IDENTIFICATION AND CHARACTERIZATION OF *FUSARIUM GRAMINEARUM* PATHOGENESIS GENES AND DETERMINATION OF AGGRESSIVENESS

BY

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THESIS

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ABSTRACT

Fusarium graminearum, the causal agent of Fusarium head blight of wheat, is a devastating pathogen that causes yield and quality losses to its host. *F. graminearum* produces mycotoxins in the grain that cause reduced milling and baking qualities, granary rejection, and livestock feed refusal. Research has been conducted to identify genes associated with deoxynivalenol, the most important mycotoxin produced by *F. graminearum*, yet little is known about other pathogenesis compounds or pathways used by the pathogen to infect wheat.

To identify essential fungal pathogenesis genes and determine whether host resistance impacts aggressiveness of a given isolate, a paired strategy of isolate and transcriptome characterization of naturally infected wheat lines was implemented. In the summer of 2016, naturally infected spikelets that symptomatically resembled Fusarium head blight were collected from soft red winter wheat with varying levels of resistance. Collected *Fusarium* isolates were surface sterilized, grown on potato dextrose agar with rifamycin for six days, and single spored. Of the original collected isolates, twelve were utilized as a representative sample to ascertain aggressiveness.

Species identification was completed for the twelve isolates by sequencing the translation elongation factor 1-alpha gene (EF1-α) using EF1/EF2 primers. DNA was trimmed and blasted for species similarities using the Fusarium ID database. Of the twelve isolates, seven identified as *F. graminearum* (Schwabe), three as *F. armeniacum*, and two were non-determinant.

Aggressiveness was categorized through the utilization of two field assays, one greenhouse assay, mycotoxin assays, and a spore quantification assay. Pathogenesis assays were conducted with the representative *Fusarium* isolates and a negative control. During anthesis, isolates were inoculated in the center spikelet of wheat heads from two cultivars and were replicated per assay. Fungal aggressiveness was determined through disease severity with information taken at 14, 21, and 28 days post inoculation with area under the disease progress curves calculated from severity data. After threshing inoculated heads for each aggressiveness assay, *Fusarium* damaged kernels were collected, processed, and measured for mycotoxin contamination with Reveal Q+ for DON.

Fusarium isolates caused varying levels of infection on inoculated soft red winter wheat. Disease severity differed based on cultivar but was higher on highly susceptible cultivars. Aggressiveness varied among the isolates by origin of collection and level of host resistance from which the isolate was collected from. Spore quantification gave little indication into each isolate's potential aggressiveness upon inoculation. Currently, aggressiveness is defined as a quantifiable amount of disease caused by a pathogen. To date, there is no single index measurement that syndicates the individual measurements of aggressiveness. The goal of this research was to combine disease severity translated into area under the disease progress curve, *Fusarium* damaged kernels, and mycotoxin quantification through deoxynivalenol into a single index and quantifiable measurement of aggressiveness. A principal component analysis was conducted on the collective aggressiveness traits from each assay to create a multivariate description of isolate aggressiveness. This index was then employed in a cluster analysis to classify isolates, as described by origin of collection and level of host resistance from which each isolate was collected, into clusters based on the index value.

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This thesis is dedicated to those I love most: my mother, my sister, my brother, and my significant other, Cody M. Reed, for their countless love and support. I would like to thank my mother for instilling the idea that education is the key to success and continuously being my cheerleader. I am so proud to be her daughter and strive to be impactful every day. To my sister for encouraging me to enjoy and savor my time especially during my graduate studies. She has taught me so many skills in my lifetime and I am truly grateful. To my brother for reminding me that laughter is the greatest seasoning you can add to life and to go with the flow.

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I value the mountain of encouragement my colleagues, friends, and family have given me over the years, when times were great, when times were rough, and when I considered leaving. I wouldn't be the woman, daughter, sister, significant other, and scientist I am today without your love and support.

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CHAPTER 1: INTRODUCTION TO *FUSARIUM GRAMINEARUM*

Introduction

Fusarium graminearum (Schwabe) (syn. *Gibberella zeae*) is a homothallic, facultative parasite within the *Ascomycota* fungal phylum that causes a multitude of diseases on several hosts. Many species are affected by *F. graminearum*, with the most economic importance being Gibberella ear and stalk rot on maize, seed decay and damping-off of soybean, and Fusarium head blight (FHB) or scab in small grains, particularly wheat, barley, rye, and triticale.

F. graminearum is regarded as the most important pathogen on wheat (*Triticum aestivum* L.) for a multitude of reasons. FHB can be found in all wheat and other small grains producing regions worldwide and is a highly devasting disease due to its manifold infectious capabilities (Imathiu, Edwards, Ray, and Back, 2014). *F. graminearum* not only causes yield reduction due to pathogen infection but also results in mycotoxin deposition within the grain (Imathiu et al., 2014). In 2012, the Molecular Plant Pathology journal listed *F. graminearum* as fourth in the top ten destructive fungal pathogens based on scientific and economic importance (Dean et al., 2012).

Disease Cycle

At the start of the season, *F. graminearum* poses as a biotrophic fungus by allowing the host to survive during fungal nutrient uptake and then becoming necrotrophic later in the season (Bushnell, Hazen, and Pritsch, 2003; Singh et al., 2016). *F. graminearum* overwinters as perithecia buried in the prior year's corn or small grains debris (Figure 1.1). As the warm, moist spring approaches, two types of spores germinate to cause host infection: ascospores and

macroconidia. Ascospores (sexual spores) are ejected from overwintered perithecia and are dispersed by wind and air, whereas macroconidia (asexual spores) are derived from sporodochia and conidiophores and are rain disseminated. Infection predominantly takes place during anthesis when either spore type lands on open anthers (Bushnell et al., 2003; Schroeder and Christensen, 1963). Flowering generally lasts three to five days long. Once contact is made, the spore germinates, penetrates the host, and grows into the reproductive tissue, making its way through the host. In the primary stages of infection, individual spikelets undergo premature bleaching. As further infection occurs, surrounding spikelets become bleached, leading to a fully symptomatic wheat head. As mycelia colonizes tissue, the rachis begins to discolor from a healthy green tissue to slightly black. Later in the season, mycelium and sporodochia on the glumes of wheat appear light pink or salmon in coloration. Kernels become diseased and appear shriveled with a white to pink discoloration known as tombstoning (Shaner, 2003). As the growing season comes to an end, purple to black perithecia (Gaffoor et al., 2005) form on symptomatic glumes, causing the visual cue of scab and becoming the source for the following year's inoculum.

Importance on Wheat

FHB can be found in all wheat and other small grains producing regions worldwide (Figure 1.2) and is a highly devasting disease (Goswami and Kistler, 2004; Imathiu et al., 2014; Singh et al., 2016; Turkington, Petran, Yonow, and Kriticos, 2014). As severity increases, yield is inversely correlated (Salgado, Madden, and Paul, 2015). In the United States, $7.67 billion was lost due to FHB in wheat and barley between 1993 and 2001 (Singh et al., 2016). China has experienced multiple severe and moderate epidemics in the last seventy years with yield losses as ranging from 5 – 10% in moderate years to 20 – 40% in severe epidemics, with 100% yield losses

reported some years (Singh et al., 2016). *F. graminearum* also induces grain quality issues such as low seed weight, lack of germination, reduced milling and baking qualities, granary/elevator rejection, mycotoxin buildup within the grain, and livestock feed refusal.

Mycotoxins

Aside from drastically reducing yields, *F. graminearum* also produces two groups of secondary metabolites. Group one includes trichothecenes such as nivalenol (NIV), deoxynivalenol (DON), and modified forms of DON: 3-acetyl DON (3-ADON) and 15-acetyl DON (15-ADON) (Desjardins, 2006; McCormick, 2003; Mirocha, Xie, and Filho, 2003). Group two are biosynthesis inhibitors and estrogenic mimics such as zealerone (ZEA), T-2 toxin, and HT-2 toxin. Currently, sixteen genes controlling DON production in the pathogen have been described (Amarasinghe and Fernando, 2016). Apart from DON, little is known about other pathogenesis compounds or pathways used by *F. graminearum* to cause disease in wheat.

According to the United Nations Food and Agriculture Organization (FAO), an estimated 25% of world food crops are contaminated with mycotoxins (Smith, Solomons, Lewis, and Anderson, 1995). FDA standards have been created to control the amount of DON permitted in human and animal consumable products. Upon consumption of these diseased kernels or contaminated products, humans and animals such as cows, poultry, swine, and other feed animals can exhibit fusariotoxicoses. Ingestion of these compounds can cause emesis, feed refusal, digestive issues, male feminization, and weight loss (Čonková, Laciaková, Kováč, and Seidel, 2003). Extreme fusariotoxicoses can cause carcinogenic, estrogenic, mutagenic, hemorrhagic, neurotoxic, and immunosuppressive effects (Chilaka, De Boevre, Atanda, and De Saeger, 2017). In developing

regions, *F. graminearum* has been associated with human illnesses (Goswami and Kistler, 2004; Singh et al., 2016; Turkington et al., 2014) due to the lack of economic resources to control the pathogen and its mycotoxin contamination in food products.

Control

Host resistance, cultural, and chemical practices are the best tactics to employ when controlling FHB. Utilizing crop rotations outside of corn-corn, corn-wheat-soybean, corn-wheat, or wheat-wheat allow overwintering structures in crop debris to break down and thus reduce the following year's quantity of inoculum and disease pressure. Another strategy to effectively control FHB and mycotoxin production within the grain is timely fungicide application. Research has noted that the use of demethylation inhibitors (DMI) applied at anthesis provide effective control of FHB (Ahmed, Mesterházy, and Sági, 1996; Audenaert, Vanheule, Höfte, and Haesaert, 2013; Bissonnette, Kolb, Ames, and Bradley, 2018; P.A. Paul et al., 2008). Research has also shown that fungicides in the quinone outside inhibitor (QoI) class have adverse control effects in comparison to DMI fungicides (Bissonnette et al., 2018; P. A. Paul et al., 2018; Pierce A. Paul et al., 2018; Pirgozliev, Edwards, Hare, and Jenkinson, 2003). When QoI fungicides are applied to wheat between booting and anthesis, DON concentrations increase within the grain (Bissonnette et al., 2018; P. A. Paul et al., 2018; Pierce A. Paul et al., 2018; Pirgozliev et al., 2003). Currently, the following DMI fungicides can be used to best control FHB: metconazole (Caramba) by BASF Agricultural, prothioconazole (Proline) by Bayer Crop Science, and tebuconazole with prothioconazole (Prosaro) by Bayer Crop Science (Bissonnette et al., 2018).

