubated at room temperature for 5 minutes.

The samples were centrifuged at 13,320 x g in a microfuge for 17 minutes at 4°C. The aqueous phase was transferred to a new Eppendorf and 500 µL of cold isopropanol was added and mixed to precipitate the RNA. Samples were then left to precipitate overnight in isopropanol or 70% Ethanol DEPC at 20°C. Tubes were centrifuged for 12 minutes at 13,320 x g at 4°C. Supernatant was discarded, and the tube was dabbled with a new Kimwipe to remove excess. The pellet was washed by centrifuging with 1 mL 70 % Ethanol DEPC for 7 minutes at 13,320 x g at 4°C. Tubes were air dried for 10 minutes and any excess liquid was removed by pipetting. The pellet was resuspended in 50-100 µL of DEPC water. Follow-up cleaning was performed by using Enzymax LLC columns (Lexington KY). An equal volume of 70% Ethanol DEPC was added to the RNA, and the solution was loaded onto the column and spun down for one minute at 13,320 x g at 4°C. Flowthrough was discarded and 350 µL of 75% Ethanol DEPC was then added to the column and spun down for 1 minute. This step was repeated once more. The column was spun down for 2 additional minutes to remove excess ethanol. RNA was eluted by adding 50 µL of DNase/RNase free water pre-warmed at 65°C to the center of the column and incubated for 5 minutes. Tube was centrifuged at max speed. Samples were immediately stored at -80°C.

5.2.5 RNAseq.

Library construction and transcript analysis were provided by Novogene Co (Sacramento, CA). RNA quality and quantity were evaluated by the nanodrop method prior to shipment, and then subjected to further quality control (QC) by Novogene. Messenger RNA was purified from total RNA using poly-T oligo-attached magnetic beads. Directional libraries were prepared for each sample after end repair, A-tailing, adapter ligation, size selection, enzyme digestion, amplification, and purification. The library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. Clustering of the index-coded samples was performed according to the manufacturer’s instructions. After cluster generation, the library preparations were sequenced on an Illumina platform (Illumina NovaSeq 6000) and paired-end reads (PE150, 11 G raw data per sample) were generated.

5.2.6 Data Analysis.

Data analysis was provided by Novogene. Raw reads in FASTQ format were initially processed through fastp to remove adapter contamination or low-quality reads (**Table 5.2**). Reference genome and gene model annotation files (R King, Urban, & Hammond-Kosack, 2017; Robert King, Urban, Hammond-Kosack, Hassani-Pak, & Hammond-Kosack, 2015) were downloaded from the NCBI genome website browser (GCA_900044135.1). Paired-end clean reads were mapped to the reference genome by using HISAT2 software (D. Kim, Paggi, Park, Bennett, & Salzberg, 2019).

Differential expression analysis between each pair of treatments (three biological replicates per condition) was performed with the DESeq2 R package (Anders et al., 2010). The resulting P values were controlled for False Discovery Rate (FDR) (Benjamini & Hochberg, 1995). Genes with an adjusted P value < 0.05 were differentially expressed.

5.3 Results

5.3.1 RNA recovery and Quality of RNA libraries and RNAseq.

Total RNA of sufficient quality and quantity for analysis was recovered for most of the samples in experiment 1 (**Table 5.2**). Recovery of RNA in experiment 2 was less successful, with only two treatments yielding sufficient quantity and quality across all replications for analysis (**Table 5.2**). Water controls yielded good quality RNA for all reps in both experiments, but they were not included in the RNAseq analysis to save money, and since I mainly wanted to focus on the fungal genes here. These RNA samples were stored at -80°C and may be analyzed later to investigate the host gene expression patterns in more detail.

Mapping rates to the fungal reference genome were relatively low overall, especially at 2 dai (**Table 5.3**). An average of 6,256,122 reads per sample were mapped, but the numbers ranged widely, from 27,760 to 27,670,183. Unique mapping rates averaged 1.41% and ranged from 0.33-4.14% for 2dai. At 4 dai, the average was 10.56%, ranging from 0.58-26.11%. The percentage of mapped reads was mostly related to the specific

treatment and not to the replication, as replicates were similar with only two exceptions (B1 and C3 corresponding to Δ*mat1-2-1* strain 2-1 and Δ*mat1-2-1* strain 2-2, both at 2 dai). The percentage of mapped reads for the second experiment was lower than for the first (average of 1.88% versus 8.04%). Most mapped reads were exonic as expected (average of 77.4%), with the remainder intronic (0.2%) or intergenic (22.4%), suggesting a possibility of error in the annotation models.

5.3.2 Correlation coefficient.

Correlation of gene expression levels between samples gives an indication of the reproducibility of the experiment, and of the degree of similarity among various treatments. The closer the correlation coefficient is to 1, the more similar the samples are. The correlation coefficient matrix for the samples in my experiment is shown in (**Figure 5.1**). Darker colors indicate higher levels of correlation: the matrix demonstrates that the degree of similarity among replications of the same treatment in the same experiment was mostly high. It also indicates that overall similarity among the different treatments within the same experiment was also high, but that results from the two experiments were more divergent. A principal component analysis (PCA) shows a similar result, with most treatments from experiment 1 clustered together, while experiment 2 was more separate, and showed more divergence between the wild type and *mat1-2-1* deletion strain (**Figure 5.2**).

5.3.3 Comparisons of co-expression of genes among samples.

More than 11,000 expressed fungal genes were detected *in planta*, out of 14,164 total predicted genes encoded by *F. graminearum*. A Venn diagram comparing the PH-1 wild type strain at 2 and 4 dai in the two experiments showed that results were comparable, especially at 4 dai. (**Figures 5.3**). The 2 dai samples from experiments 1 and 2 differed by 14%, and the 4 dai samples by only 8%. In experiment 1, a comparison of the three deletion strains and the wild type at either 2 dai **(Figure 5.4)** or 4 dai (**Figure 4.5**) demonstrated in each case that about 80% of expressed genes were shared among all

the samples while approximately 20% were unique, or shared among only some of the treatments.

5.3.4 Differentially Expressed Gene Expression Analysis.

Volcano plots of pairwise comparisons among the treatments illustrate patterns of statistically significant up and down regulated genes (DEG) (**Figure 5.5 – 5.7**). Comparisons of different MAT deletion mutants to the wild type revealed similar patterns of variation at 2 or 4 dai for each pair. At 2 dai, there were no significant DEG between the whole locus MAT1 deletion and the other MAT deletions, and only four DEG between the MAT1 deletion and the wild type. There were more differences between the *mat1-1-1* and *mat1-2-1* deletions. At 4 dai, there were more differences among all the strains. The *mat1-1-1* and *mat1-2-1* deletions mostly had genes that were up regulated versus down regulated compared with the wild type at 2 dai, but that pattern was reversed at 4 dai.

As expected, the *MAT1-1-1* and *MAT1-2-1* genes were differentially expressed at 2 and 4 dai in plants inoculated with the MAT deletion mutants, but only in the first experiment (**Table 5.4**). The *MAT1-1-1* gene was more highly expressed by the *MAT1-2-1* deletion strain versus the wild type at both 2 and 4 dai.

The *TRI5* trichothecene biosynthetic cluster has been reported to be upregulated *in planta* during infection of wheat heads (D. W. Brown, McCormick, Alexander, Proctor, & Desjardins, 2001; Hohn & Beremand, 1989; Kimura et al., 2003; McCormick & Alexander, 2002). Several genes belonging to the trichothecene biosynthetic cluster were differentially expressed in my study, but only in the second experiment (**Table 5.4**). *TRI3*, *TRI4*, *TRI5*, *TRI6*, *TRI8*, *TRI9*, *TRI11*, *TRI12*, *TRI13*, *TRI14,* and *TRI101* were all upregulated at 4 dai compared with 2 dai in the wild type, and all but *TRI6*, *TRI9*, and *TRI13* were also upregulated in the *mat1-2-1* deletion strain between 2 and 4 dai (**Table 5.4**). The genes did not differ significantly in expression between the deletion mutant and the wild type, except for *TRI4* and *TRI5* at 2 dai.

Gene ontology and KEGG analyses for the DEG among the pairwise comparisons from experiment 4, which had the highest numbers of mapped reads, are presented in (**Figures 5.9 – 5.14**). The KEGG analyses showed that all the strains underwent shifts in

genes associated with primary metabolism between 2 and 4 dai. The patterns were generally similar, except that the *mat1-2-1* strain did not show a significant shift in secondary metabolism like the others. This difference also showed up in the pairwise comparisons at 2 and 4 dai. The gene ontology analyses indicated strongly significant shifts in hydrolytic activities and extracellular activities between 2 and 4 dai for the wild type, but the pairwise comparisons indicated that the MAT deletions differed from the wild type in these functions, which are likely to be important for host colonization and pathogenicity.

5.4 Discussion

The transcriptome analysis gave indications of some statistical variation among the treatments, even though mapping percentages were relatively low overall. This low percentage is expected given the relative difference in fungal versus plant biomass, and indeed I saw that the percentage of mapped reads increased between 2 and 4 dai, indicating that fungal biomass increased during that time. With low numbers of mapped reads, particularly in the second experiment, the ability to identify differentially regulated genes will be limited, but useful information can still be gained. Increased confidence in the value of the experiments was given by my ability to differentiate expression of the MAT genes themselves as expected between the wild type and the MAT deletion mutants. Since I only saw this difference in the first experiment, it may be that the data are more reliable from that experiment. On the other hand, I was not able to detect significant variation in the *TRI* gene cluster between 2 and 4 dai in that experiment, while I did detect that expected difference in the second experiment. This indicates that there was a difference in the two experiments and that in the first experiment, growth may have been slower such that there was less differentiation between 2 and 4 dai in comparison with the second experiment. It is not unexpected for different experiments to vary, since they are done with different batches of inoculum, different plants grown under different conditions, and with the infection also occurring under different conditions. Thus, it may be beneficial to consider results of both experiments, at least for the wild type and *mat1-*

2-1 deletion strains that are included in both. Unfortunately, the RNA was not of sufficient quality in the second experiment to include the other treatments.

Based on the overall analysis presented here, I saw that the transcriptome of the MAT deletion strains did not differ dramatically from that of the wild type, with more than 80% of the genes expressed shared among all of them. This suggests that the impact of the MAT genes, at least at these time points, is relatively small. KEGG analysis indicated that the *mat1-2-1* mutant may differ from the rest in the production of secondary metabolites, and that all the MAT deletion mutants may express fewer hydrolytic enzymes and secreted proteins during early infection. These factors could affect the ability of the mutants to colonize the plants successfully. It will be interesting in the future to undertake a more detailed manual analysis of the data, to understand more about the specific identities and roles of these differentially expressed genes, and to look more directly at the ability of the strains to colonize the host tissues.

Table 5.1 Strains and Treatments Included in the Transcriptome Analysis.

Sample Code	Strain	Treatment	DAI	Experiment	Rep
A1	1_3	Δ*mat1-1-1*	2	1	1
A2	1_3	Δ*mat1-1-1*	2	1	2
A3	1_3	Δ*mat1-1-1*	2	1	3
A4	1_3	Δ*mat1-1-1*	4	1	1
A5	1_3	Δ*mat1-1-1*	4	1	2
A6	1_3	Δ*mat1-1-1*	4	1	3
B1	2_1	Δ*mat1-2-1*	2	2	1
B2	2_1	Δ*mat1-2-1*	2	2	2
B3	2_1	Δ*mat1-2-1*	2	2	3
B4	2_1	Δ*mat1-2-1*	4	2	1
B5	2_1	Δ*mat1-2-1*	4	2	2
B6	2_1	Δ*mat1-2-1*	4	2	3
C1	2_2	Δ*mat1-2-1*	2	1	1
C2	2_2	Δ*mat1-2-1*	2	1	2
C3	2_2	Δ*mat1-2-1*	2	1	3
C4	2_2	Δ*mat1-2-1*	4	1	1
C5	2_2	Δ*mat1-2-1*	4	1	2
C6	2_2	Δ*mat1-2-1*	4	1	3
D1	0_1	ΔMAT1	2	1	1
D2	0_1	ΔMAT1	2	1	2
D3	0_1	ΔMAT1	2	1	3
D4	0_1	ΔMAT1	4	1	1
D5	0_1	ΔMAT1	4	1	2
D6	0_1	ΔMAT1	4	1	3
F1	PH-1	Wild Type	2	1	1
F2	PH-1	Wild Type	2	1	2
F3	PH-1	Wild Type	2	1	3
F4	PH-1	Wild Type	4	1	1
F5	PH-1	Wild Type	4	1	2
F6	PH-1	Wild Type	4	1	3
F7	PH-1	Wild Type	2	2	1
F8	PH-1	Wild Type	2	2	2
F9	PH-1	Wild Type	2	2	3
F10	PH-1	Wild Type	4	2	1
F11	PH-1	Wild Type	4	2	2
F12	PH-1	Wild Type	4	2	3

Table 5.2 Summary of Quality Control.
Including coding system, RNA Integrity Number, reads, bases, percentage of error and quality score of 20 and 30.