Resistance

Through the development of breeding strategies to deter infection, small grain resistance types have been categorized as follows: Type 1 resistance is defined as resistance to the initial infection of *F. graminearum* (Mesterházy, 1995; Schroeder and Christensen, 1963) and commonly referred to as incidence (Bushnell et al., 2003). Incidence is predominantly measured as the percentage of heads that are symptomatic. Type 2 resistance is described as partial resistance due to a limiting factor that inhibits fungal spread within the head, i.e. progression from spikelet to spikelet (Mesterházy, 1995; Schroeder and Christensen, 1963). Many breeding programs use Sumai 3 and related wheat lines as a source of Type 2 resistance (Bushnell et al., 2003). To show Type 2 resistance, *F. graminearum* is inoculated into a single, center spikelet and observed daily to determine if surrounding spikelets become symptomatic. Type 3 resistance pertains to limiting the secondary metabolite mycotoxin, DON, within the grain (Bushnell et al., 2003). As mentioned earlier, mycotoxin build-up within the grain is rejected at elevators due to the detrimental effects on animals that feed on the infected grain. In contrast to natural mechanisms of resistance, Type 4 resistance includes genetic modification of wheat to inhibit DON accumulation by creating lines that are resistant to the effects of trichothecenes. Finally, type 5 resistance was defined by Mesterházy (1995) as the capability of wheat to be a high yield producer despite being in presence of the disease. This type was determined through visual symptoms and did not correlate to grain infection. Thus, resistance can be separately defined for wheat such as head, culm, grain, etc. (Mesterházy, 1995).

Disease progression and colonization can be quantified in numerous ways. FHB infection can be measured through: disease severity of individual spikelets (a visualization of the number of

symptomatic spikelets) (Kuhnem, Del Ponte, Dong, and Bergstrom, 2015), incidence (a visualization of the number of symptomatic heads per area), damaged kernels (number of kernels that appear shriveled or tombstoned), and mycotoxin content (chemical quantification) (Shaner, 2003). Goswami and Kistler (2005) determined that aggressiveness can be derived from higher trichothecene accumulation rather than the type of mycotoxin derivative. Highly aggressive *F. graminearum* isolates tended to also progress further than the presence of hyphal strands (Goswami and Kistler, 2005).

Given the quantitative nature of the traits governing *Fusarium* aggressiveness, breeding is of the utmost importance. *F. graminearum* aggressiveness is quantitatively inherited (Voss, Bowden, Leslie, and Miedaner, 2010), and at present, 176 quantitative trait loci (QTL) have been associated with resistance (Löffler, Schön, and Miedaner, 2009). Talas et al. (2016) identified 50 quantitative trait nucleotides (QTNs) for aggressiveness and 29 QTNs for DON production. Effectors, proteins expressed by phytopathogens for infection to occur, are believed to be necessary for pathogenicity to occur.

Objectives

The question has yet to be examined if *F. graminearum* isolate aggressiveness is specific to the level of resistance derived from the wheat line. For instance, if an isolate was collected from a highly susceptible wheat line, does that isolate only have the capability to infect other highly susceptible wheat lines, or is aggressiveness non-descript. Using pathogenomics, the utilization of genomic information to understand plant and host disease interactions, it is possible to recognize pathogenesis genes and their correlation to host infection. Through the collection of biological samples, the goal is to capture genes necessary for infection to occur and identify them through RNA sequencing. In addition, field and greenhouse assays will be utilized to determine levels of isolate aggressiveness collected from wheat lines with various levels of resistance. Currently, there is no measurement that conglomerates the individual measurements of aggressiveness such as disease severity translated into area under the disease progress curve, *Fusarium* damaged kernels, and deoxynivalenol. The final goal of this research was to combine these individual traits to determine a quantifiable definition of aggressiveness. A principal component analysis was conducted on the collective aggressiveness traits from each assay to create a multivariate description of isolate aggressiveness. Isolates were binned into aggressive, moderately aggressive, and non-aggressive groups based on Ward's Minimum Variance Measure of Dissimilarity and the index created through use of principal cluster analysis-based index.

Figures

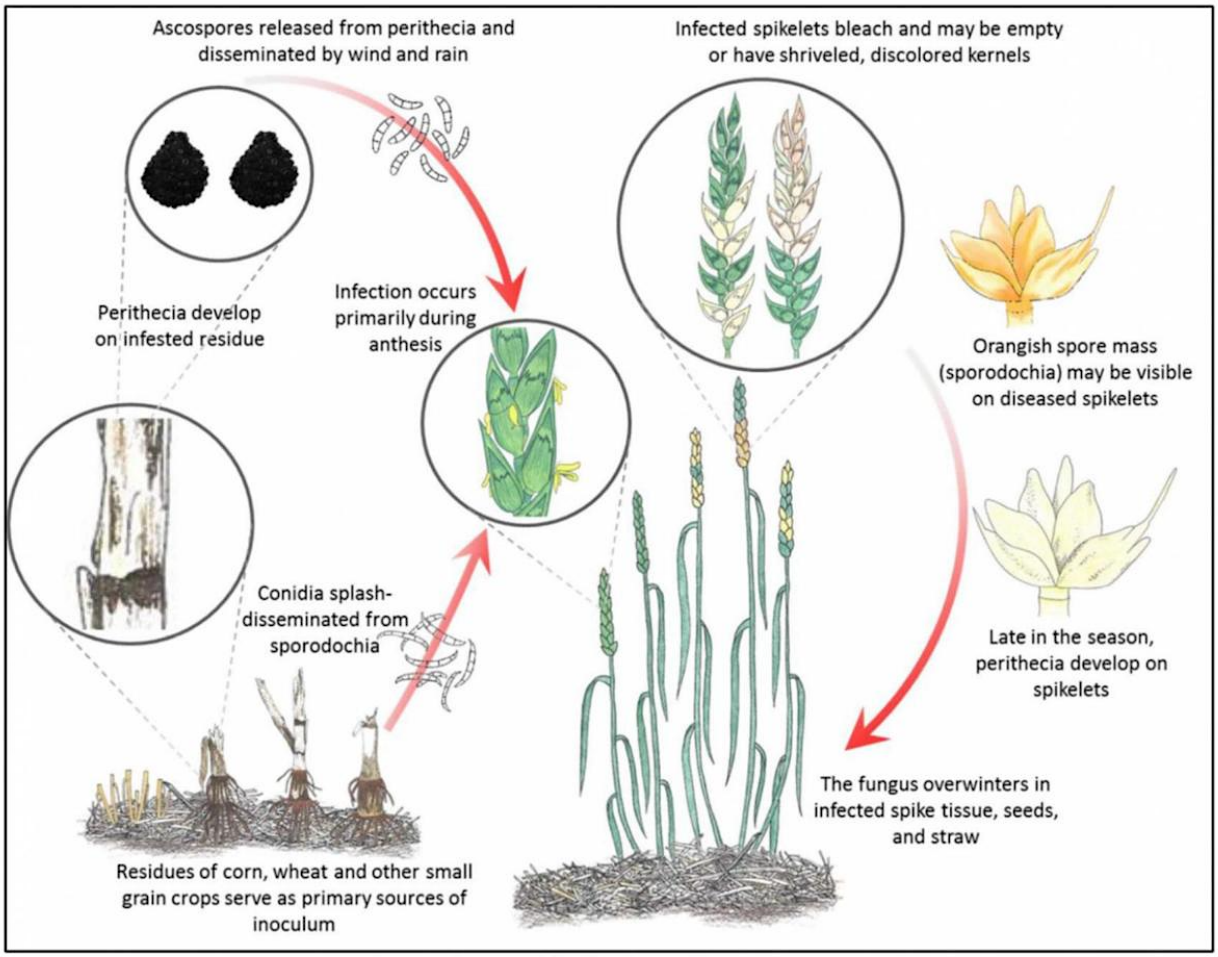
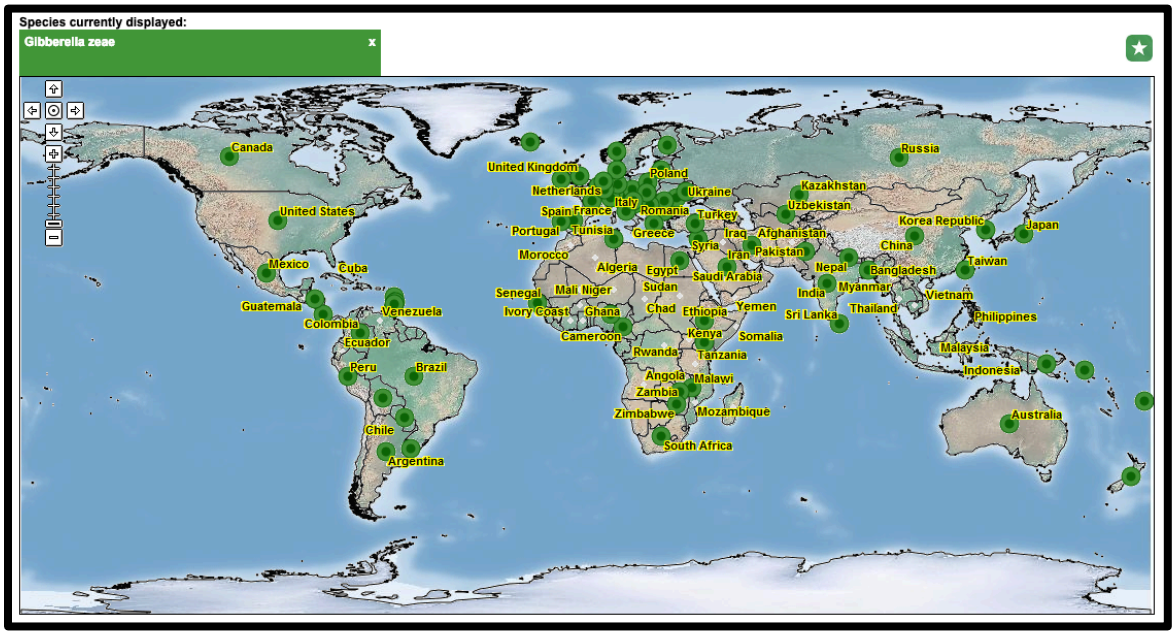


Figure 1.1: *Fusarium graminearum* disease cycle. Courtesy of Ohio State University-Extension.

a) Worldwide distribution map of *F. graminearum*.



b) U.S. distribution map of *F. graminearum*.

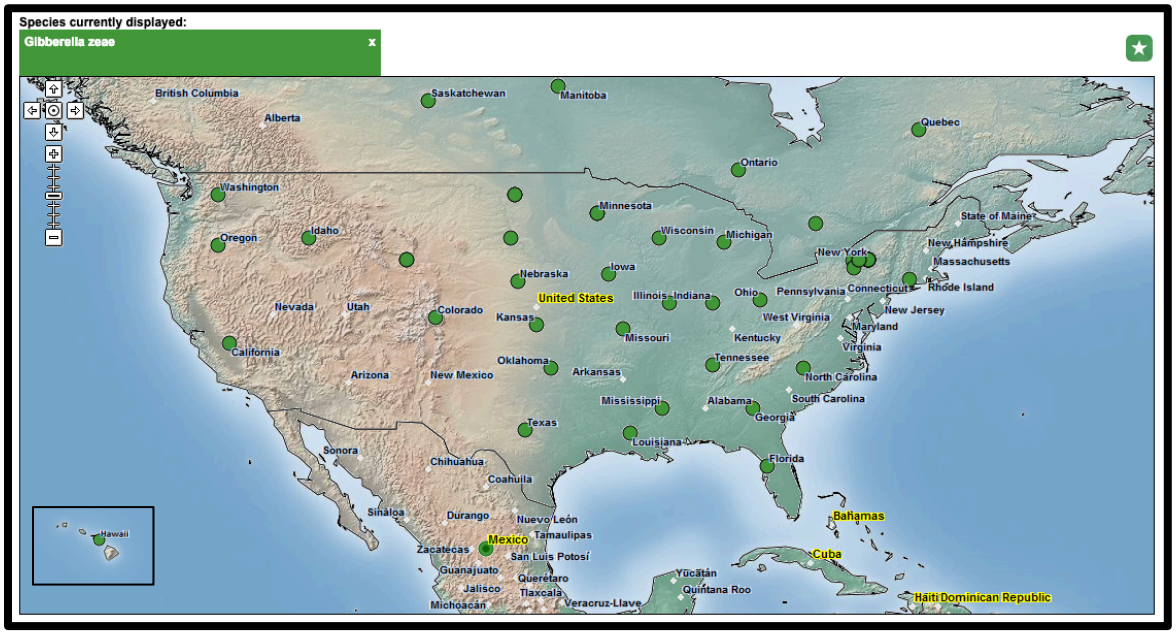


Figure 1.2: Distribution map of where *F. graminearum* (*G. zeae*) can be found a) worldwide and b) within the United States. Maps courtesy of Plantwise Knowledge Bank.

CHAPTER 2: CHARACTERIZATION OF FIELD COLLECTED *FUSARIUM*

Introduction

Fusarium graminearum, along with 21 other *Fusarium* species, is contained within the *F. sambucinum* species complex lineage 1, FSAMSC-1 (Gale, 2003; Kelly et al., 2016). Species within FSAMSC-1 play the largest role in causing Fusarium head blight as well as producing trichothecenes. This study focuses on identifying what *Fusarium* species cause FHB, with main focus on *F. graminearum*, from naturally infected field samples in Illinois. To properly identify field collected isolates and ensure *F. graminearum* is within the sample set, DNA extraction was conducted through single locus genotyping (SLGT) and compared to the USDA-ARS Mycotoxin Prevention and Applied Microbiology Research Unit multi-locus genotyping (MLGT) method. SLGT calls for identification through amplification of one primer whereas MLGT undergoes amplification using more than one primer with an average of three to five. Each laboratory uses their own combination of genes for species identification dependent of pathogen characterization. Published reports suggest that TRI3 (15-O-acetyltransferase), TRI10 (trichothecene 3-O-acetyltransferase), TRI12 (trichothecene efflux pump), EF1-α (elongation translation factor 1-α), RED (reductase), and MAT (mating type) are among the most used genes to determine *Fusarium* species (Boutigny, Ward, Ballois, Iancu, and Ioos, 2014; Cuomo et al., 2007; Kelly and Ward, 2018).

Aside from species identification, RNA sequencing analysis was conducted to compare the transcriptomes of moderately resistant, moderately susceptible, and highly susceptible cultivars and to identify pathogenesis genes that are required for infection on wheat. Results on the transcriptome analysis are reported in Fall, Salazar, et al., in press (2019).

Materials and Methods

Sample Collection

In the summer of 2016, in order to collect a variety of *Fusarium* samples, research sites were established in the following Illinois locations: Brownstown, St. Jacob, Carmi, Urbana, and Savoy (Figure 2.1). Within each site, five wheat lines were planted in a randomized complete block design using the University of Illinois' wheat breeding program plots. Wheat lines included the following: two moderately resistant lines (IL11-28222 and IL07-19334), a moderately susceptible line (IL10-19464), one susceptible line (Kaskaskia), and one highly susceptible line (Pioneer 25R47). Ten naturally infected heads were identified for each line and two spikelets were collected from each head. One spikelet was kept on ice in a microcentrifuge tube for fungal isolation and the other adjoining spikelet was immediately placed into a 1.5mL microcentrifuge tube containing 500µL of RNA*later.* RNA*later* (Sigma-Aldrich, Catalog Number R0901) is a storage buffer that penetrates fresh tissue to stabilize RNA for later extraction. A potential total of 250 *Fusarium* samples could have been collected (five locations, five wheat lines, ten samples, two subsamples).

Sample Processing

All field collected samples underwent processing in order to create single spore stocks for future assays. Each spikelet was surface sterilized by washing in 70% ethanol for five minutes, moved to 10% bleach, washed for five minutes, and finally rinsed twice in sterile double distilled water. After rinsing, individual chaff or glumes from the sterilized spikelet were placed on potato dextrose agar plus rifamycin (PDA+) plates and grown for four days at 25°C. Once mycelia colonized the PDA+ plates, a 5mm mycelia covered agar plug was placed in a capped,

autoclaved glass test tube with 2mL of carboxymethylcellulose sodium salt, low viscosity broth (CMC) (Sigma-Aldrich, Catalog Number C5678) and placed on a shaker. Test tubes were shaken at 125rpm at 25°C for two days. During this two-day timeframe, CMC broth encouraged spore formation while shaking discouraged mycelial growth. Once free-floating spores were formed in the CMC broth, 2mL of sterile double distilled water were added to each test tube and agitated by vortexing or shaking vigorously. Contents of the test tube were poured onto new PDA+ plates, liquid was spread carefully using a sterile bent glass rod, and plates were kept slightly ajar to remove moisture for spore germination. After plates dried, they were incubated for a 16hour period at 25°C. Four germinating spores or hyphal tips, if spores were not readily available, were selected, placed equidistant on a new PDA+ plate, and grown for four days at 25°C. Once mycelial colonies formed from the germinating spores, one randomly selected colony was transferred to a new PDA+ plate with autoclaved popcorn kernels. After seven days, kernels were collected in a 2mL microcentrifuge tube, given an isolate identification label, and frozen at -80°C for future assays. Some samples were compromised with various secondary pathogens during processing and omitted from stock creation.

Species Identification

DNA Extraction

A randomly selected subsample of twelve isolates (one from each sampled field and mixture of wheat lines) was chosen from the processed isolates to represent the population. Mycelia grown from PDA+ plates were collected from the representative isolates and a positive *F. graminearum* control, PH-1 (King, Urban, and Hammond-Kosack, 2017) or NRRL 31084 (USDA-ARS

Culture Collection (NRRL); Peoria, IL)). DNA was extracted using the FastDNA Spin Kits (MP Biomedicals, Catalog Number 116540000) protocol with small adjustments.

A ceramic bead was added to a FastDNA Spin Kit tube with sample filling half of the tube. 1mL of CLS-Y extraction buffer, 80µL of PVP solution, and another ceramic bead was added to the tube. Tubes were sealed tightly, placed into a cell disruptor, and ran at 6rpms for 60seconds and repeated three times until a homogenous mixture was made. Tubes were removed and mixed by inverting so foam head could blend with sample. Tubes were incubated at room temperature for three minutes and then centrifuged for six minutes at 14,000g. After centrifugation, supernatant was transferred to a new 1.5mL tube and centrifuged again for five minutes at 14,000g. Following centrifugation, 600µL of supernatant were transferred to a new tube along with 600µL of well mixed binding matrix and incubated at room temperature for five minutes. Tubes were centrifuged for one minute at 14,000g, supernatant discarded, re-centrifuged for one minute at 14,000g, and remaining supernatant pipetted out. The binding matrix pellet was gently resuspended with 500µL of SEWS-M, transferred to a spin module, and centrifuged for one minute at 14,000g. Following centrifugation, 80% ethanol was added to spin filter, the catch tube was emptied, and spun again for one minute at 14,000g. Tubes were centrifuged a third time for one minute at 14,000g where catch tubes were replaced with recovery tubes. DNA was eluted by resuspending the binding matrix in the spin filter with 100µL of DES grade water and incubated for five minutes in a 55°C water bath. After heating, tubes were centrifuged for one minute at 14,000g to pull DNA into the recovery tube. Final tubes were stored at 4°C for immediate use.

Isolated DNA was quantified using a NanoDrop OneC Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific, Catalog Number ND-ONEC-W). High quality DNA contained concentrations higher than 50ng/μL, an A260/280 score between 1.5 and 2.0, and void of read defects such as bubbling.

PCR Assay with EF1-α

PCR assays were conducted by amplifying the translation elongation factor 1-alpha gene (EF1-α) using EF1/EF2 primers (Karlsson et al., 2016; O'Donnell, Kistler, Cigelnik, and Ploetz, 1998; O'Donnell et al., 2010) to identify if the representative isolates were part of the *Fusarium* genus. EF1 primer sequence was ATGGGTAAGGARGACAAGAC and EF2 primer sequence was GGARGTACCAGTSATCATGTT (Karlsson et al., 2016; O'Donnell et al., 1998, 2010).

The total reaction volume per isolate consisted of 7.5μL of ddH_2O, 12.5μL of goTAQ Green PCR buffer, 1μL of EF1 forward primer at 10μm concentration, 1μL of EF2 reverse primer at 10μm concentration, and 3μL of target DNA at 25ng/μL. For PCR amplifications, the thermocycler was programmed for one cycle of two minutes at 95ºC, followed by 35 cycles of 30seconds at 95ºC, 30seconds of 53ºC, and one minute at 72ºC, after the 35 cycles, one cycle of ten minutes at 72ºC is needed, and finally, product can rest in the thermocycler at 10ºC until processing.

To identify banding at the EF1-α region, gel electrophoresis was conducted on a 1% agarose gel with TAE buffer and ran at 90V for 30 minutes. Each well contained 10μL of PCR product and 2μL of EZ-Vision, Dye-as-Loading-Buffer, 6X (VWR, Catalog Number 97064) and ran with

EasyLadder I (BioLine, Catalog Number BIO-33045) for easy band length identification. DNA purification after PCR cleanup was completed using Wizard SV Gel and PCR Clean-Up System (Promega, Catalog Number A9281).

In an SV minicolumn with a collection tube, 15μL of PCR product were added to an equal amount of membrane binding solution and incubated at room temperature for one minute. Tubes were centrifuged at 16,000g for one minute with flowthrough discarded. Following centrifugation, 700μL of membrane wash solution + ethanol was added to the column, centrifuged at 16,000g for one minute, and had flowthrough discarded. Next, 500μL of membrane wash solution + ethanol was added to the column, centrifuged at 16,000g for five minutes, and had flowthrough discarded. The collection tube was emptied and recentrifuged for one minute with the lid open to allow evaporation of residual ethanol. To elute DNA, the minicolumn was transferred to a new 1.5mL microcentrifuge tube, 30μL of nuclease free water was added to the column, incubated for one minute at room temperature, and centrifuged at 16,000g for one minute.

Purified DNA was quantified using a NanoDrop OneC Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific, Catalog Number ND-ONEC-W). High quality DNA contained concentrations higher than 50ng/μL, an A260/280 score between 1.5 and 2.0, and void of read defects such as bubbling.

Sanger Sequencing & BLAST Analysis

DNA from the representative isolates were submitted to the Core DNA Sequencing Facility at the University of Illinois at Urbana-Champaign and 5μL of each sample from the purified PCR product along with 10μL of EF1 primer were used for sequencing. DNA sequence output was used for sequence-based species identification. The following online databases were initially used for identification: Westerdijk Fungal Biodiversity Institute, FUSARIUM-ID, Joint Genome Institute, EnsemblFungi, and NCBI. After a preliminary assessment, all BLAST searches were conducted through FUSARIUM-ID (Geiser et al., 2004).