Code	RIN	Raw reads	Clean reads	Raw bases	Clean bases	Error rate (%)	Q20 (%)	Q30 (%)
A1	8.8	47361981	47107870	14.2	14.1	0.03	97.76	93.48
A2	9.3	49352029	48678428	14.8	14.6	0.02	98.07	94.43
A3	9.1	41647366	41038514	12.5	12.3	0.03	97.8	93.72
A4	3.2	42945817	42169475	12.9	12.7	0.03	97.61	93.28
A5	3.3	47405784	46808505	14.2	14	0.03	97.8	93.84
A6	8.7	49232066	48579405	14.8	14.6	0.02	98.15	94.68
B1	9.2	49546907	48886289	14.9	14.7	0.03	97.91	94.07
B2	9.4	52059064	51612245	15.6	15.5	0.02	98.23	94.75
B3	9.4	53337016	52925547	16	15.9	0.03	97.98	94.04
B4	8.8	50308845	49816280	15.1	14.9	0.03	97.43	93.05
B5	9.5	48209958	47482883	14.5	14.2	0.02	98.05	94.27
B6	8.9	64313516	63515703	19.3	19.1	0.03	97.28	92.72
C1	6.6	46521709	46231685	14	13.9	0.02	98.1	94.3
C2	9	49098973	48495862	14.7	14.5	0.03	97.96	94.17
C3	9.3	37154394	36561280	11.1	11	0.02	97.97	94.22
C4	7.8	52185079	51283069	15.7	15.4	0.03	97.37	93.01
C5	3.1	55884375	55584232	16.8	16.7	0.03	98.01	94.09
C6	9.3	68859962	68513820	20.7	20.6	0.03	98	94.07
D1	8.7	48272768	47904153	14.5	14.4	0.02	98.06	94.27
D2	4.6	48539370	48168445	14.6	14.5	0.03	97.92	93.98
D3	9	27552578	26660448	8.3	8	0.02	98.07	94.47
D4	4.1	51106114	50633130	15.3	15.2	0.02	98.03	94.21
D5	5.5	51922538	51582318	15.6	15.5	0.02	98.11	94.42
D6	8.6	49057043	48626988	14.7	14.6	0.02	98.1	94.36
F1	9.1	48417139	47977505	14.5	14.4	0.02	98.07	94.27
F2	9.4	48918638	48246969	14.7	14.5	0.03	97.73	93.59
F3	9.4	48598736	47945825	14.6	14.4	0.03	97.5	93.13
F4	8.8	49407789	48978228	14.8	14.7	0.03	97.71	93.55
F5	6.2	55154532	54733908	16.5	16.4	0.03	97.98	94.11
F6	7.7	49812658	49318051	14.9	14.8	0.03	97.8	93.82
F7	8.8	49790880	49396028	14.9	14.8	0.03	97.97	94.09
F8	9.1	49243642	48822186	14.8	14.6	0.03	97.13	92.37
F9	9.1	61716592	61110476	18.5	18.3	0.03	97.03	92.15
F10	2.9	46452197	46054751	13.9	13.8	0.02	98.13	94.44
F11	8.6	48189270	47789307	14.5	14.3	0.03	97.79	93.68
F12	8.7	53064632	52596578	15.9	15.8	0.02	98.08	94.41

Table 5.3 Mapping rates to the fungal reference genome at 2 dai.
Total reads give the initial output of high quality sequencing reads. Mapped reads refer to the number of reads mapped to the *Fusarium graminearum* reference sequence. Poor quality replications are highlighted in red.

Code	Total reads	Total mapped reads	Uniquely mapped reads	Multiple mapped reads	Total mapping rate	Uniquely mapping rate	Multiple mapping rate
A1	94215740	1474066	1427742	46324	1.56%	1.52%	0.05%
A2	97356856	2457365	2341644	115721	2.52%	2.41%	0.12%
A3	82077028	3455580	3396893	58687	4.21%	4.14%	0.07%
A4	84338950	13985747	12808890	1176857	16.58%	15.19%	1.40%
A5	93617010	14145223	13625177	520046	15.11%	14.55%	0.56%
A6	97158810	11745470	11492524	252946	12.09%	11.83%	0.26%
B1	97772578	333907	318945	14962	0.34%	0.33%	0.02%
B2	103224490	491625	476715	14910	0.48%	0.46%	0.01%
B3	105851094	551663	532447	19216	0.52%	0.50%	0.02%
B4	99632560	601651	581051	20600	0.60%	0.58%	0.02%
B5	94965766	1246145	1206458	39687	1.31%	1.27%	0.04%
B6	127031406	5663092	5417603	245489	4.46%	4.26%	0.19%
C1	92463370	1944236	1719797	224439	2.10%	1.86%	0.24%
C2	96991724	1754270	1694146	60124	1.81%	1.75%	0.06%
C3	73122560	277760	269172	8588	0.38%	0.37%	0.01%
C4	102566138	14895727	14562566	333161	14.52%	14.20%	0.32%
C5	111168464	15716253	15369515	346738	14.14%	13.83%	0.31%
C6	137027640	9474236	8615754	858482	6.91%	6.29%	0.63%
D1	95808306	1469870	1338305	131565	1.53%	1.40%	0.14%
D2	96336890	2265737	2168810	96927	2.35%	2.25%	0.10%
D3	53320896	763743	735098	28645	1.43%	1.38%	0.05%
D4	101266260	19287795	18889108	398687	19.05%	18.65%	0.39%
D5	103164636	27670183	26937089	733094	26.82%	26.11%	0.71%
D6	97253976	8106859	7958633	148226	8.34%	8.18%	0.15%
F1	95955010	1565888	1522079	43809	1.63%	1.59%	0.05%
F10	92109502	3680029	3583656	96373	4.00%	3.89%	0.10%
F11	95578614	3296887	3190017	106870	3.45%	3.34%	0.11%
F12	105193156	6878866	6610388	268478	6.54%	6.28%	0.26%
F2	96493938	1965776	1926312	39464	2.04%	2.00%	0.04%
F3	95891650	1778387	1731859	46528	1.85%	1.81%	0.05%
F4	97956456	15550224	14795163	755061	15.87%	15.10%	0.77%
F5	109467816	12418095	12218733	199362	11.34%	11.16%	0.18%
F6	98636102	16477738	15111825	1365913	16.71%	15.32%	1.38%
F7	98792056	594438	485744	108694	0.60%	0.49%	0.11%
F8	97644372	740517	718270	22247	0.76%	0.74%	0.02%
F9	122220952	495327	468258	27069	0.41%	0.38%	0.02%

Table 5.4 Selected DEGs from F. graminearum expressed in experiment 2.
MAT genes were only differentially expressed in experiment 1, and the trichothecene biosynthetic cluster (TRI) genes were only differentially expressed in experiment 2.

	ΔMAT vs WT 4dai EXP1	Δmat121 vs WT 2dai EXP1	Δmat121 vs WT 4dai EXP1	Δmat121 vs ΔMAT 4dai EXP1	Δmat111 vs Mat121	WT 2dai vs 4dai EXP2	Δmat121 2dai vs 4dai EXP2	Δmat121 vs WT 2dai EXP2
TRI3 FGSG_03534						-5.1	-7	
TRI4 FGSG_03535						-3.5	-7.8	-6.8
TRI5 FGSG_03537						-3.8	-8.8	-6.3
TRI6 FGSG_03536						-3		
TRI8 FGSG_03532						-3.8	-7.7	
TRI9 FGSG_03539						-4.1		
TRI11 FGSG_03540						-3	-6	
TRI12 FGSG_03541						-6.2	-7.4	
TRI13 FGSG_03542						-4.9		
TRI14 FGSG_03543						-3.9	-4.4	
TRI101 FGSG_07896						-3.4	-4.5	
FGSG_08893 MAT1-2-1	-7.4		-7.1					
FGSC_08892 MAT1-1-1		8.9	5.7	10.6	-9.4			

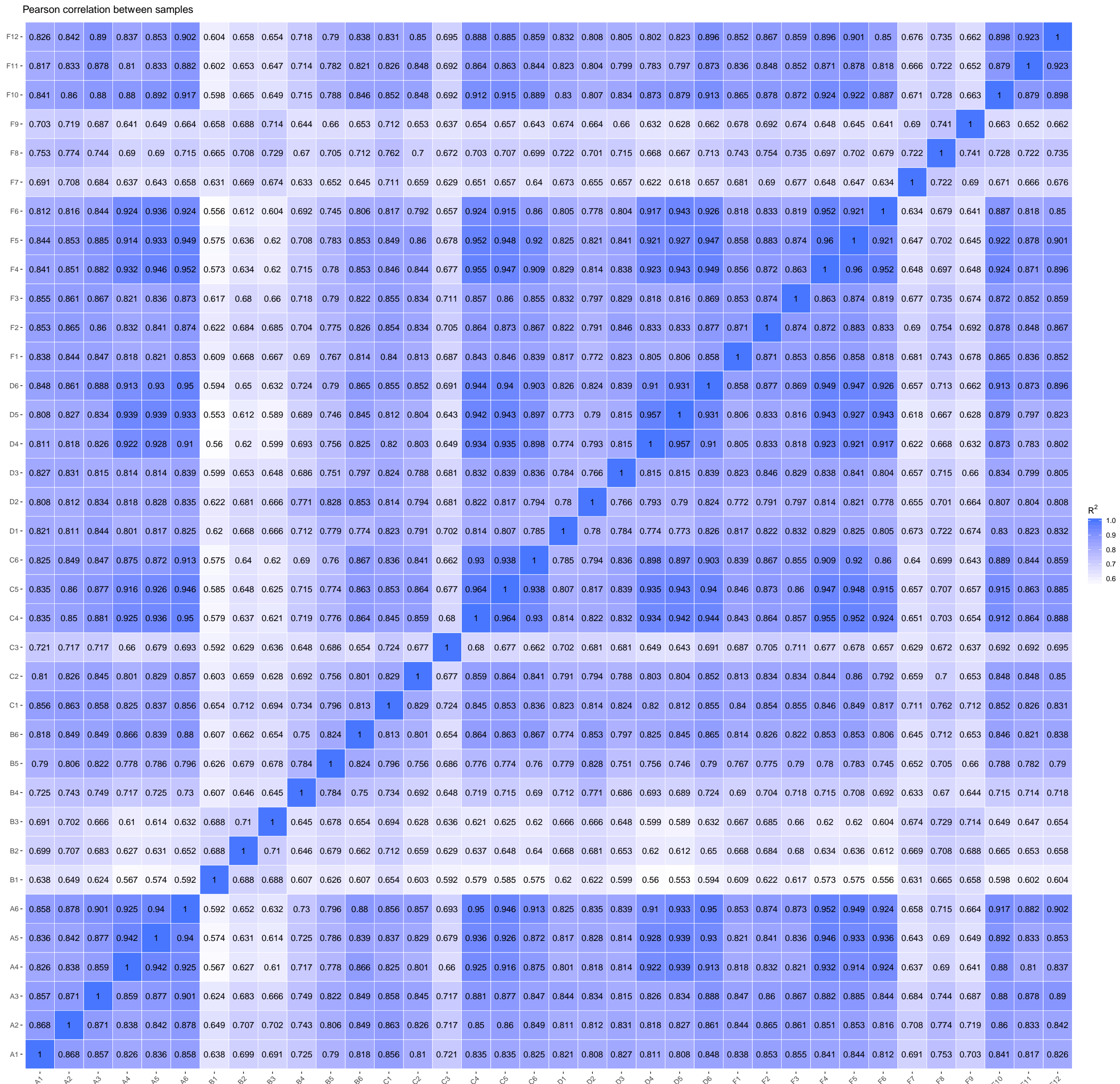


Figure 5.1 Correlation coefficient matrix of samples for each MAT deletion strain and wild type by using Pearson's Coefficient analysis.