Representative isolate sequences were trimmed by removing all uncalled nucleotides (N) before and after a 5N repeating sequence towards 3' respectively. Next, the top and bottom 160bps were removed and the remainder base pairs were used for analysis. For more information, see Appendix A. Reference isolates with the highest similarity percentage were used to determine species identification. Sequencing and BLAST analysis were repeated a second time to correctly identify species.

Multi-Locus Genotyping

All processed isolates were sent to the USDA-ARS Mycotoxin Prevention and Applied Microbiology Research Unit in Peoria, IL for species identification using MLGT. Some isolates were compromised with various secondary pathogens and omitted from the MLGT analysis. All processed isolates were conducted using methods written in Kelly and Ward (2018). Of the 175 processed isolates, 164 were sent to the USDA-ARS Mycotoxin Prevention and Applied Microbiology Research Unit in Peoria, IL.

RNA Extraction and Sequencing

Total RNA was extracted from the twelve representative isolates using TRIzol (Thermo-Fisher, Catalog Number 15596026), RNAeasy MinElute Kits (Qiagen, Catalog Number 74204), and modified method from The Maize Genetics and Genomics Database (Lawrence, Dong, Polacco, Seigfried, and Brendel, 2004).

To ensure RNA contamination did not occur, mortar and pestles were baked in an oven at 180°C for a minimum of three hours and allowed to cool. Under a flow hood, 1mL of TRIzol was pipetted into 1.5mL microcentrifuge tubes and left with the cap open. Liquid nitrogen was poured into the unwrapped mortar along with thawed sample in the RNA*later*, quickly ground into a fine talc-like powder, added to the 1mL of TRIzol, vortexed, and incubated for five minutes at room temperature, vortexing frequently. It is crucial to not allow the ground tissue to thaw in the mortar since RNAases can rapidly break down RNA as it is yet to be protected by the TRIzol. Each tube had 200µL of chloroform added with the TRIzol, vortexed for 15seconds, incubated for one minute at room temperature, and vortexed again for 15seconds. Tubes were centrifuged in a 4°C incubator for ten minutes at 15,000g to separate phases. Following centrifugation, 700µL of Qiagen RLT buffer were added to a new tube. Next, 200µL were removed from the top layer of the prior centrifuged tube to the new RLT buffer tube. The remainder of the supernatant can be placed into a new tube and frozen at -20°C to serve as a backup in case initial yield is low. Using the 200µL of sample now combined with 700µL RLT buffer, 500µL of 100% ethanol was added and mixed by vortexing. Half of the sample (~700µL) was added to a Qiagen MinElute spin column placed in a 2mL microcentrifuge tube, spun for

one minute at 10,000rpm, flow through discarded, and repeated with the remainder of the sample. The MinElute column was moved to a new 2mL catch microcentrifuge tube and 500µL of RPE buffer was added to the column. Tubes were centrifuged at 10,000rpm for one minute and flow through discarded. Following centrifugation, 750µL of 80% ethanol was added to the spin column, centrifuged at 10,000rpm for one minute, and flow through discarded. The prior step was repeated to ensure removal of all guanidine salts that may inhibit downstream applications. Tubes were centrifuged again at top speed for five minutes with the cap off to remove all trace amounts of ethanol. RNA was eluted by transferring the spin column to a new 1.5mL microcentrifuge tube with 10µL of RNAase free water and spun at top speed for one minute. Another 10µL of RNAase free water was added to the column and spun at top speed for one minute. Purified RNA was quantified using a NanoDrop OneC Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific, Catalog Number ND-ONEC-W). High quality RNA contained concentrations higher than 100ng/µL, an A260/280 score near 2.0, and void of read defects such as bubbling.

To provide a visual display of rRNA bands, gel electrophoresis was conducted on a 1.2% agarose gel with TAE buffer and ran at 190V for 30 minutes. Each well contained: 1µL of GelRed Prestain Loading Buffer, 6X (Biotium, Catalog Number 41009) and 5µL of 100ng/µL RNA product with TAE buffer. The gel was run with EasyLadder I (BioLine, Catalog Number BIO-33045) for easy band length identification. Upon completion, the gel was photographed using a UV light box. Isolated RNA was quantified using a NanoDrop OneC Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific, Catalog Number ND-ONEC-W). High quality

RNA contained concentrations higher than 100ng/μL, an A260/280 score near 2.0, and void of read defects such as bubbling.

RNA was submitted to the University of Illinois' Roy J. Carver Biotechnology Center to prepare RNA sequencing libraries using Illumina Truseq Kit and to sequence using the HiSeq4000 100nt paired-end reads (Illumina). Paired-end reads were aligned to a recently completed *F. graminearum* PH-1 genome (King et al., 2017) to determine genes that aligned to the fungal genome. All bioinformatics were completed using the BioCluster at the University of Illinois' Institute for Genomic Biology. Differential gene expression analysis was conducted with the services of HPCBio at the University of Illinois' Institute for Genomic Biology. Pairwise comparisons of gene expression were conducted by controlling for level of host resistance (moderately resistant, moderately susceptible, and highly susceptible wheat lines) and origin of the collected isolate. This allowed for the identification *F. graminearum* genes that govern and are necessary for pathogenicity to occur.

Results and Discussion

Sample Collection

A total of 197 samples were collected from the various field sites (Figure 2.1). Across locations, more samples were collected from Brownstown, St. Jacob, and Carmi, Illinois suggesting that in the summer of 2016, Fusarium were at a higher pressure in southern counties. As expected, more samples were collected from moderately and highly susceptible lines (Table 2.1). This was expected since pathogens are more likely to cause disease on hosts with less resistance.

Sample Processing

Of the 197 collected samples, 175 isolates were processed and cataloged (Table 2.2). Processing results mirror that of sample collection with the exception that the most lost isolates derived from the Carmi location, specifically from the moderately susceptible cultivar group. Given that Urbana did not yield any samples collected from either moderately resistant cultivars, the location was not used for further experimentation. To accurately capture the *Fusarium* genetic diversity and take into account time and resources, a smaller subsample was surveyed from the total processed isolates. Three isolates were randomly chosen from each of the remaining locations: one isolate collected from the moderately resistant cultivar (IL11-28222), one from the moderately susceptible cultivar (IL10-19464), and finally one from the highly susceptible cultivar (Pioneer 25R47).

The twelve representative isolates that were used for the remainder of the study are BMR, BMS, BHS, JMR, JMS, JHS, CMR, CMS, CHS, SMR, SMS, and SHS (Table 2.3). Each selected isolate denotes the origin of collection as well level of host resistance the isolate was collected from, otherwise denoted as level. For example, BMR isolate was collected from a moderately resistant wheat cultivar from Brownstown, Illinois.

Species Identification

DNA Extraction

High quality DNA was extracted from all twelve representative isolates and the *F. graminearum* positive control. Most concentrations were higher than 50ng/μL, an A260/280 score between 1.5 and 2.0, and void of read defects such as bubbling (Table 2.4).

PCR Assay

After gel electrophoresis of the PCR products of the EF1–α gene, proper banding was observed for all isolates and the control except BMS, JMS, and JHS (Figure 2.2). Non-banding may be due to quality of the DNA, protein contamination, or to the isolates not belonging to the *Fusarium* genus. PCR cleanup was conducted on all representative isolates that produced banding. After cleaning, most isolates had DNA concentrations higher than 50ng/µL, an A260/280 score between 1.5 and 2.0, and void of read defects such as bubbling (Table 2.5).

Sanger Sequencing & BLAST Analysis

First and second replicates of sequenced DNA (Table 2.6 and 2.7) yielded roughly 200 – 400bps after trimming. After both BLAST analyses were conducted on the twelve representative isolates: seven identified as *F. graminearum* (Schwabe), three as *F. armeniacum* (Burgess et al., 1993), and two were non-determinant given issues during PCR amplification (Table 2.8). Given that the St. Jacob series of isolates were not able to be identified, they were excluded from the remainder of the study. To complete Koch's Postulates, all *Fusarium* isolates were re-isolated from infected glumes after threshing and found that they maintained their morphological characteristics in culture.

F. armeniacum was first reported in Minnesota (Kommedahl et al., 1979) and subsequently Australia, South Africa, China (Ellis et al., 2012), and Argentina (Nichea et al., 2015). To date, *F. armeniacum* has been reported to cause seed and root rot on soybeans (Ellis et al., 2012), cultured from asymptomatic corn (Leslie and Summerell, 2006), and living as a saprophyte in

natural Argentinean grasses (Nichea et al., 2015). *F. armeniacum* has yet to be reported to cause FHB in small grains, specifically wheat. A first report has been written that delves into *Fusarium armeniacum* causing FHB on soft red winter wheat (SRWW) in Illinois (Salazar, in review, 2018).

Multi-Locus Genotyping

At the facility, 24 isolates were omitted from the analysis due to secondary pathogen infection. Inferences were made on the 140 isolates processed by the USDA facility and were deposited in their database. 93.6% of the total isolates were part of the *F. sambucinum* species complex (FSAMSC), in which *F. graminearum* is a member of (Figure 2.3). Ninety percent of the total isolates were identified as *F. graminearum* (Figure 2.4). After analysis, five species were identified: *F. acuminatum*, *F. armeniacum* (Burgess et al., 1993), *F. circinatum*, *F. graminearum* (Schwabe), and *F. reticulatum* (Figure 2.5). Eighty nine percent of the isolates produced 15-ADON as predicted by their genotypes.

The majority of the isolates came from moderately susceptible, susceptible, and highly susceptible levels as opposed to the moderately resistant level (Figure 2.6). Upon comparison of the selected twelve isolates using SLGT and MLGT, two more isolates were identified through SLGT, and CMS was identified to be *F. armeniacum* rather than *F. graminearum* (Table 2.9).

RNA Extraction and Sequencing

After RNA extractions, all isolates produced high quality concentrations (Table 2.10) to be used for gel electrophoresis. Upon gel electrophoresis, all isolates yielded strong banding at the 28S and 18S regions (Figure 2.7).

Differential gene expression analysis was conducted with the services of HPCBio at the University of Illinois' Institute for Genomic Biology. Pairwise comparisons of gene expression were conducted by controlling for level of host resistance (moderately resistant, moderately susceptible, and highly susceptible wheat lines) and origin of the collected isolate. This allowed for the identification *F. graminearum* genes that govern and are necessary for pathogenicity to occur. More work on the field pathogenomic assay can be found in Fall, Salazar, et al., Accepted (2018).

Conclusion

Isolate collection and processing results suggest that across Illinois wheat fields in 2016, underwent high levels of disease pressure from multiple *Fusarium* species. After SLGT with the twelve representative isolates, seven identified as *F. graminearum* (Schwabe), three as *F. armeniacum* (Burgess et al., 1993), and two were non-determinant. *F. armeniacum* was first reported in Minnesota (Kommedahl et al., 1979) and subsequently Australia, South Africa, China (Ellis et al., 2012), and Argentina (Nichea et al., 2015). To date, *F. armeniacum* has been reported to cause seed and root rot on soybeans (Ellis et al., 2012), cultured from asymptomatic corn (Leslie and Summerell, 2006), and living as a saprophyte in natural Argentinean grasses (Nichea et al., 2015). *F. armeniacum* has yet to be reported to cause FHB in small grains,

specifically wheat. A first report has been written that delves into *F. armeniacum* causing FHB on soft red winter wheat in Illinois (Salazar, in review, 2018). The USDA's MLGT analysis was able to detect more *Fusarium* species, the species complex they belong to, as well as the mycotoxin chemotype produced. Of the isolates processed by the USDA facility, 93.6% of the total isolates were part of the *F. sambucinum* species complex (FSAMSC), in which *F. graminearum* is a member of. Ninety percent of the total isolates were identified as *F. graminearum.* After analysis, five species were identified: *F. acuminatum*, *F. armeniacum* (Burgess et al., 1993), *F. circinatum*, *F. graminearum* (Schwabe), and *F. reticulatum*. Eighty nine percent of the isolates produced 15-ADON as predicted by their genotypes.

Tables and Figures

Table 2.1: Total number of collected samples per origin and wheat resistance level.

	Moderately resistant 1 (IL11-28222)	**Moderately resistant 2** (IL07-19334)	**Moderately susceptible** (IL10-19464)	**Susceptible** (Kaskaskia)	**Highly susceptible** (Pioneer 25R47)	**Total**
Brownstown	10	10	10	9	10	**49**
St. Jacob	10	10	10	10	10	**50**
Carmi	10	10	10	10	10	**50**
Urbana	0	0	10	2	10	**22**
Savoy	2	0	10	4	10	**26**
Total	**32**	**30**	**50**	**35**	**50**	**197**

Table 2.2: Total number of processed isolates per origin and wheat resistance level.

	Moderately resistant 1 (IL11-28222)	**Moderately resistant 2** (IL07-19334)	**Moderately susceptible** (IL10-19464)	**Susceptible** (Kaskaskia)	**Highly susceptible** (Pioneer 25R47)	**Total**
Brownstown	10	10	10	9	10	**49**
St. Jacob	8	10	8	10	9	**45**
Carmi	10	9	4	5	5	**33**
Urbana	0	0	10	2	10	**22**
Savoy	2	0	10	4	10	**26**
Total	**30**	**29**	**42**	**30**	**44**	**175**

Table 2.3: Twelve representative isolates from Illinois fields.

Origin	**Resistance Level**	**12 Representative *Fusarium* Isolates**
Brownstown	Moderately resistant 1	BMR
Brownstown	Moderately susceptible	BMS
Brownstown	Highly susceptible	BHS
St. Jacob	Moderately resistant 1	JMR
St. Jacob	Moderately susceptible	JMS
St. Jacob	Highly susceptible	JHS
Carmi	Moderately resistant 1	CMR
Carmi	Moderately susceptible	CMS
Carmi	Highly susceptible	CHS
Savoy	Moderately resistant 1	SMR
Savoy	Moderately susceptible	SMS
Savoy	Highly susceptible	SHS

Table 2.4: DNA concentrations for representative isolates and *F. graminearum* control.

Isolate	Concentration (ng/µL)	A260/280	A260/230
PH-1.1	52.97	1.80	0.39
PH-1.2	43.09	1.76	0.34
BMR	169.72	1.51	0.56
BMS	301.72	1.76	0.64
BHS	170.05	1.52	0.54
JMR	132.72	1.63	0.67
JMS	299.59	1.67	0.72
JHS	194.72	1.79	0.64
CMR	450.32	1.92	1.01
CMS	138.09	1.66	0.53
CHS	209.40	1.53	0.49
SMR	207.77	1.42	0.90
SMS	112.68	1.55	0.52
SHS	69.78	1.64	0.42

Table 2.5: DNA concentrations after PCR cleanup for isolates and *F. graminearum* control. Dash indicates lack of product.

Isolate	Concentration (ng/μL)	A260/280	A260/230
PH-1.1	51.23	1.77	1.83
PH-1.2	53.43	1.77	1.31
BMR	56.88	1.79	1.20
BMS	-	-	-
BHS	53.31	1.76	1.34
JMR	46.24	1.76	1.34
JMS	-	-	-
JHS	-	-	-
CMR	57.68	1.84	1.39
CMS	59.97	1.77	1.89
CHS	63.78	1.76	1.81
SMR	65.36	1.79	1.69
SMS	56.04	1.74	1.64
SHS	53.03	1.78	1.70

Table 2.6: First replicate of sequenced DNA. Dash denotes lack of product.

Isolate	Total number of sequenced bps	Trimmed sequence for BLAST analysis
PH-1.1	1442	293
PH-1.2	1136	352
BMR	1152	348
BMS	-	-
BHS	1152	359
JMR	1074	347
JMS	-	-
JHS	-	-
CMR	1173	424
CMS	1159	370
CHS	1147	366
SMR	1139	348
SMS	1105	288
SHS	1129	183

Table 2.7: Second replicate of sequenced DNA. Dash denotes lack of product.

Isolate	Total number of sequenced bps	Trimmed sequence for BLAST analysis
PH-1	1431	356
BMR	1388	364
BMS	1630	294
BHS	1440	369
JMR	1459	380
JMS	1479	358
JHS	-	-
CMR	1483	355
CMS	1500	352
CHS	1493	359
SMR	1449	357
SMS	1471	360
SHS	1456	359

Table 2.8: Identification of representative *Fusarium* isolates using sequencing database, FUSARIUM-ID. Dash denotes database was unable to determine a reference species.

Isolate	Rep1 FUSARIUM-ID database		Rep2 FUSARIUM-ID database	
	Reference Species	**Percent Similarity**	**Reference Species**	**Percent Similarity**
PH-1.1	*F. graminearum*	99.65	*F. graminearum*	100
PH-1.2	*F. graminearum*	100		
BMR	*F. graminearum*	100	*F. graminearum*	99.15
BMS	-	-	-	-
BHS	*F. graminearum*	100	*F. graminearum*	99.72
JMR	*F. graminearum*	100	*F. graminearum*	98.94
JMS	-	-	*F. armeniacum*	98.32
JHS	-	-	-	-
CMR	*F. armeniacum*	98.01	*F. armeniacum*	98.3
CMS	-	-	*F. armeniacum*	98.29
CHS	*F. graminearum*	100	*F. graminearum*	100
SMR	*F. graminearum*	100	*F. graminearum*	100
SMS	*F. graminearum*	98.61	*F. graminearum*	98.05
SHS	*F. graminearum*	95.32	*F. graminearum*	99.44

Table 2.9: Comparison of identification of the 12 representative isolates between SLGT and MLGT analysis with differing species highlighted in gray. Dash denotes database was unable to determine a reference species.

Isolate	M.M. Salazar Analysis Species Identified	USDA-ARS Analysis Species Identified
BMR	*F. graminearum*	*F. graminearum*
BMS	-	-
BHS	*F. graminearum*	*F. graminearum*
JMR	*F. graminearum*	*F. graminearum*
JMS	*F. armeniacum*	-
JHS	-	-
CMR	*F. armeniacum*	-
CMS	*F. armeniacum*	*F. graminearum*
CHS	*F. graminearum*	*F. graminearum*
SMR	*F. graminearum*	*F. graminearum*
SMS	*F. graminearum*	*F. graminearum*
SHS	*F. graminearum*	*F. graminearum*

Table 2.10: RNA concentrations from representative isolates after extraction.

Isolate	Concentration (ng/μL)
BMR	267.97
BMS	253.63
BHS	478.54
JMR	324.41
JMS	210.27
JHS	904.65
CMR	86.77
CMS	557.43
CHS	775.4
SMR	385.71
SMS	290.25
SHS	187.3

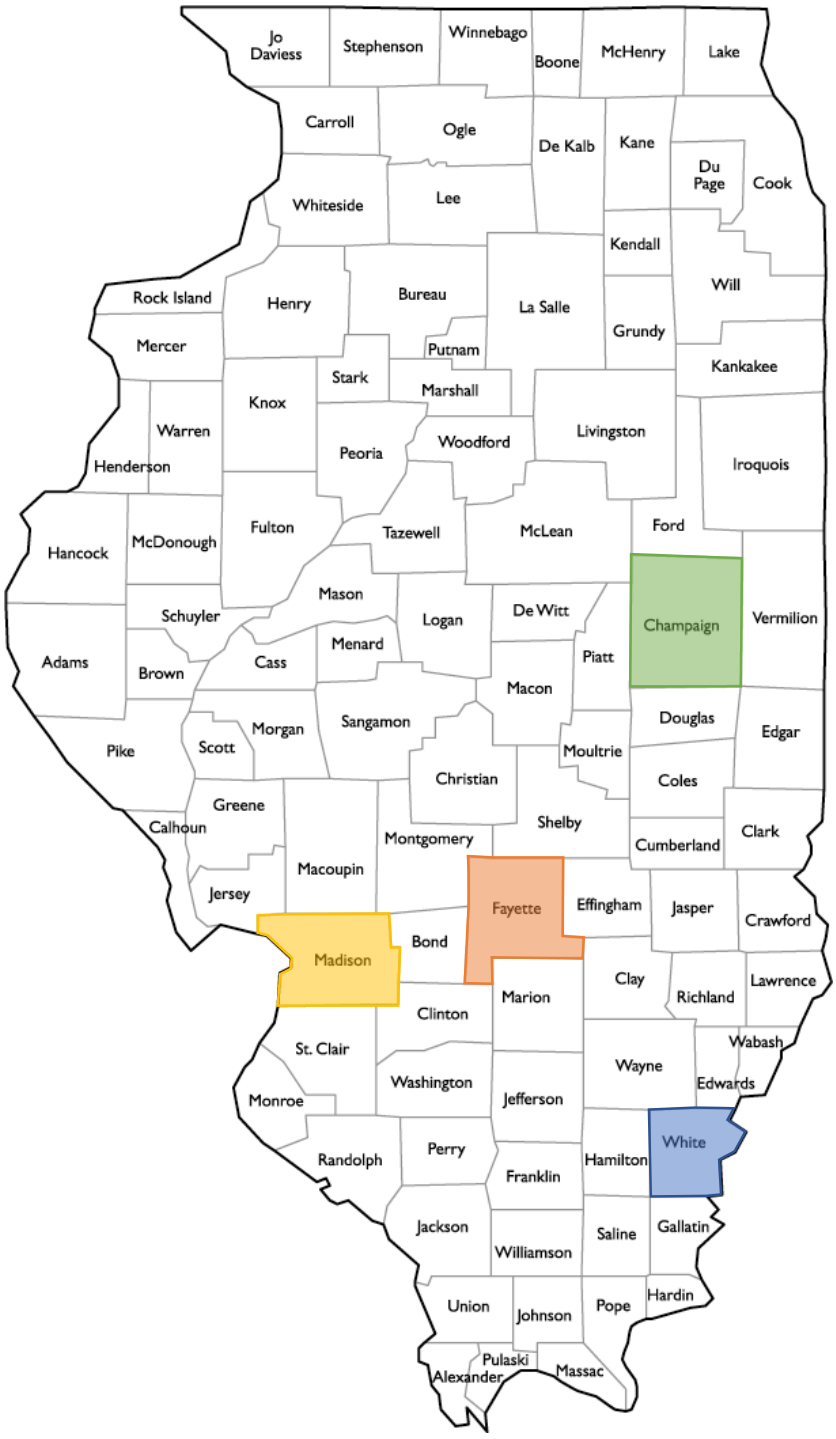


Figure 2.1: Highlighted Illinois counties indicate locations where *Fusarium* samples were collected. Map outline courtesy of WorldAtlas.