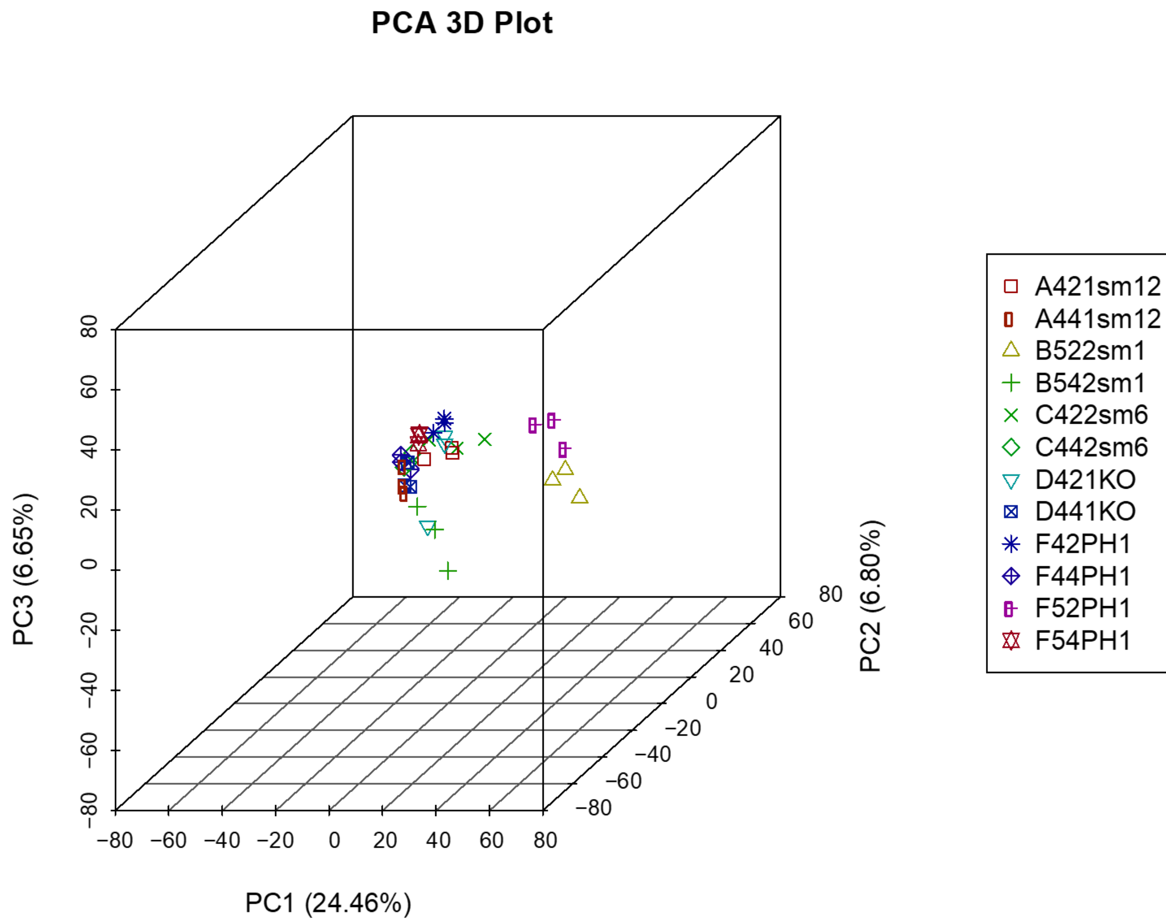


Figure 5.2: Principal Components Analysis of *Fusarium graminearum* MAT deletion strains and wild type PH-1 RNA expression in point -inoculated wheat spikelets.

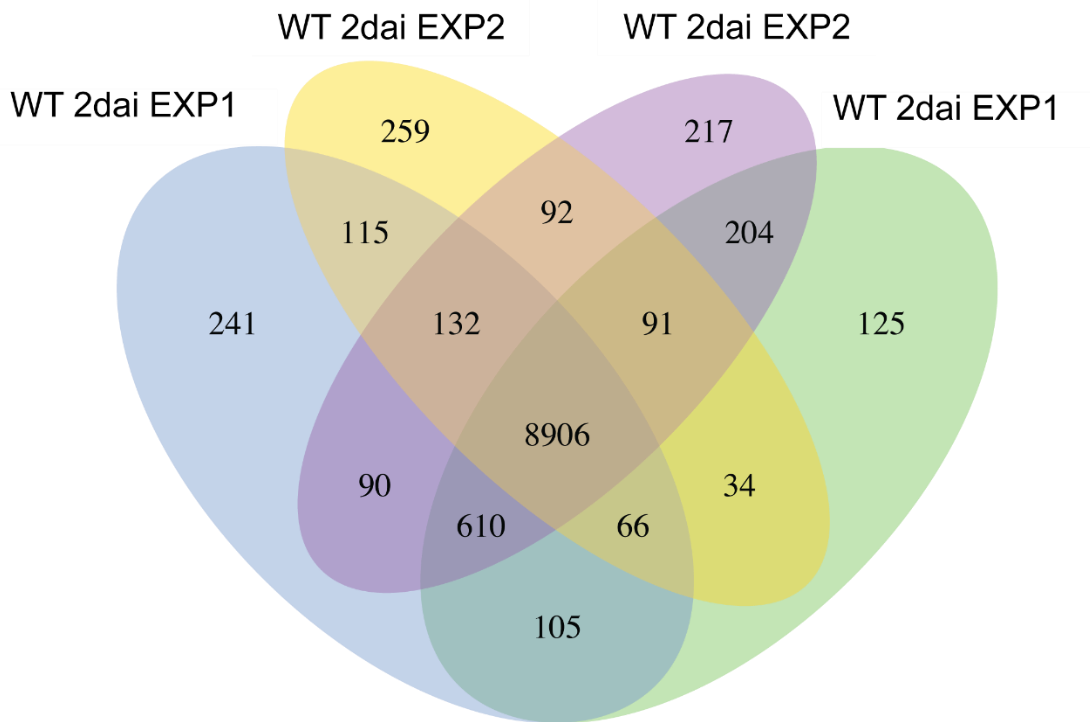


Figure 5.3 Co-expression Venn diagram among all PH-1 wild type (WT) treatments from 2 and 4 dai in experiment 1 and 2.

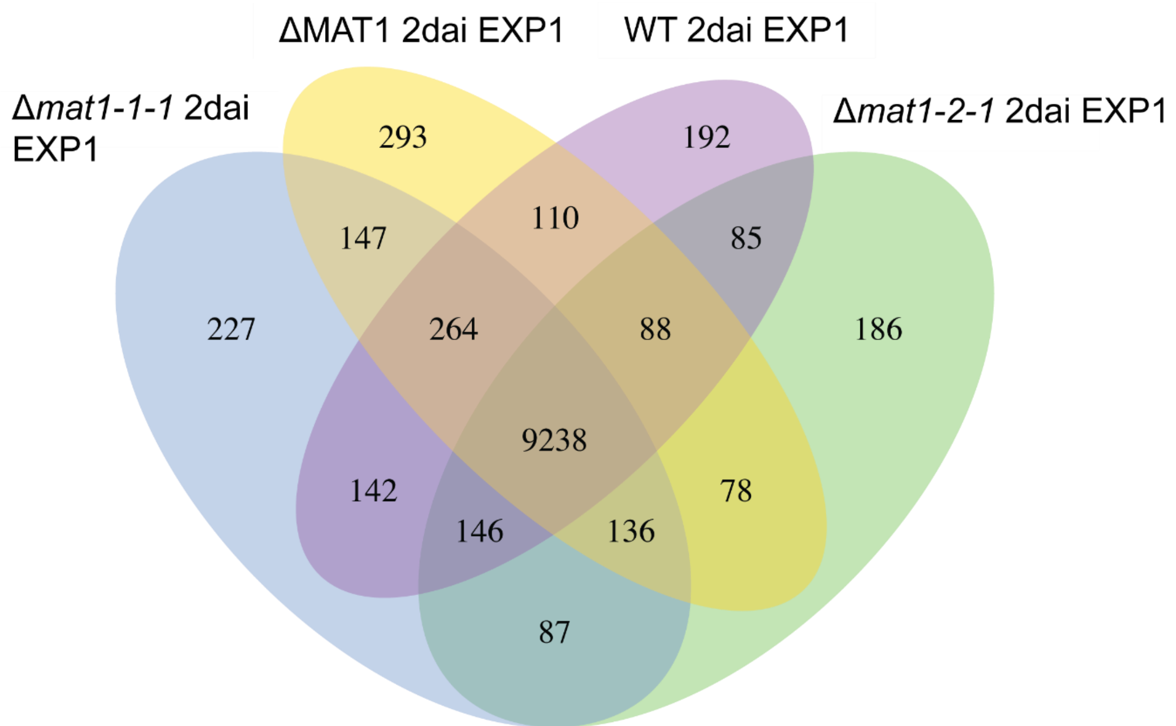


Figure 5.4 Co-expression Venn diagram between all MAT deletion strains and the PH-1 wild type (WT) 2 dai in experiment 1.

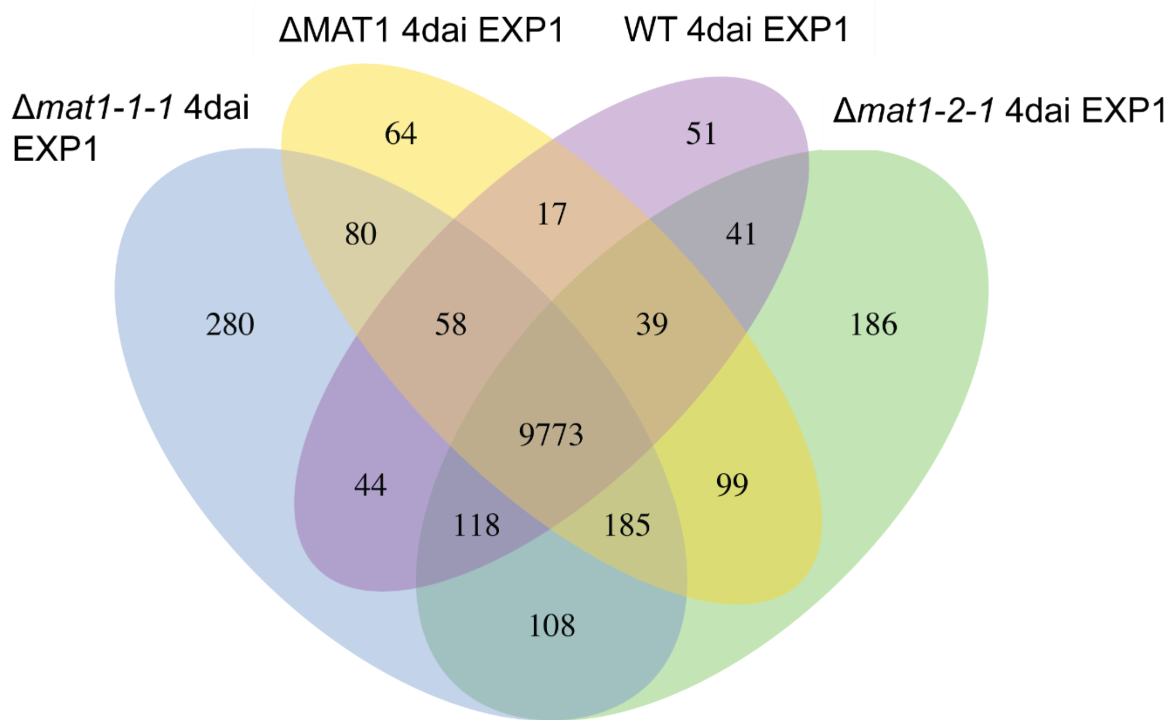


Figure 5.5: Co-expression Venn diagram between all MAT deletion strains and the PH-1 wild type (WT) 4 dai in experiment 1.

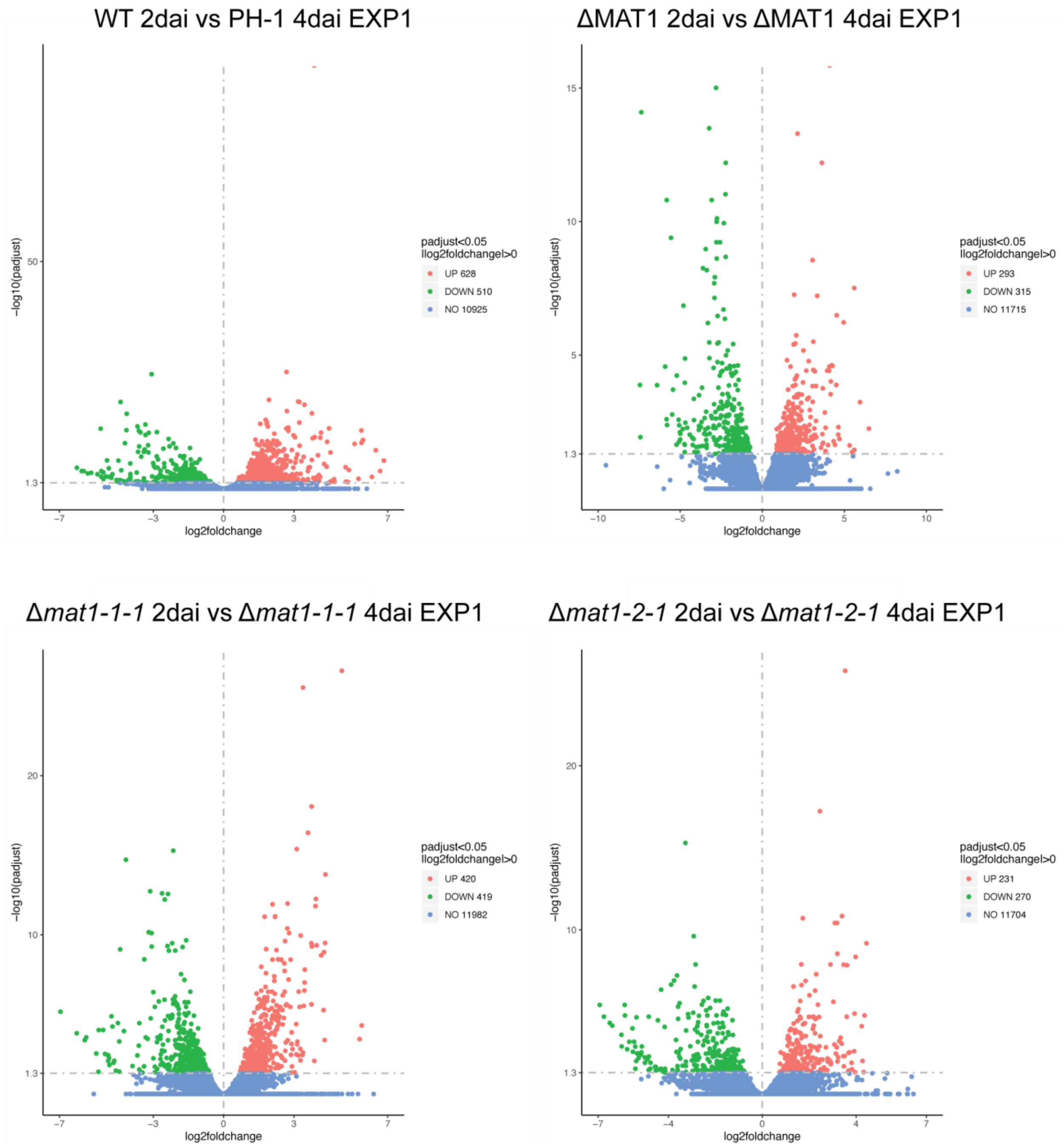


Figure 5.6 Volcano plots, wild type and MAT strains compared between 2 and 4 dai.
Showing up (red) and down (green) regulated genes. Blue dots show genes that were not differentially regulated.

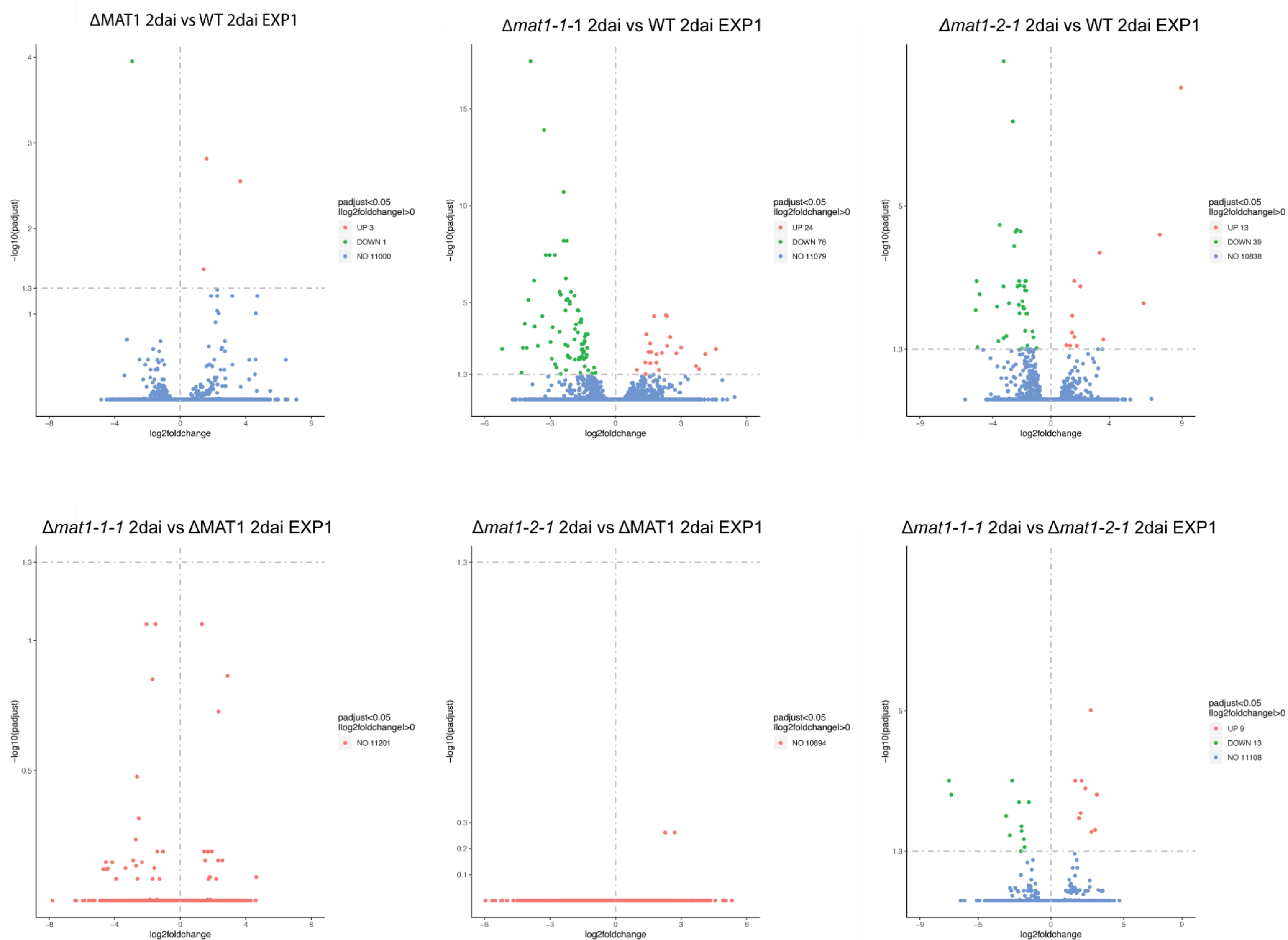


Figure 5.7 V**olcano plots, wild type versus MAT strains compared at 2 dai .**
Showing up (red) and down (green) regulated genes. Blue dots show genes that were not differentially regulated.

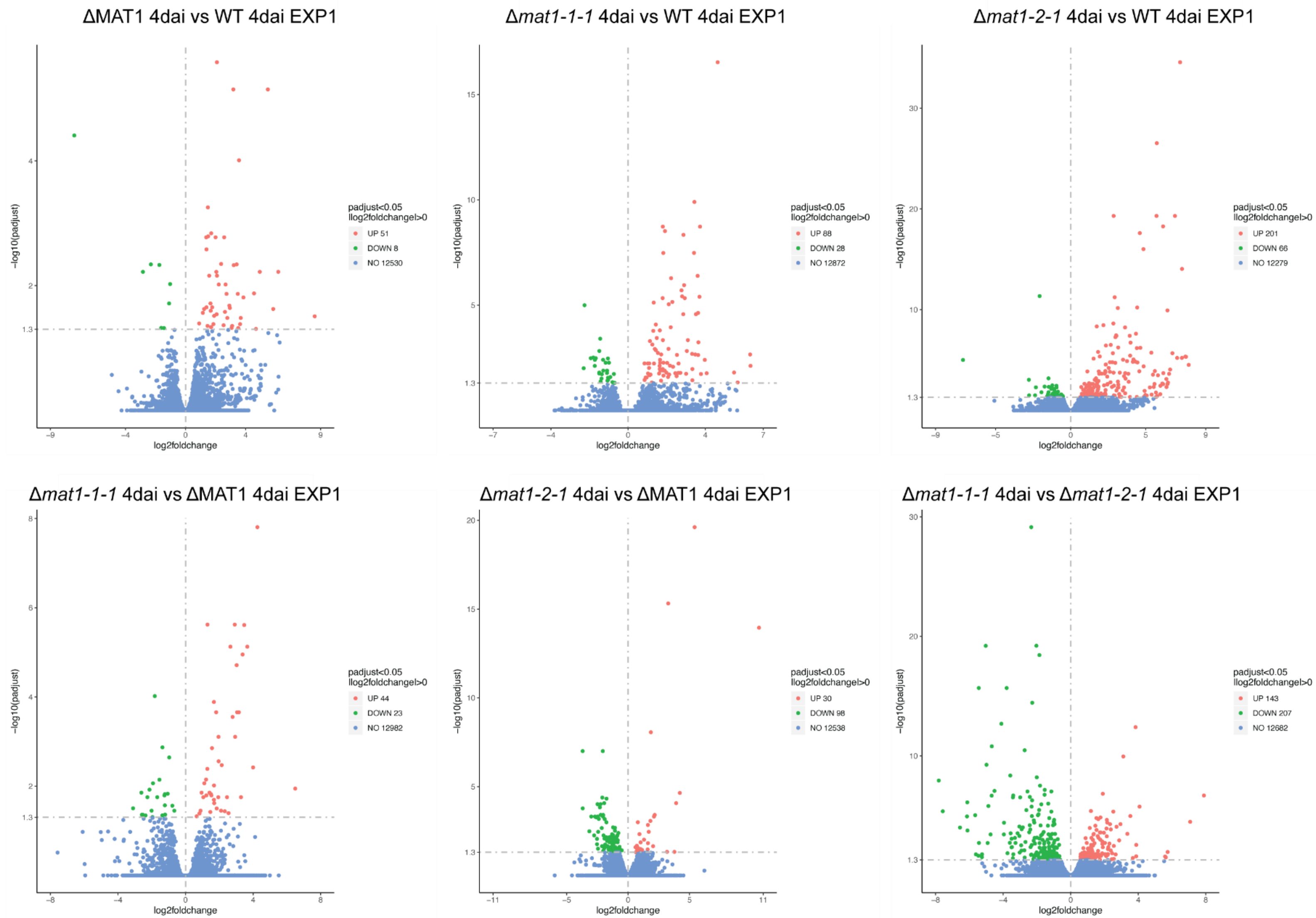


Figure 5.8 Volcano plot, wild type versus MAT strains compared at 4 dai.
Showing up (red) and down (green) regulated genes. Blue dots show genes that were not differentially regulated.

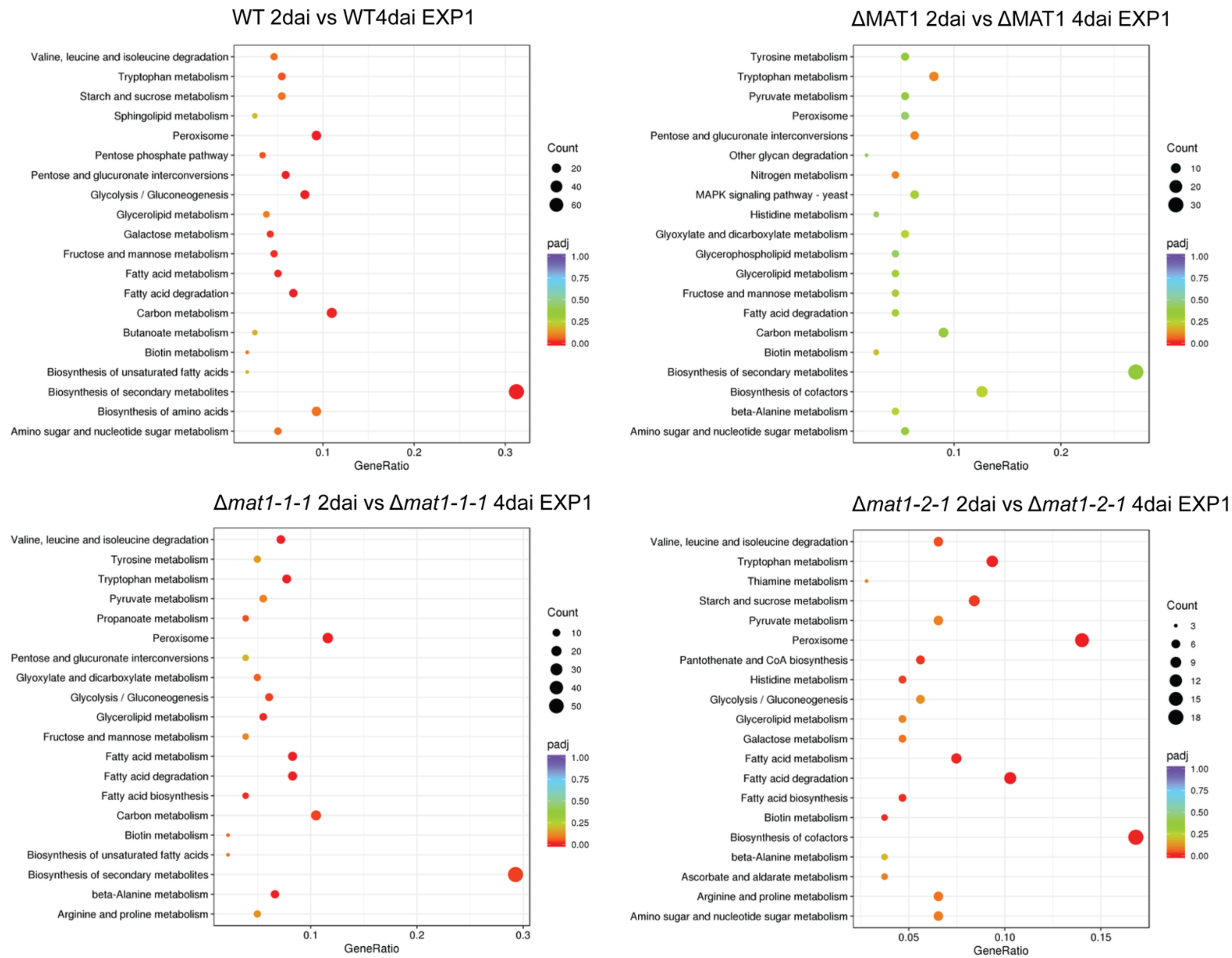


Figure 5.9 KEGG dot blots showing enrichment of genes involved in biological processes and molecular functions at 2 dai vs 4 dai in Experiment 1.
The size of the dot indicates the number of enriched genes, and the color of the dot shows the level of significance.