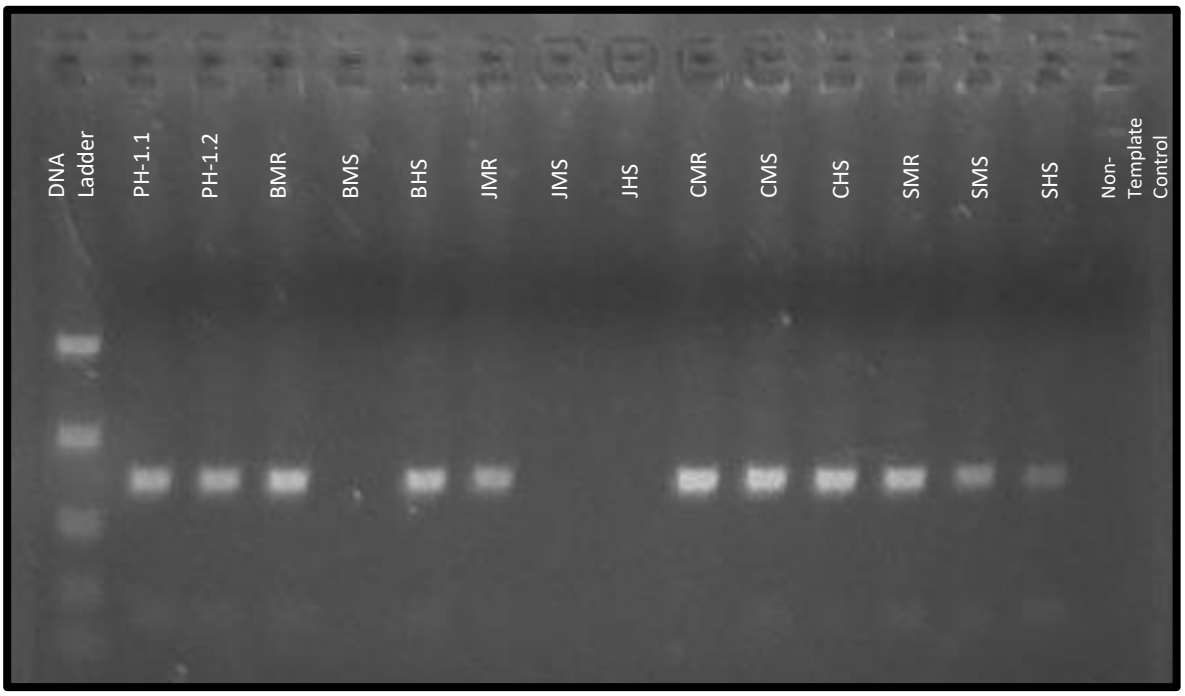


Figure 2.2: PCR products from the EF1-α gene showing banding on a 1% agarose gel. All isolates produced correct banding except BMS, JMS, and JHS.

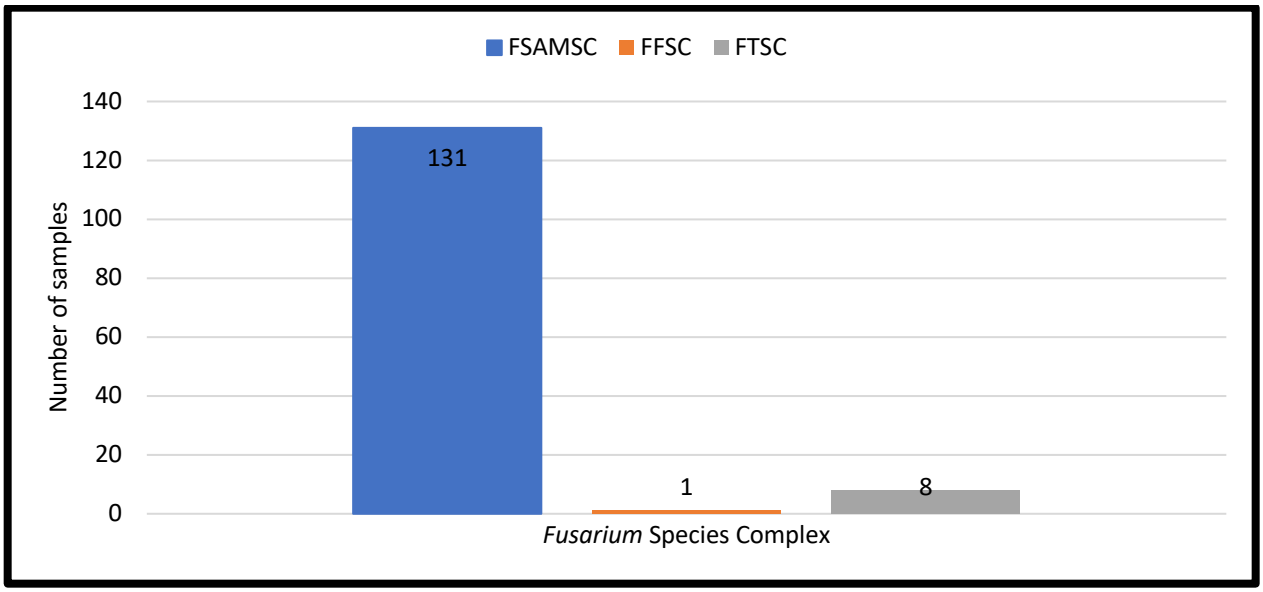


Figure 2.3: MLGT results describe *Fusarium* species complex within Illinois fields. Analysis shows 93.6% of the collected isolates were determined to be part of FSAMSC which *F. graminearum* is part of.

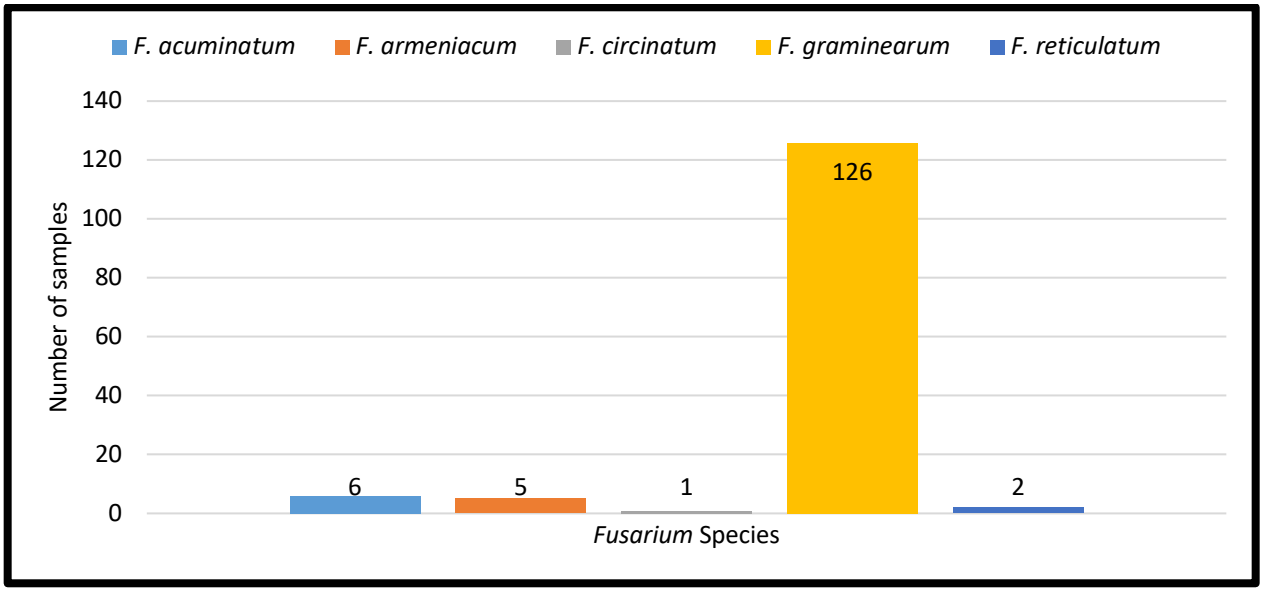
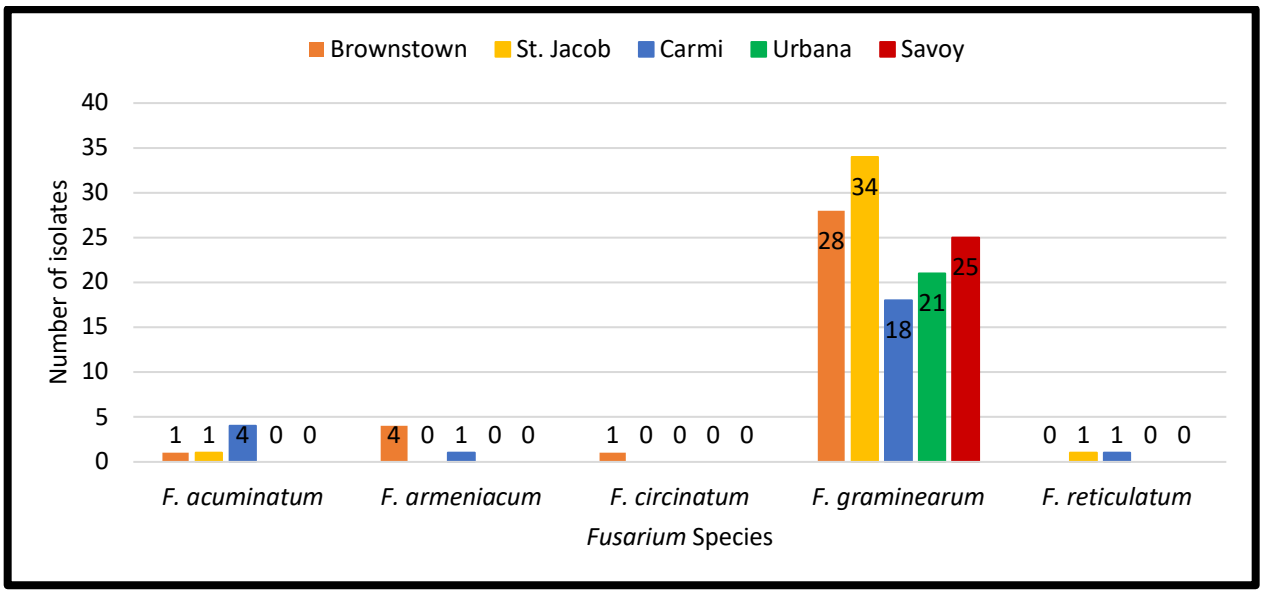


Figure 2.4: MLGT results describe *Fusarium* speciation within Illinois fields. Analysis shows 90% of the collected isolates were identified to be *F. graminearum*.

a) *Fusarium* species based on origin of collection.



b) *Fusarium* species based on resistance level.