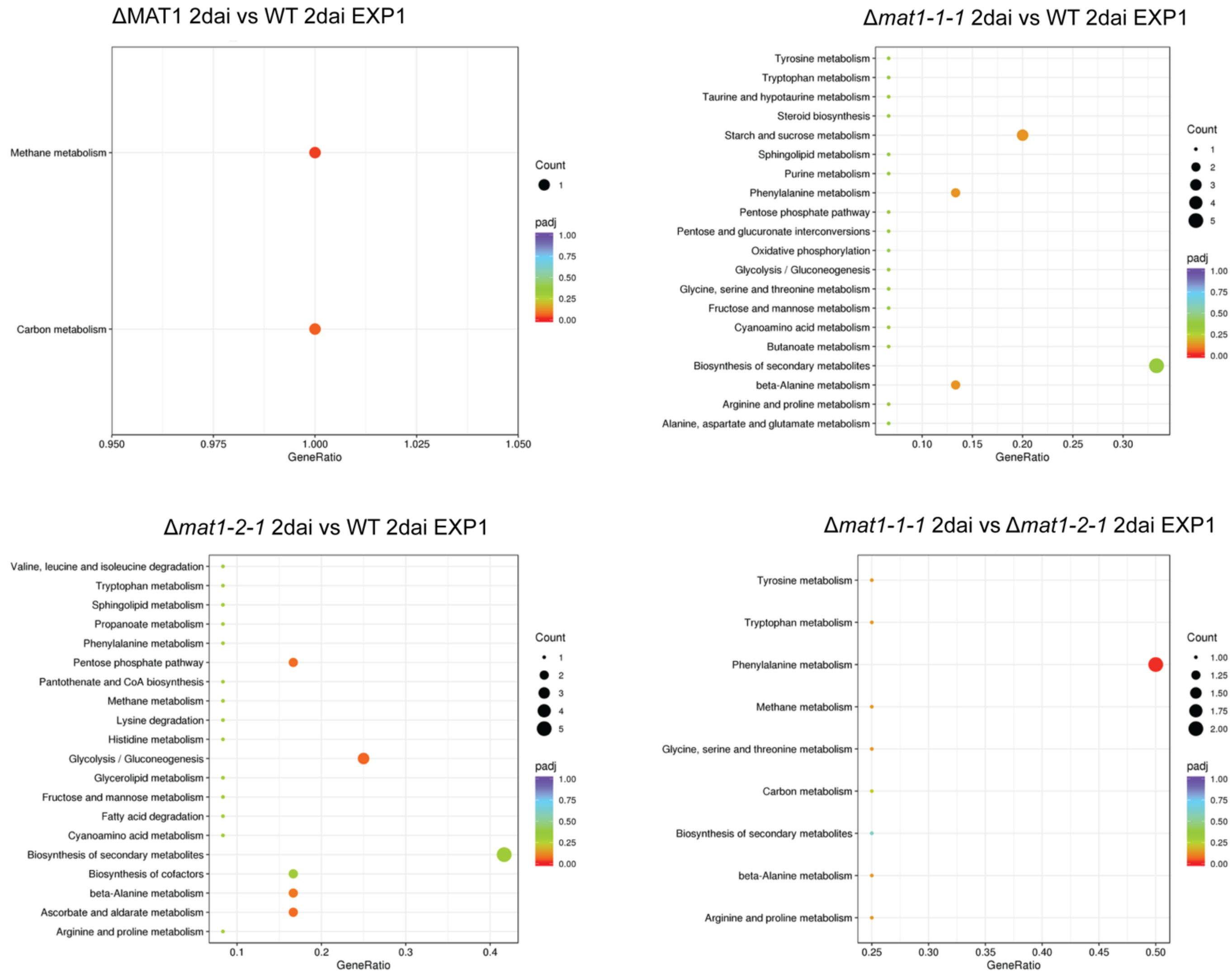


Figure 5.10 KEGG dot blots showing enrichment of genes involved in biological processes and molecular functions compared among different treatments at 2 dai in Experiment 1.
The size of the dot indicates the number of enriched genes, and the color of the dot shows the level of significance.

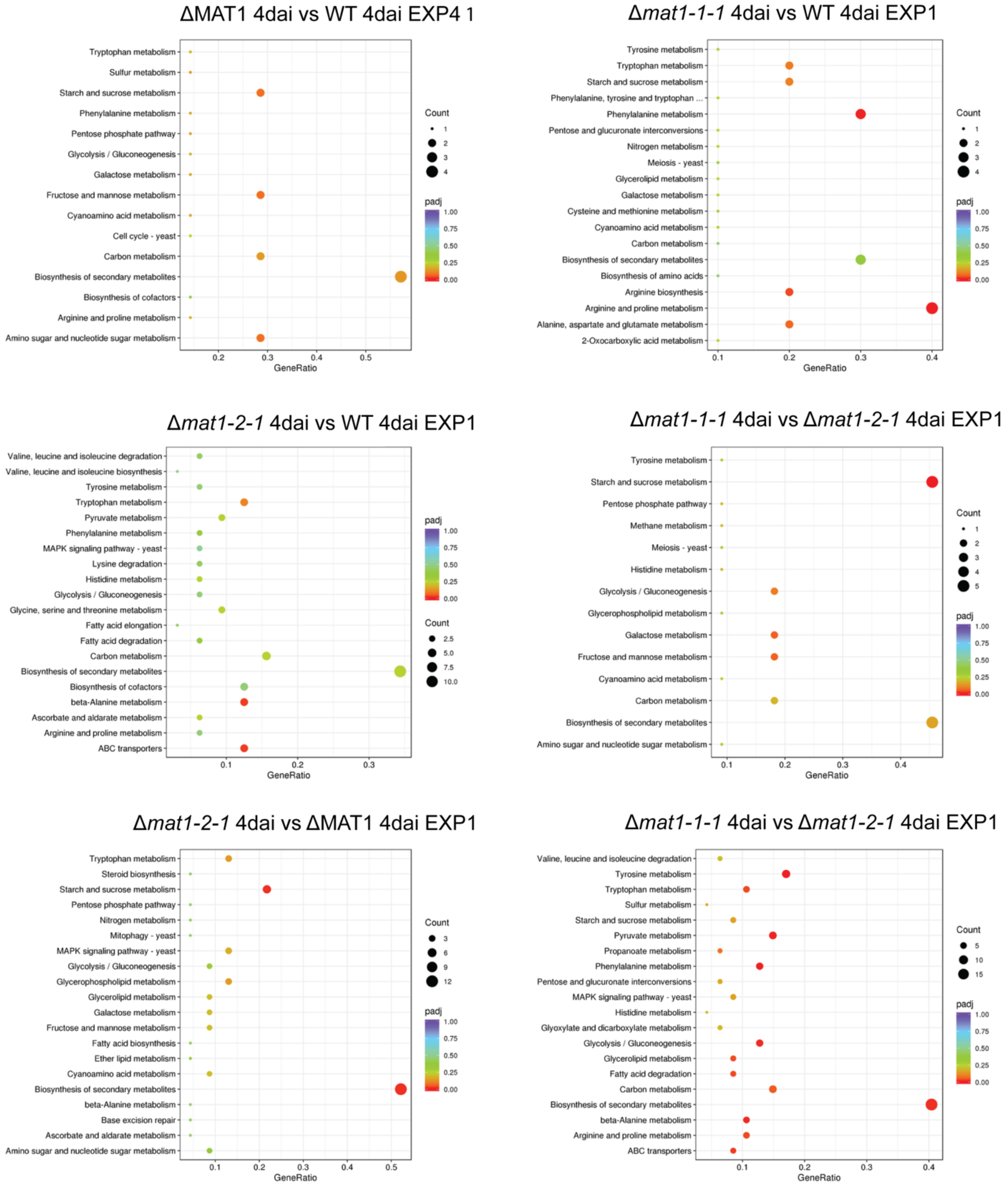


Figure 5.11 KEGG dot blots showing enrichment of genes involved in biological processes and molecular functions compared among different treatments at 4 dai in Experiment 1.

The size of the dot indicates the number of enriched genes, and the color of the dot shows the level of significance.

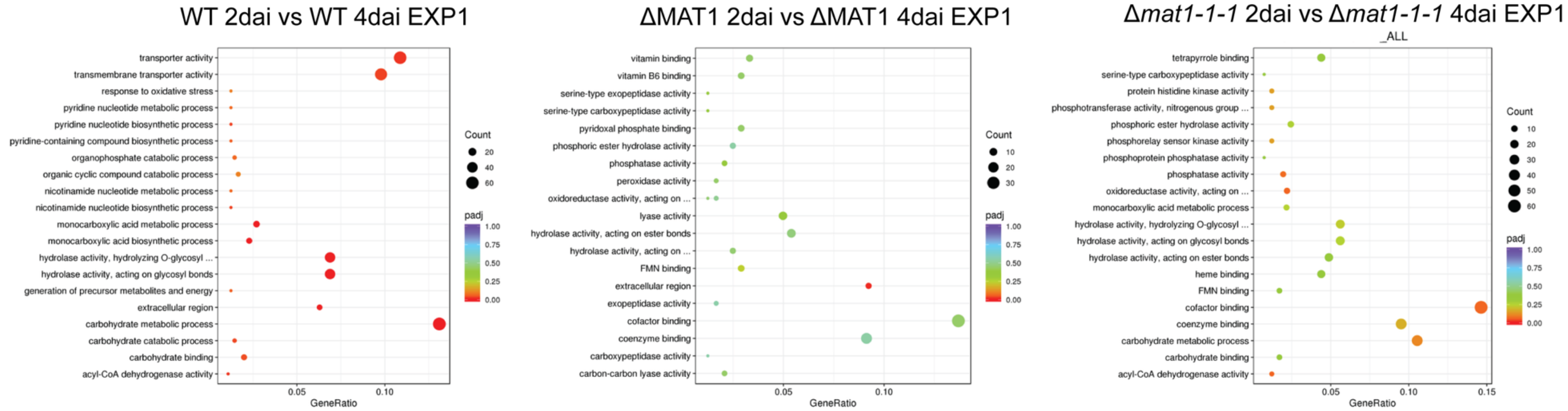


Figure 5.12 Gene Ontology dot blots showing enrichment of genes involved in biological processes and molecular functions compared at 2 dai versus 4 dai in Experiment 1.
The size of the dot indicates the number of enriched genes, and the color of the dot shows the level of significance.

ΔMAT1 2dai vs WT 2dai EXP1

Δ*mat1-1-1* 2dai vs WT 2dai EXP1

Δ*mat1-2-1* 2dai vs WT 2dai EXP1

Δ*mat1-1-1* 2dai vs Δ*mat1-2-1* 2dai EX1

Figure 5.13 Gene ontology dot blots showing enrichment of genes involved in biological processes and molecular functions compared among different treatments at 2 dai in Experiment 1.

The size of the dot indicates the number of enriched genes, and the color of the dot shows the level of significance.

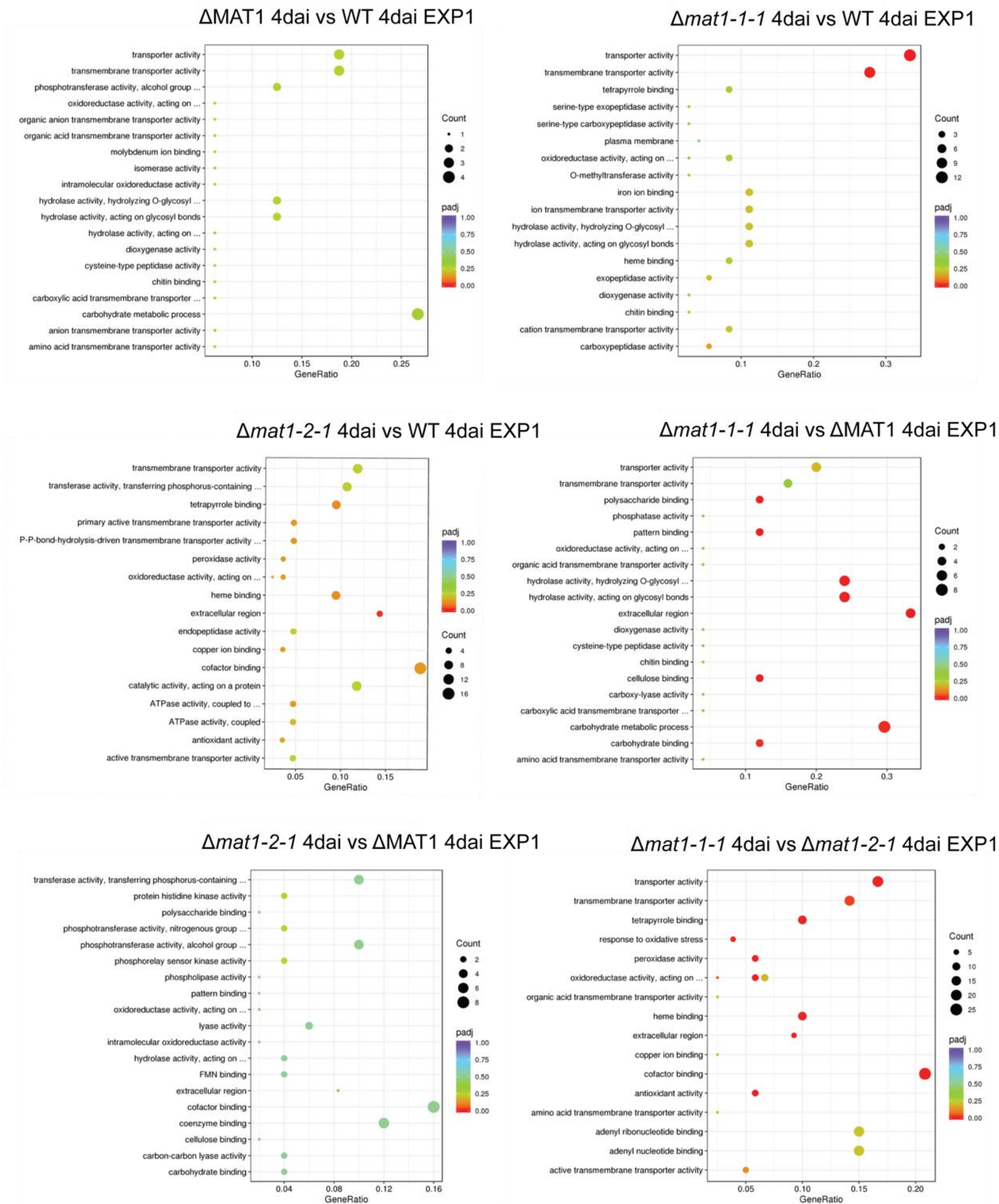


Figure 5.14 Gene ontology dot blots showing enrichment of genes involved in biological processes and molecular functions compared among different treatments at 4 dai in Experiment 1.

The size of the dot indicates the number of enriched genes, and the color of the dot shows the level of significance.

APPENDIX: DEVELOPMENT OF FAST FLOWERING MINI MAIZE (FFMM) FOR STUDIES OF GIBBERELLA EAR ROT AND OTHER FUNGAL EAR ROT DISEASES.

A.1 Introduction

Maize ear rots are among the most important diseases of maize worldwide (Kedera, Ochor, Ochieng, & Kamidi, 1994; Mutitu, 2003; Shurtleff, 1980). In Kentucky, the most common maize ear rot pathogens are *F. graminearum*, the cause of FHB which also causes Gibberella ear rot (GER); *F. verticillioides*, which causes Fusarium ear rot (FER); and *Stenocarpella maydis*, which causes Diplodia ear rot (DER) (Ullstrup, 1977). FER and GER symptoms include a tan or brown discoloration of kernels, usually scattered in groups. Signs include white to pink mold on the kernels especially at the tips of the ears. (Shurtleff, 1980). DER symptoms include dessication of infected kernels, and signs include white mycelium growing in between kernels thoroughout the ear (Flett, Bensch, Smit, & Fourie, 1996). The Fusarium ear rot pathogens don't just cause yield losses, but also accumulation of mycotoxins that are harmful to humans and livestock (Chen et al., 2019; A. Desjardins & Proctor, 2007; Munkvold, 2017). For example, outbreaks of GER in Canada between 1972-1977 (Sutton, Baliko, & Funnell, 1980) resulted a high incidence of zearalenone in grains, causing estrogenism in swine (Funnell, 1979; Sutton et al., 1980). All of the ear rot pathogens can overwinter on host residues (Kommedahl & CE, 1981; Nyvall & Kommedahl, 1970). Coinfection by ear rot pathogens can occur, but not much is known about how they interact together.

Because they are diseases of mature ears, ear rots are difficult to study in the greenhouse or growth chamber. As a result, we don't know very much about the details of infection or colonization, especially on a molecular level, in comparison with foliar diseases of maize. A model system for maize that had some of the advantages of Arabidopsis (e.g. small size but with normal rates of ear production, fast generation time) would be beneficial for studies of ear rots. The Fast Flowering Mini-Maize (FFMM) inbreds developed by (McCaw & Birchler, 2017; McCaw, Wallace, Albert, Buckler, & Birchler, 2016) seem to be ideal, as they

can be grown in the greenhouse, seed to seed, within 60 days and they remain very compact at maturity, usually less than a meter tall. They produce several small, but normal ears per plant. There is a whole-genome sequence, and a transformation system has also been developed (McCaw et al., 2021), increasing the potential value of FFMM as a model for understanding ear rot host-pathogen interactions at a molecular level.

Another difficulty with studying ear rots is that disease evaluation and characterization relies on rating systems like (Reid, Mather, Bolton, & Hamilton, 1994). These methods are effective for basic studies, e.g. comparison of disease management protocols, but they are not quantitative, and not much is known about the relationship between these disease scales and the degree of fungal colonization or mycotoxin production. A novel digital phenotyping system for maize ears was developed by (Warman et al., 2021). The system consists of an apparatus that rotates a maize ear on its axis and allows for digital recording that is then processed through a pipeline to produce a two-dimensional projection of the maize ears. These images can then be digitally analyzed to quantitatively characterize the maize ears in detail.

The principal goal of the project described in this Appendix was to establish a system to study the pathology of maize ear rots using the FFMM and the digital phenotyping system. Some parts of the study were done with Elisabeth Rintamaa, an undergraduate intern who worked with me on optimizing inoculation methods for *F. graminearum* and *S. maydis* on Fast Flowering Mini Maize ears during the summer of 2021.

A.2 Materials and methods

A.2.1 Plant Growth:

The inbred maize line “Fast-Flowering Mini Maize A'' (FFMM) was used for this study. Seeds were obtained from Dr. Birchler at the University of Missouri, and increased by self-pollination according to (McCaw et al., 2021). Three maize seeds

were planted in a mixture of topsoil (Maury silt loam) and PromixBC grown substrate (3:2) in plastic 11-inch pots. Pots were filled with soil halfway, then 5.5 g of Ferrous sulfate heptahydrate, 10.5 g of 20-10-20 fertilizer, and 7.5 g of Osmocote were added, and pots were filled with soil to the top. The seeds were lightly covered with a moist layer of soil mixture. Seeds were germinated in the greenhouse at an ambient temperature of 25°C - 28°C, with a 14/10 photoperiod provided by "Hortilux" LU430S/HTL/EN high pressure sodium lights. After germination, seedlings were thinned to one plant per pot. Plants were fertilized with 150 ppm of N:P:K (20:10:20) fertilizer formulation every other day, beginning two weeks after planting, and then daily once they reached maturity (anthesis). Ear shoots were bagged as soon as they appeared, and silks were cut one to two days prior to pollen release so that they would grow a bit to maximize fertilization. Tassels were vigorously shaken over a paper bag to release and collect pollen. The pollen was filtered through a soil sieve to separate it from the anthers, and then gently decanted over the silks. The ears were then lightly tapped to remove extra pollen and covered with the labeled ear bags. Pollination was repeated twice for each ear. About 24 to 25 days after pollinating, ears were harvested and left on the greenhouse bench to dry for a minimum of 10 days.

A.2.2 Fungal Growth

All fungal strains used for this study are listed in **Table A.1.** Fungal strains were routinely grown at 23°C with constant light (Sylvania F032/741/ECO). Mutant strains were single-spored and stored on silica gel at -20°C or -80°C (Tuite, 1969, after Perkins, 1962). Strains were cultured on PDA for 5 days, before collecting colonies with sterile toothpicks and transferring to sporulation inducing media. Asexual spores (macroconidia) were produced on mungbean agar (40 g mungbean and 10 g Bacto® Agar per L) and/or in carboxymethylcellulose (CMC) liquid shaking cultures at 250 rpm, both at 23°C for 10 days. Spores were harvested from mungbean agar cultures by applying 2 mL of sterile water to the surface of the Petri plate and rubbing gently with a sterile plastic micro-pestle.

Asexual spore suspensions from mungbean and CMC were filtered through a double layer of sterile cheesecloth to remove mycelia and collected in a sterile 50 mL Falcon tube. Spores were counted by using a hemocytometer. For use as inoculum, spores were centrifuged at 3330 x g, then washed once in sterile water and resuspended at a concentration of 1×10^5 per mL. *Stenocarpella maydis* isolates were recovered on PDA from silica gel stocks stored at -20°C or -80°C (Tuite, 1969, after Perkins, 1962). Strains were cultured on PDA for 5 days, before collecting colonies with sterile toothpicks and transferring to Oatmeal Agar (OA) to induce sporulation. After 5 days, the mycelium was flattened by rubbing it with a sterile glass rod in 2.5% Tween 80. This accelerated the sporulation process significantly, with spores being produced in less than a week, versus several weeks without.

A.2.3 Gibberella Ear Rot and Diplodia Ear Rot Inoculations

Five days after pollination, ears were inoculated with *F graminearum* and *S. maydis* in one of two ways. In the first method (top inoculation) 1 mL of a 1 x 10^5 per mL spore suspension was applied to the silk channel. Inoculated ears were covered with ear bags and then with tassel bags. In the second method (bottom inoculation) a 3 mm plug taken directly from a fungal culture on PDA was applied to a small lesion in the ear shank created with a scalpel and secured with parafilm. Two weeks after inoculating, ears were removed from the plants, shucked, and analyzed.

A.2.4 Quantitative Evaluation of Disease Severity and Mycotoxin

After harvesting, all ears were photographed. Ears with enough kernels were placed on a rotating apparatus for digital imaging. Videos were used to create a 2D image using ImageJ and FFmpeg. The 2D images could then be used for quantification in ImageJ (Warman et al., 2021). The overall area of kernels and

cob was measured, along with the area of visible disease. The percentage of diseased area was used as a quantitative measure of disease severity.

Harvested ears infected with *F. graminearum* were dried in the greenhouse and kept in a cold room (4°C) until analysis. Mycotoxin production by each isolate and the water control was determined by pooling the samples from each experiment, with each bulked sample considered as a replicate. The maize ears 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, where the amount of DON and its acetylated forms (15ADON and 3ADON), NIV, and ZEA were quantified by using a gas chromatography–mass spectrometry method as described (Fuentes et al., 2005; C. J. Mirocha et al., 1998).

A.2.5 Data Analysis

All experiments were conducted as a completely randomized design. Data were visualized and analyzed by 95 % Confidence Intervals (CI) and by Scott Knott. CI was done by using ‘mean_cl_boot’ from the ‘Hmisc’ package, which implements basic nonparametric bootstrapping to obtain confidence limits for a population of means without assuming a normal distribution (Harrell Jr & Harrell Jr, 2019). CI was performed for multiple experimental replications. If confidence limits of mutants overlapped with the wild type PH-1, these were considered similar. For the Scott Knott, data from multiple replicated experiments were combined. The Scott Knott was used to group the isolates according to the means of measurements and counts (Jelihovschi et al., 2014). All analyses were run in R (R Core Team 2019).

***A.3* Results**

A.3.1 GER and DER pathogenicity assay

Mini-maize ears were susceptible to wild type and *mat1-1-1* and *mat1-2-1* deletion strains of *F. graminearum* (**Figure A.1**). The ears exhibited variable levels of GER symptoms, and many ears seemed to have little or no infection. The most common and visible symptom was necrotic cob tip, but this symptom could also be seen on water controls, possibly due to other fungi attracted to the accumulation of pollen which is high in sugars. The characteristic pink mold was visible on some, but not all the ears. Using a GFP-expressing strain of *F. graminearum*, mycelial growth could be observed mostly colonizing the glumes between kernels (**Figure A2)**. Ears were highly susceptible to *S. maydis*, with most exhibiting nearly 100% DER symptoms, including the characteristic white mycelium growing in between kernels (**Figure A.3**).

A.3.2 Mycotoxin accumulation in ears.

Confidence intervals showed all strains were like PH-1 in both experiments (**Figure A.4**). Varying amounts and types of mycotoxin accumulated in ears infected by wild type and MAT deletion strains of *F. graminearum,* but there was no strong association with the type of deletion (**Figure A.5**). Most of the MAT deletion strains produced less mycotoxin than the wild type, although three of the strains (one *mat1-2-1* deletion and two *mat1-1-1* deletions) produced more. Interestingly, levels of mycotoxin did not seem to be related to the amount of visible disease on the ears.

A.3.3 Quantitative Evaluation of Visible Disease on Corn Ears

A phenotyping apparatus that could rotate a maize ear through 360° was built and kindly donated by Mr. Daniel Murphy (**Figure A.6**). A quantitative evaluation was not performed for the experiment comparing the MAT mutants. Flattened projections were developed for co-inoculations of *F. graminearum* PH1 and *S. maydis* (**Figure A.7**) with the summer intern. Disease Severity for GER was

relatively low, but the DER severity was much higher and provided more consistent results (**Figure A.7**)

A.4 Discussion

The *F. graminearum* wild type PH-1 and MAT deletion strains were all pathogenic to FFMM, but the symptoms produced were variable among replications. In at least some cases, ears were completely engulfed with mycelium and killed prior to grain fill, thus it does appear that FFMM is very susceptible to the pathogen, and ears with lower levels of disease are probably escapes that may be due to environmental factors that I was unable to fully control (e.g. humidity, etc). Pollination was suboptimal on some ears, possibly due to environmental factors e.g. the high temperatures in the greenhouse, or recovery of insufficient pollen from the tassels. Ears that were poorly pollinated had very few kernels and didn't show typical symptoms. The *F. graminearum* mostly colonized the top of the ears when it was applied to the silks, while shank inoculations produced lower disease severity, of 10% or less. In nature, *F. graminearum* spores most commonly land on emerging silks and cause ear tip infections (Hesseltine & Bothast, 1977; Koehler, 1942).