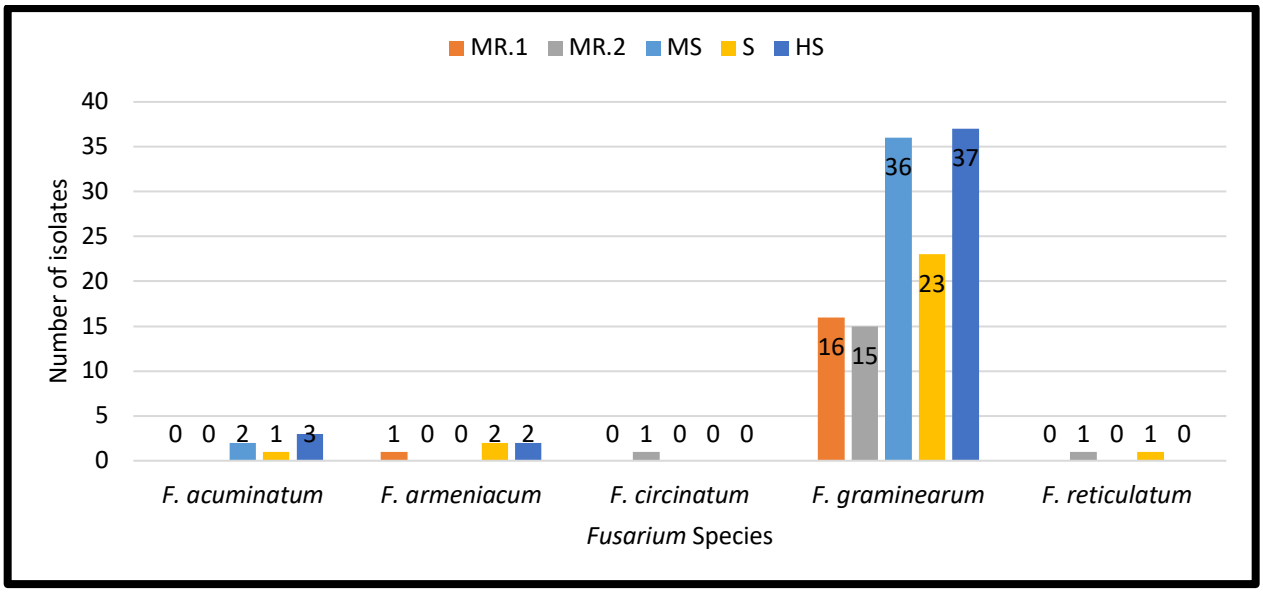


Figure 2.5: Processed isolates based on (a) origin of collection and (b) level of host resistance. Key describes moderately resistant line 1 (IL11-28222), moderately resistant line 2 (IL07-19334), moderately susceptible line (IL10-19464), susceptible line (Kaskaskia), and highly susceptible line (Pioneer 25R47).

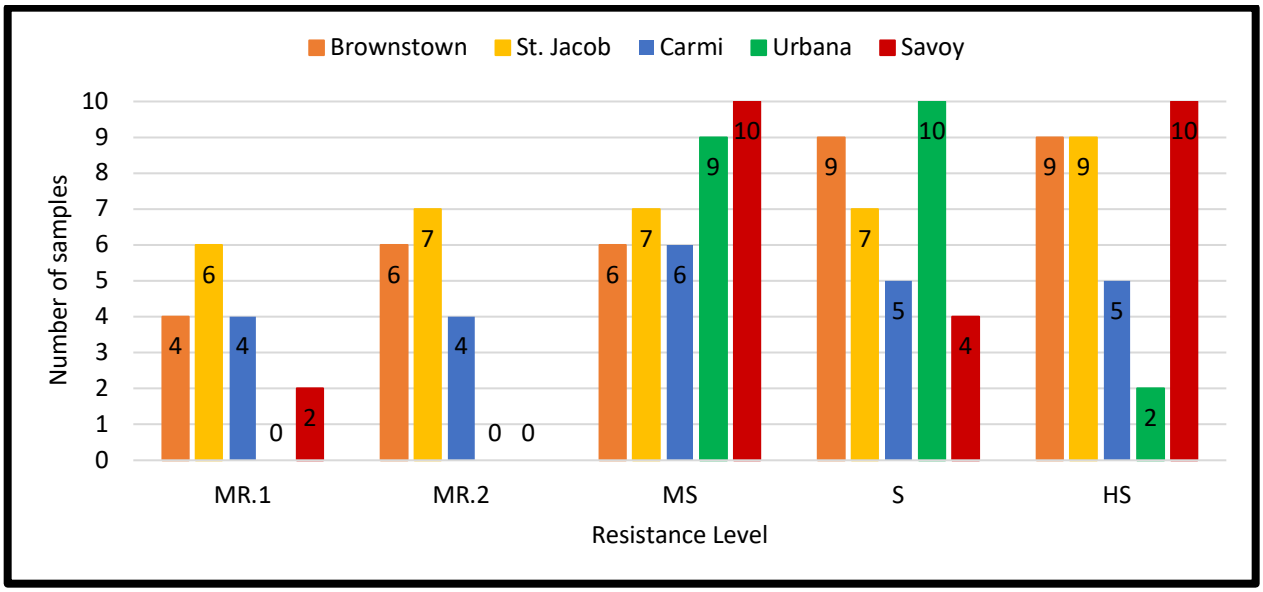


Figure 2.6: Processed isolates based on origin of collection and resistance level. Resistance level axis describes moderately resistant line 1 (IL11-28222), moderately resistant line 2 (IL07-19334), moderately susceptible line (IL10-19464), susceptible line (Kaskaskia), and highly susceptible line (Pioneer 25R47).

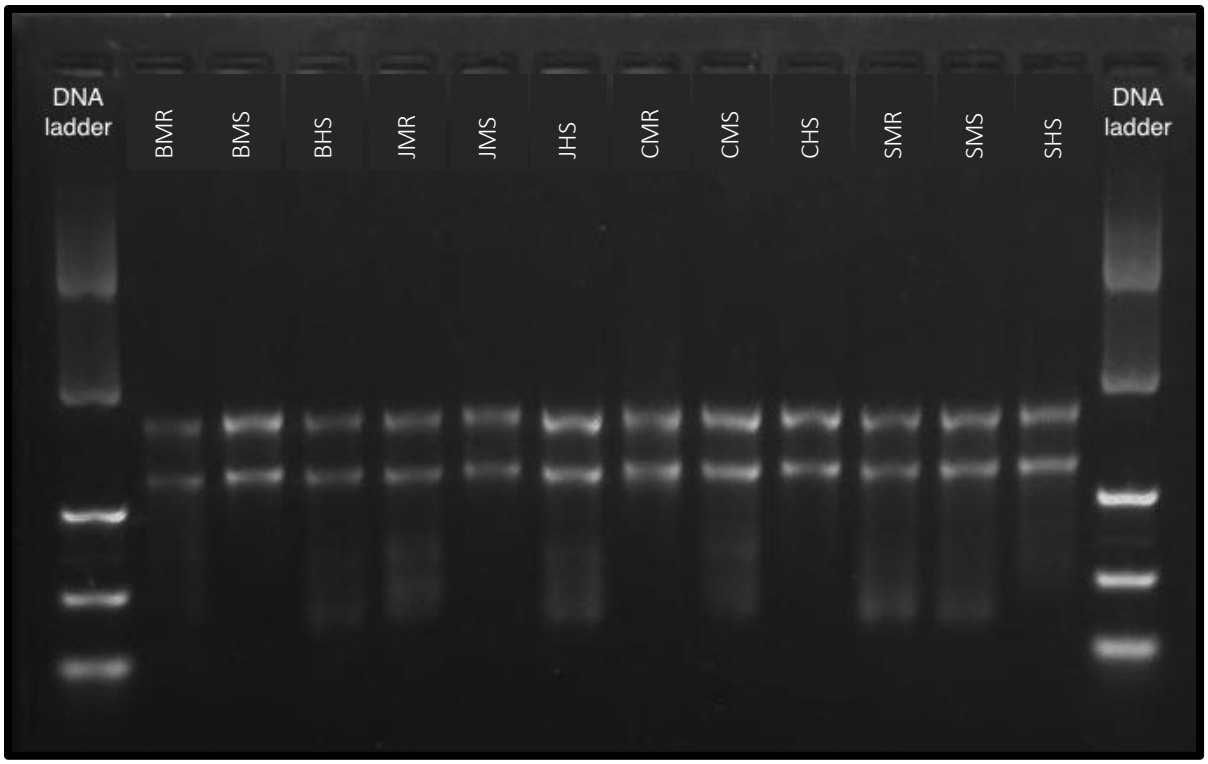


Figure 2.7: Extracted RNA showing strong banding at 28S and 18S on a 1.2% agarose gel.

CHAPTER 3: UNIVARIATE AND MULTIVARIATE ANALYSIS OF AGGRESSIVENESS IN *FUSARIUM*

Introduction

Aggressiveness is currently described as a quantifiable amount of disease caused by a pathogen. Disease progression and colonization can be quantified in numerous ways. FHB infection can be measured through disease severity (DS), area under the disease progress curve (AUDPC), incidence (INC), *Fusarium* damaged kernels (FDKs), mycotoxin quantification such as deoxynivalenol (DON), and spore quantification. DS is a visualization of the number of symptomatic spikelets (Kuhnem et al., 2015). AUDPC utilizes DS data and measures the progression of disease through time. INC is a visualization of the number of symptomatic heads per area or plot. FDKs are a quantification of symptomatic kernels that appear shriveled or tombstoned. Of all the mycotoxins produced by the *Fusarium* genus, trichothecenes like DON (Desjardins, 2006) are the most studied. It is hypothesized that the more aggressive an isolate is the higher the DON accumulation should be (Bai and Shaner, 2004). To determine functional pathogen aggressiveness, isolates were inoculated on wheat lines with varying resistance levels of resistance in field and greenhouse experiments as well as grown on media to quantify spore production.

Currently, there is no measurement that conglomerates the individual measurements of aggressiveness. The final goal of this research was to combine these individual traits to determine a quantifiable definition of aggressiveness. A principal component analysis (PCA) was conducted on the collective aggressiveness traits from each assay to create a multivariate description of isolate aggressiveness (Butts-Wilmsmeyer, Seebauer, Singleton, and Below, 2019;

Johnson, 1998a). A PCA utilizes principal components or uncorrelated variables that are derived from correlated response variables (Johnson, 1998b). Multivariate analyses aim to accomplish two objectives: 1) understand the dimensionality, or spatial viewing, of the data, and 2) identify significant variables (Johnson, 1998b). Principal components are output in decreasing order of importance explaining each level of correlation, where the first principal component accounts for the largest amount of variability possible and each subsequent component describes the remaining variability to completion (Johnson, 1998b). Component vector loadings are derived from normalized eigenvectors, as they explain comparisons of each variable within vector loadings rather than across loadings (Johnson, 1998b). When determining the number of principal components to use for a cluster analysis, variable dimensionality needs to be visualized through the number of principal components with variances larger than zero (Johnson, 1998a, 1998b). Applying the above outputs, a hierarchical tree diagram can be utilized to visualize the similarity and dissimilarity between clusters of observations (Johnson, 1998a). In this instance, isolates were binned into aggressive, moderately aggressive, and non-aggressive levels based on Ward's Minimum Variance Measure of Dissimilarity and the index created through use of principal cluster analysis-based index. This comprehensive quantitative measure of aggressiveness can be utilized to determine a standard definition of aggressiveness across multiple disease measures.