Stenocarpella maydis was much more aggressive to the ears of the FFMM than the *F. graminearum* strains. Both top and bottom inoculations with *S. maydis* resulted in similar and substantial amounts of disease, and shank inoculations often spread to the stalks of the plants as well. The digital phenotyping worked better for the DER than for the GER because of the abundant white mycelium on the ears. In co-inoculations with *F. graminearum* and *S. maydis*, the *S. maydis* dominated and covered the ears while few or no symptoms or signs of *F. graminearum* (eg pink mycelium) could be seen.

Overall, the FFMM and the digital phenotyping platform both show excellent potential for modeling maize ear rot disease under controlled greenhouse conditions. However, the protocols will require further work to decrease variability among replicates, and optimize inoculation procedures.

Table A.1 List of strains used in this study.

Strain labels that begin with 1 are *mat1-1-1* deletion strains, while strains that begin with 2 are *mat1-2-1* deletion strains. The number after the underscore corresponds to the number of strain of the group it corresponds to.

Strains				
Code	Name (Bec, 2011)	Genotype	Transformation	Colony type
0_1	mat1 sm5	*mat1-1-1/mat1-2-1*	split-marker	Flat
0_2	mat1 sm16	*mat1-1-1/mat1-2-1*	split-marker	Flat
1_1	mat111 sm1	*mat1-1-1/MAT1-2-1*	split-marker	Flat
1_2	mat111 sm5	*mat1-1-1/MAT1-2-1*	split-marker	Flat
1_3	mat111 sm12	*mat1-1-1/MAT1-2-1*	split-marker	Flat
1_4	mat111 sm19	*mat1-1-1/MAT1-2-1*	split-marker	Flat
1_5	mat111 sm20	*mat1-1-1/MAT1-2-1*	split-marker	Flat
2_1	mat121 sm1	*MAT1-1-1/mat1-2-1*	split-marker	Flat
2_2	mat121 sm6	*MAT1-1-1/mat1-2-1*	split-marker	Flat
2_3	mat121 sm16	*MAT1-1-1/mat1-2-1*	split-marker	Velvet
2_4	mat121 sm21	*MAT1-1-1/mat1-2-1*	split-marker	Velvet
2_5	mat121 WC5	*MAT1-1-1/mat1-2-1*	whole cassette	Velvet
2_6	mat121 sm7	*MAT1-1-1/mat1-2-1*	split-marker	Flat
WT	PH-1 FT2	*MAT1-1-1/MAT1-2-1*	none	Flat
DM6.001	*S. maydis*	-	-	

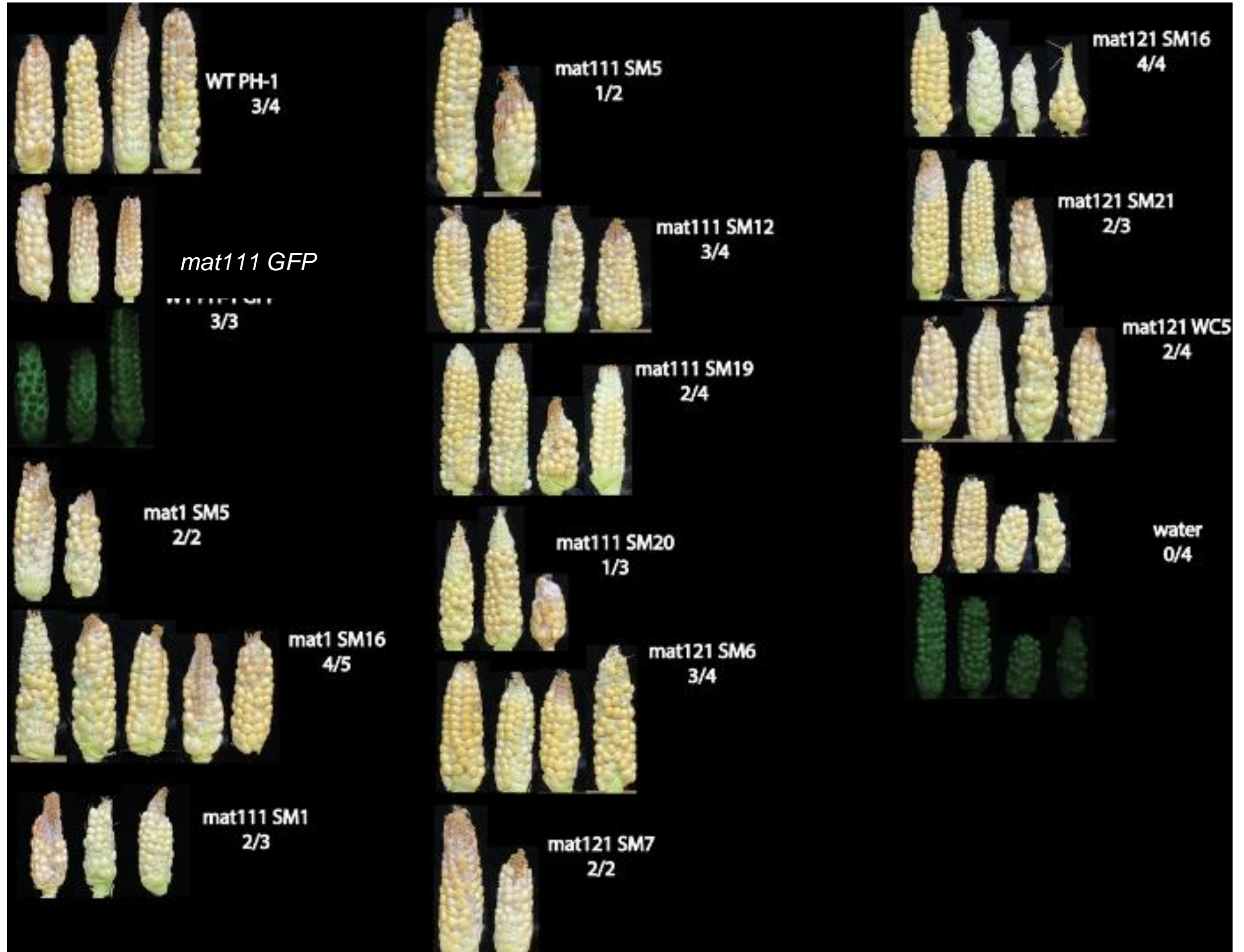


Figure A.1 GER on FFMM after 14 days. Labels to the ears are the treatments used to inoculate them, with the incidence of symptomatic ears over the total recovered ears. Ears inoculated with GFP *mat1-1-1* have visible fluorescence around the kernels. There is also some autofluorescence with the water controls.

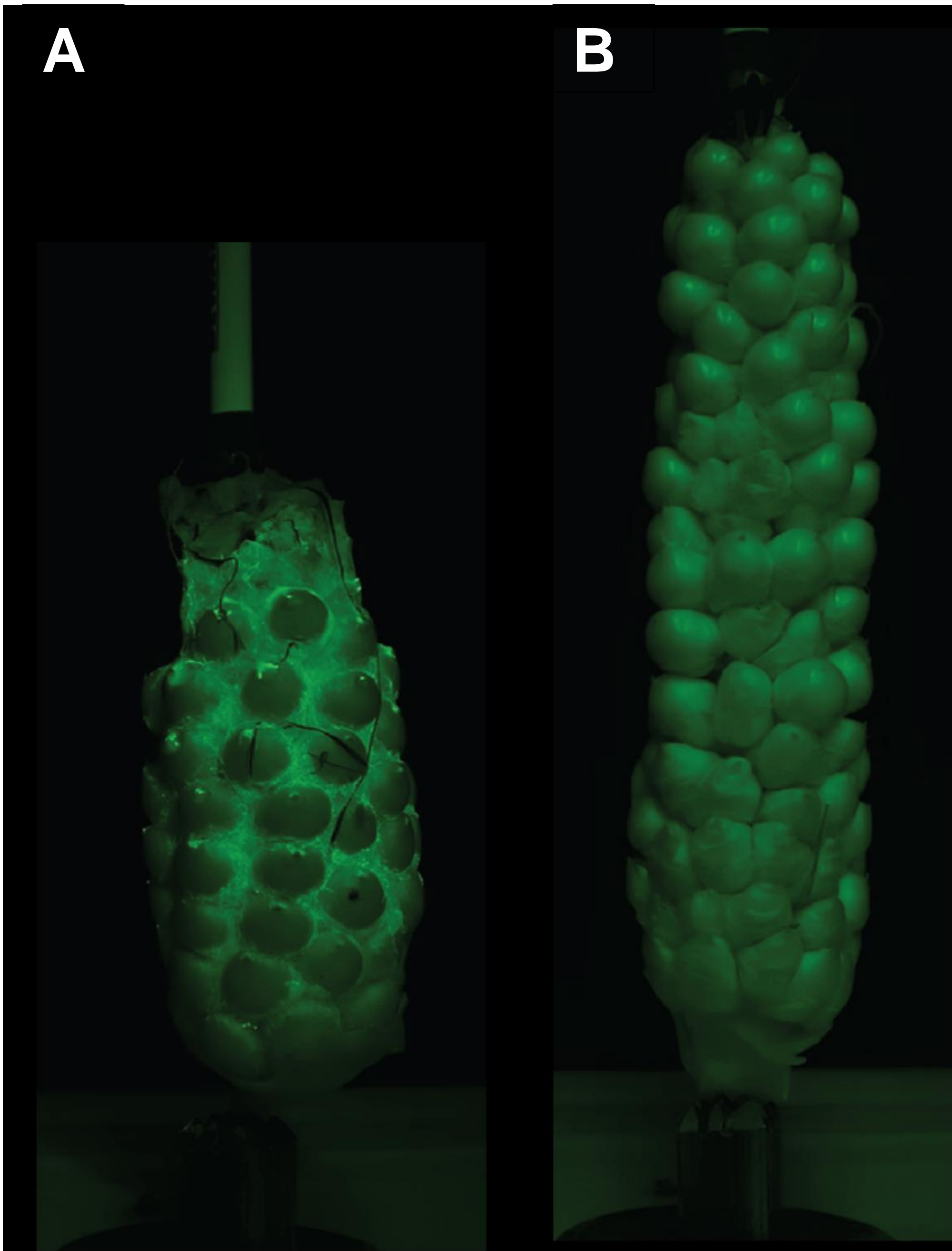


Figure A.2 Mini maize ears under fluorescence lights. Panel A shows a green fluorescence *mat1-1-1* strain with bright fluorescent mycelium growing in between the kernels. Panel B shows the mock (water) inoculation, showing fainter kernel autofluorescence.

Figure A.3 DER on FFMM after 14 days. Far left shows a water control ear, while the rest of the ears show typical DER signs.

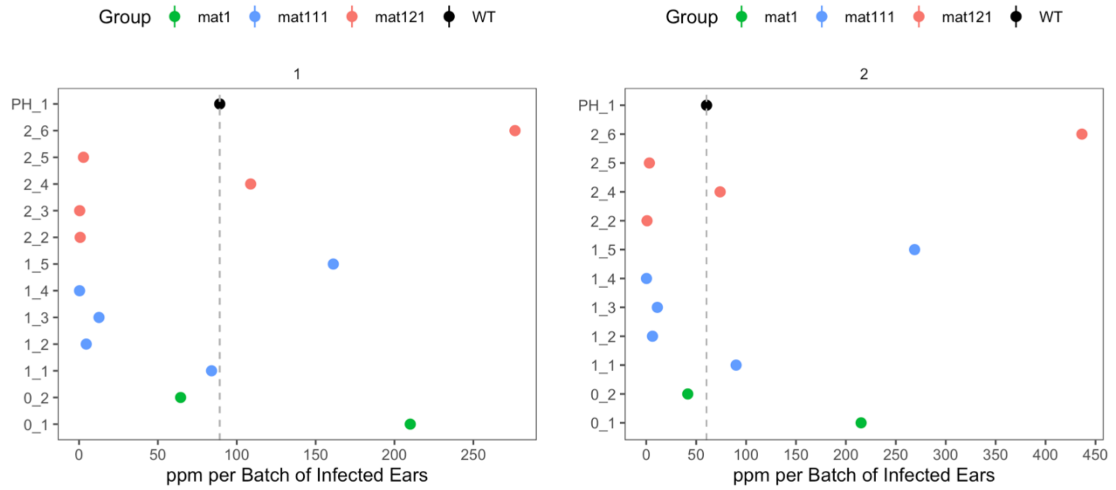


Figure A.4 Average amount (ppm) of DON on infected maize ears (95% confidence interval [CI]) of wild type and MAT deletion strains *of Fusarium graminearum* for two experiments.

Points represent mean amount (ppm) of DON on infected maize ears for each strain. Dashed lines correspond to PH-1 CI. Strains that overlap with PH-1, behave similarly to PH-1.

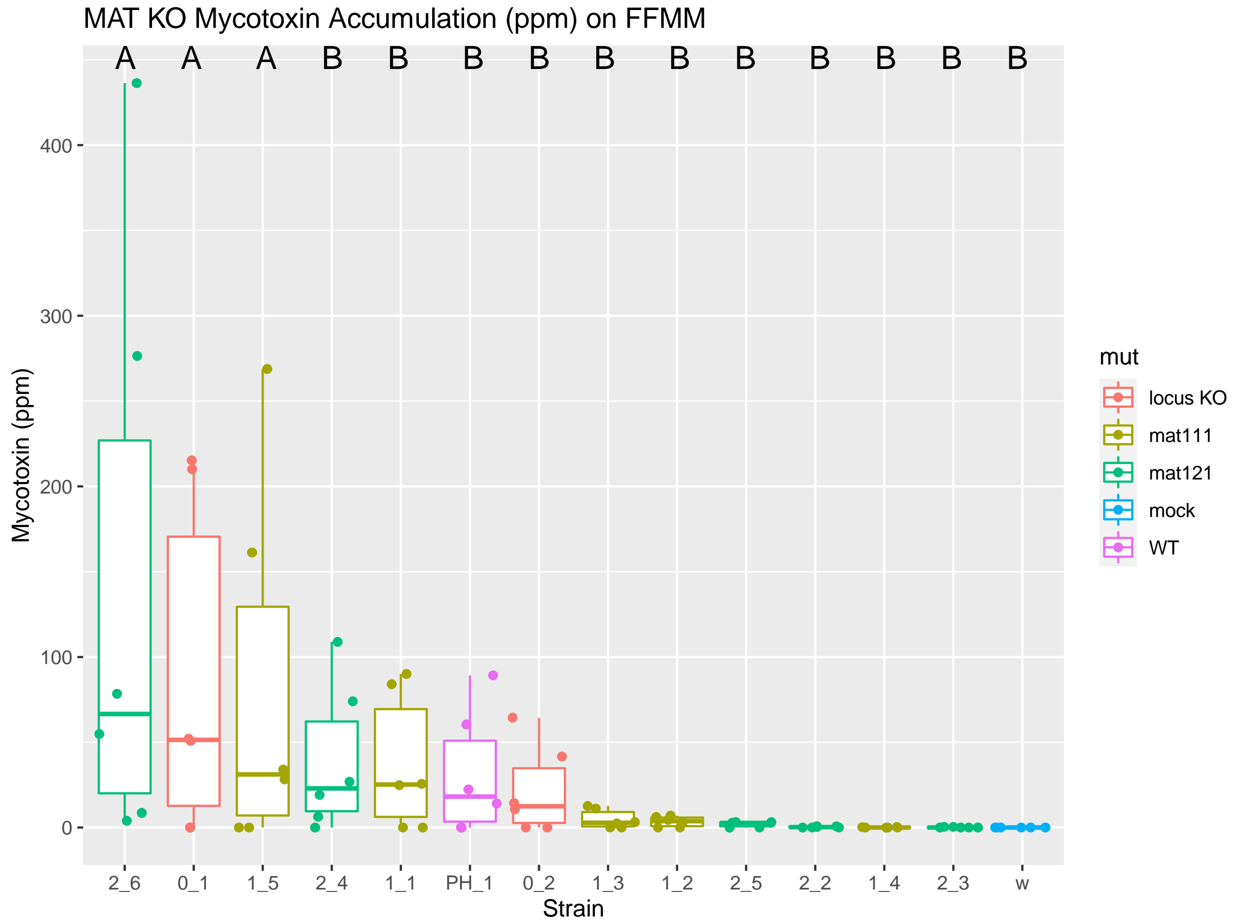


Figure A.5 Mycotoxin accumulation of one trial experiment 14 days after inoculation. Statistical comparisons were done using an ordinary one-way ANOVA using Tukey's post-hoc test to correct for multiple comparisons. Each dot shows a single data point. The horizontal bars inside the boxes indicate the mean value of that treatment. The letters on top of the bars represent the significant groupings determined by Scott Knott ($P < 0.001$, $\alpha = 0.05$).

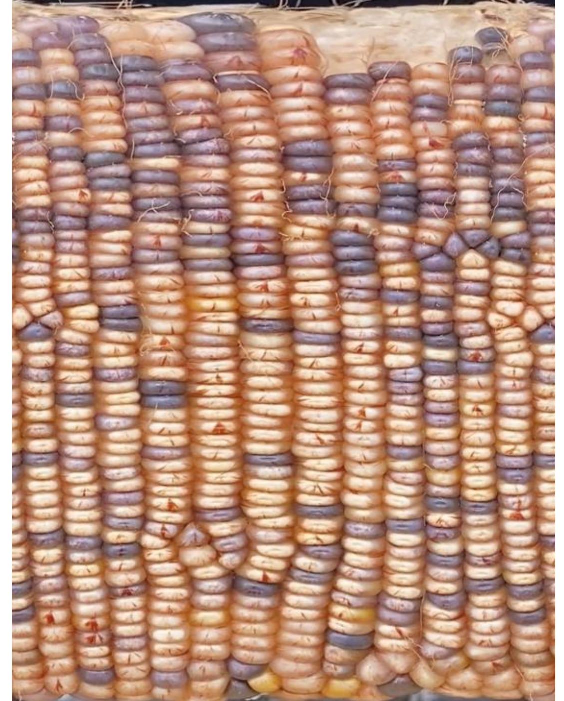


Figure A.6 Maize Ear Phenotyper, developed by Cedar Warman et al. 2021, and modified by Mr. Daniel Murphy. 2D image of an Ornamental Maize Ear, performed using the procedures described in the methodologies.

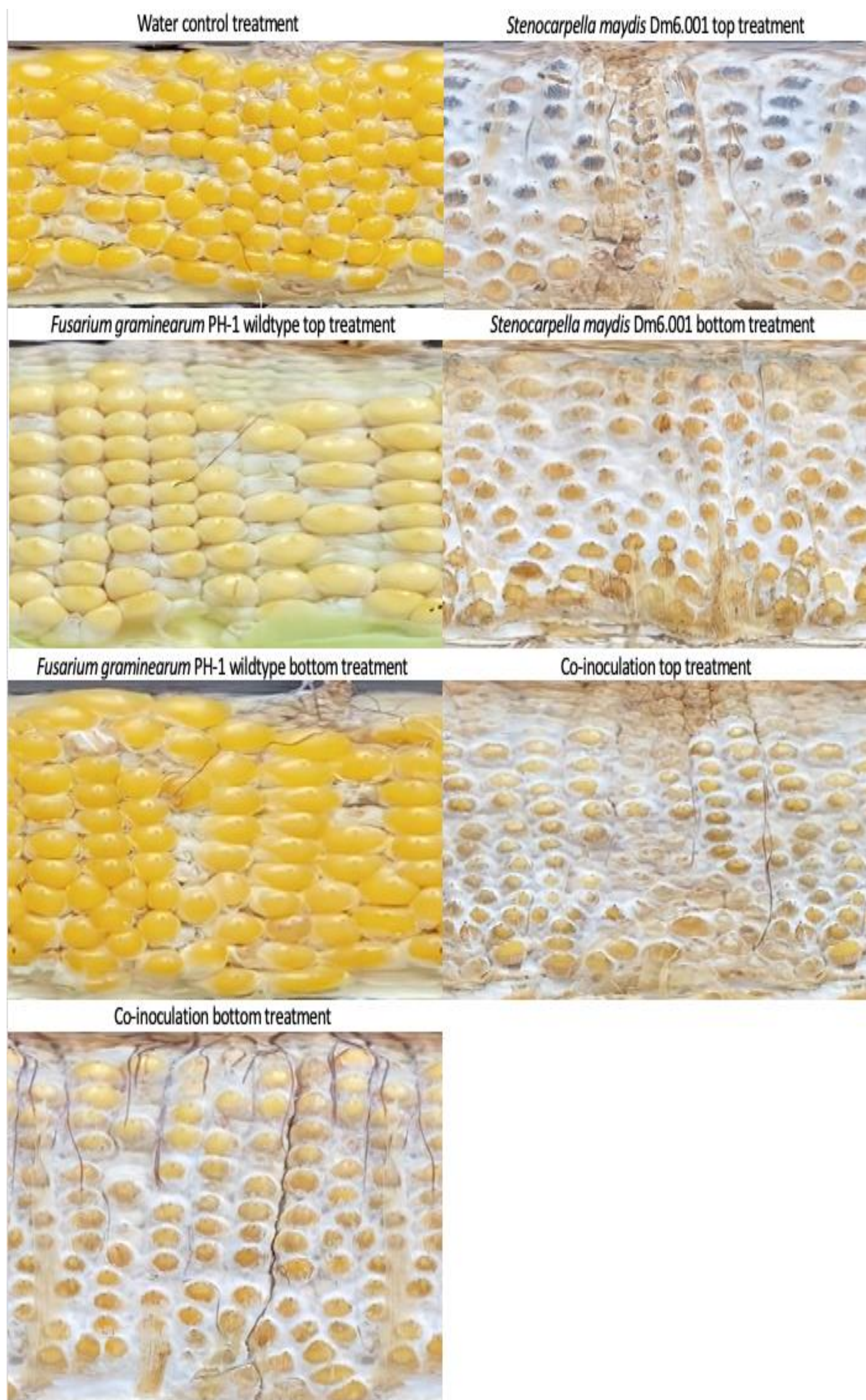


Figure A.7 GER, DER and water control alone and co-inoculated on FFMM ears, digitally projected as flattened images.

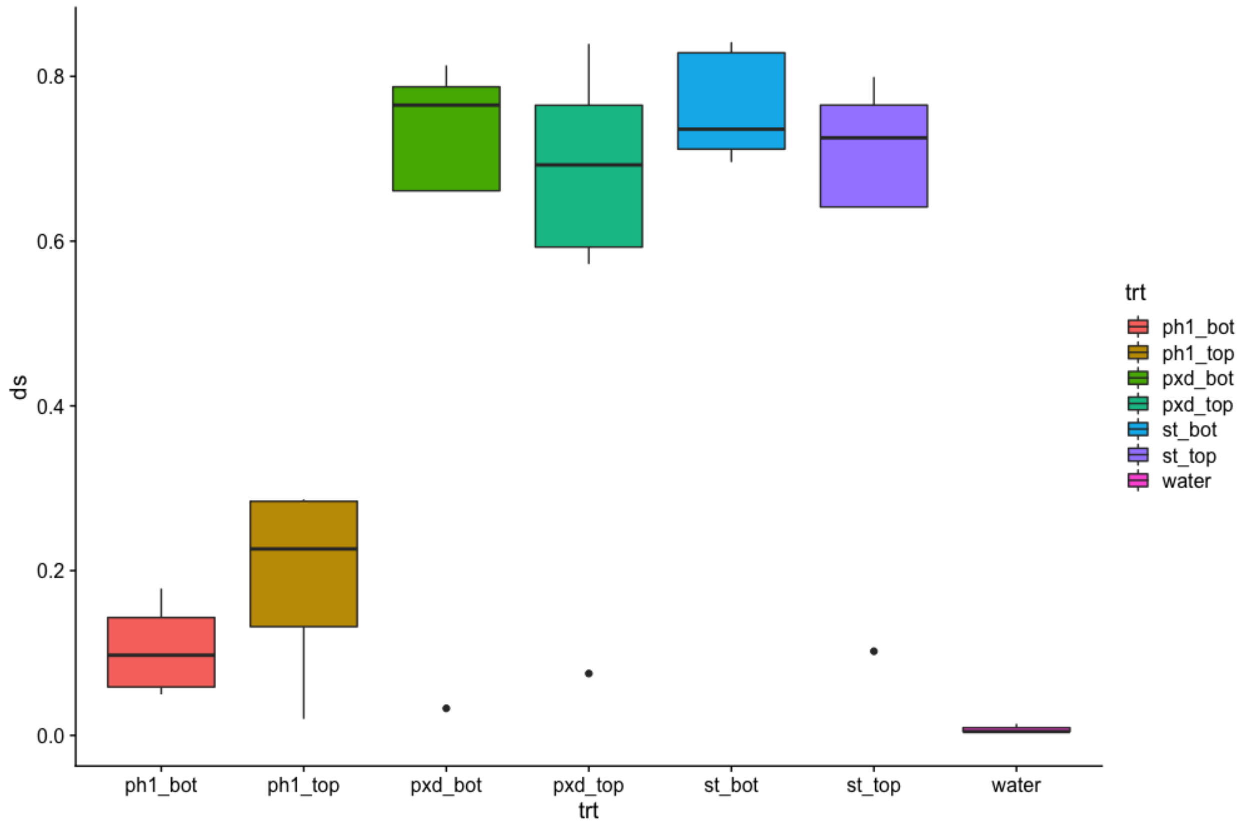


Figure A.8 Quantitative evaluation of disease severity for GER, DER and water control alone and co-inoculated on FFMM ears from projected flattened images. The labels on the bottom show which isolate was inoculated and the inoculation site: “bot” indicates inoculation of the ear shank, while “top” indicates silk inoculation. PH-1 wild type is represented as “ph1”, DM.006 wild type is represented as “st”, while the “pxd” is co-inoculation. The horizontal bars inside the boxes represent the mean values of that treatment.

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1) **Yulfo-Soto, G. E.**, Smith, H., Szarka, D., Dixon, E., Vaillancourt, L. J., & Gauthier, N. (2022). First Report of Fusarium graminearum Causing Flower Blight On Hemp (Cannabis sativa) in Kentucky. Plant disease, PDIS-06.
2) Bec, S., **Yulfo-Soto, G. E.**, & Vaillancourt, L. J. (2021). Relative efficiency of split-marker versus double-crossover replacement protocols for production of deletion mutants in strain PH-1 of Fusarium graminearum. *Fungal Genetics Reports*, *65*(1), 1.