Materials and Methods

The nine representative isolates were analyzed and characterized by origin of collection and level of host resistance, level. As a reminder, the nine isolates were collected from three wheat lines with diverse levels of resistance across three different locations in Illinois.

Univariate Analysis

Spore Quantification Assay

To determine if spore production plays a role in determining a pathogen's level of aggressiveness on a host, a quantitative assay was designed. The nine representative isolates were plated on PDA+ and allowed to grow for seven days at 25°C with natural sunlight. After ample growth, spores were gently washed from agar plates with 3mL of 1% Tween 20 and sterile bent glass rods. Collected spores were stored in 4°C and counted within a two-day window to ensure spores did not germinate in buffer. Spores were counted using a hemocytometer and diluted if necessary. The hemocytometer was cleaned and sterilized between isolates with 95% ethanol, washed with deionized water, and dried with kimwipes. Spore quantity was determined by averaging the four largest corners in the hemocytometer and repeated using both wells of the device. Once both averages were derived, a grand mean was calculated.

The assay was evaluated as a randomized complete block design (RCBD) with the following model:

$$Y_{ijk} = \mu + R_i + O_j + L_k + OL_{jk} + \varepsilon_{ijk}$$

where Y_{ijk} is the number of spores produced recorded for the ith replication, the jth origin of the collected isolate, and the kth level of host resistance ($i = 1, 2, 3;\ j = 1, 2, 3;\ \ k = 1, 2, 3$); μ is the grand population mean; R_i is the random effect of the ith replication assuming $NIID\ (0, \sigma_R^2)$; O_j is the fixed effect of the jth origin of the collected isolate; L_k is the fixed effect of the kth

level of host resistance; OL_{jk} is the fixed interaction between the jth origin of the collected isolate and the kth level of host resistance; and ε_{ijk} is the random error term assuming $NIID\ (0, \sigma_{\varepsilon}^2)$.

Statistical analysis was conducted in SAS Edition 9.4 (SAS Institute, November 2018) using MIXED, UNIVARIATE, GLM, and GLIMMIX procedures. ANOVA was conducted in PROC MIXED. Residuals were obtained from the MIXED procedure and were then analyzed using the UNIVARIATE procedure to check for the assumption of normality with Shapiro-Wilk. Original data was transformed using a log10 transformation with a qualifier to attain normality. A Brown–Forsythe Levene test was used to check the assumption of homogeneous variances. Significant differences were calculated with a Tukey's adjustment and $\alpha = 0.05$. In the presence of a significant origin by level interaction, the slice option in LSMEANS of PROC MIXED was used to examine the significance of origin and level main effects.

Inoculum Preparation

Each isolate was placed on a PDA+ plate and allowed to grow for one week at room temperature. After fungal growth, each plate was put into a laboratory grade blender with 30mL of double distilled water and blended for one minute or until the mixture became homogeneous and smooth (Wilcoxson, Kommedahl, Ozmon, and Windels, 1988). As reiterated in a Mesterhazy research paper, mycelial slurry is equally effective at causing infection as conidia are (Mesterházy, 1995). Inoculum was stored in a 50mL centrifuge tube, placed in a 4°C incubator, and used for host inoculations within a week's time.

Field Assays

Two field pathogenesis assays were conducted to determine aggressiveness under field conditions. The University of Illinois' wheat breeding program field plots in Urbana, IL were utilized where wheat plots were planted in an RCBD for each respective year's assay. In the summer of 2017 and 2018, the nine representative *Fusarium* isolates and a negative control (PDA+) were tested against two wheat cultivars: a moderately resistant (IL07-4415) and a highly susceptible (Pioneer 25R47). During anthesis (Feekes 10 growth stage), roughly 200µL of inoculum was injected via hypodermic needle into the centermost spikelet of two heads from each cultivar (Figure 3.1 a-b). Two wheat heads were inoculated to ensure data was available in case a head was lost due to the environment, animal interference, or human error. Immediately after inoculation, a waxed Seedburo Canvasback shoot bag was placed over the inoculated head (Figure 3.1 c) and stapled at the base to protect the inoculum from wind, rain, and mammals, and to increase humidity within the bag (Imathiu et al., 2014) (Mesterházy, Bartók, Mirocha, and Komoróczy, 1999). Bags were removed after 48hours and monitored daily. Wheat plots were grown using standard agronomic practices for SRWW in Illinois.

In the summer of 2017, the assay was evaluated as a split-plot in an RCBD with three replicates consisting of ten isolates inoculated on two wheat heads for two cultivars. In the summer of 2018, the assay was assessed identically as in 2017 with the exception of five replicates consisting of ten isolates inoculated on two wheat heads for two cultivars. At the end of each growing season, heads were harvested and threshed, and all seed was collected.

Greenhouse Assay

In 2017, a greenhouse pathogenesis assay was conducted to determine aggressiveness under greenhouse conditions. The nine representative *Fusarium* isolates and a negative control (PDA+) were tested against two wheat cultivars: a moderately resistant (IL07-4415) and a highly susceptible (Pioneer 25R47). Wheat cultivars were grown in a greenhouse at optimal conditions: daily average of ~24˚C, 16-hour photoperiod, watered daily, fertilized weekly, and aphid controlled. Six to eight wheat seeds of each cultivar were planted 1in deep in 3in x 2in plugs with a soil mixture made of 1 part soil : 1 part peat : 1 part torpedo sand (weed mix) with a basal tray with holes for proper water drainage. After planting, soil was watered heavily for complete saturation. Plugs were grown in a greenhouse for ten days and watered lightly when needed. Once seedlings germinated, trays were moved to a 2 – 4°C vernalization chamber with 12hr light/12hr dark fluorescent lights for eight weeks. Trays were watered on a weekly basis and checked for seedling health. After vernalization, trays were removed from the chamber, transferred to the greenhouse, and allowed to reach greenhouse room temperatures for two days. Once soil plugs were no longer cold, plugs were removed, transferred to 6inch pots, watered, and fertilized with a teaspoon of osmocote per pot. Pots were watered daily and monitored for developmental growth stages, health and wellness, and secondary pathogens. During anthesis (Feekes 10 growth stage), roughly 200µL of inoculum was injected via hypodermic needle into the centermost spikelet of one head from each cultivar (Figure 3.2 a-b). To maintain high humidity, plants were kept in a mist chamber (Figure 3.2 c) that sprayed free-floating water droplets for five seconds every ten minutes and removed from chamber after 48hrs (Imathiu et al., 2014). As referenced in *Fusarium Head Blight of Wheat and Barley*, visible FHB symptoms can be seen within three days after infection if plants are kept in a moist chamber (Bushnell et

al., 2003). Pots were grown using standard agronomic practices for SRWW. The greenhouse assay was evaluated as a split-plot in an RCBD with three replicates consisting of ten isolates inoculated on one wheat head for two cultivars. At the end of each growing season, heads were harvested and threshed, and all seed was collected.

Mycotoxin Assay

DON levels were quantified using collected grain from both field and greenhouse assays. Subsampled heads of the same isolate and cultivar from each field aggressiveness assay were combined and processed as one sample (one experimental unit was defined as both threshed heads infected from one isolate given one cultivar). Reveal Q+ for DON (Neogen, Lansing, MI, Cat. # 8385) assay strips were utilized to quantify mycotoxins. The Reveal Q+ assay strip contains specific antibodies for toxin detection and, when present, the particles concentrate to form a visible line on the test strip. If large quantities of the toxin are present within the sample, fewer particles are captured, and a visible reduction in line density becomes apparent. Using Neogen's AccuScan Gold reader, line density was quantified and translated to mycotoxin parts per million or billion (ppm or ppb).

Representative grain samples were ground using a mortar and pestle until homogenously emulsified to ensure maximum mycotoxin detection (Tuite, Shaner, and Everson, 1990). For each grain sample set, 0.1g was added to a sample cup with 1mL of distilled water. The sample was shaken vigorously for three minutes by hand. Once the sample settled, roughly 40µL was pushed through a filter syringe provided by Neogen. Next, 10µL of sample diluent was added to a provided red dilution cup along with 1µL of filtered sample and mixed by pipetting up and

down five times. Afterward, 10µL of the diluted sample was transferred to a new clear sample cup. A DON strip was added to the sample cup and allowed to sit for three minutes for strip development. The strip was removed at three minutes exactly to ensure the read wasn't overdeveloped or oversaturated. The strip was then fed into the AccuScan Gold Reader with the R-labeled cartridge adapter and DON was recorded. If samples read less than 0.05ppm DON, samples were diluted, re-ran, DON value was multiplied by the new dilution factor, and the new DON value was recorded.

Aggressiveness Traits and Analysis

Fungal aggressiveness was determined using DS, AUDPC, FDKs, and DON. DS was calculated as the percentage of FHB symptomatic spikelets per individual head. DS notes were taken at 14dpi (days post-inoculation), 21dpi, and 28dpi in the field. AUDPC was computed in R version 3.4.1 "Single Candle" using the 'agricolae' package version 1.2-4. To calculate AUDPC, DS data was used to determine the progression of fungal infection through time. FDKs were calculated as the percentage of tombstoned seed to total seed count. Reveal Q+ for DON (Neogen, Lansing, MI, Cat. # 8385) assay strips were utilized to quantify mycotoxins.

Assays were evaluated as a split-plot in an RCBD with cultivar as the whole plot (two levels) and isolate randomized within the subplot (ten isolates) in replicated blocks for each aggressiveness assay (two field and one greenhouse assay).

$$Y_{ijkl} = \mu + C_i + \beta_j + \varepsilon_{1_{ij}} + O_k + L_l + CO_{ik} + CL_{il} + OL_{kl} + COL_{ikl} + \varepsilon_{2_{ijkl}}$$

where Y_{ijkl} is the AUDPC, FDKs, DON recorded from the ith cultivar, the jth replication, the kth origin of the collected isolate, and the lth level of host resistance ($i = 1, 2$; $Field\ 2017\ j = 1, 2, 3;\ Field\ 2018\ j = 1, 2, 3, 4, 5;\ Greenhouse\ j = 1, 2, 3;\ k = 1, 2, 3;$ $l = 1, 2, 3$); μ is the grand population mean; C_i is the fixed effect of the ith cultivar; β_j is the random effect of the jth replication assuming $NIID\ (0, \sigma_\beta^2)$; $\varepsilon_{1_{ij}}$ is the whole-plot random error term assuming $NIID\ (0, \sigma_{\varepsilon_{1_{ij}}}^2)$; O_k is the fixed effect of the kth origin; L_l is the fixed effect of the lth level; CO_{ik} is the fixed interaction between the ith cultivar and the kth origin; CL_{il} is the fixed interaction between the ith cultivar and the lth level; OL_{kl} is the fixed interaction between the kth origin and the kth level; COL_{ikl} is the fixed interactions between the ith cultivar, the kth origin, and the lth level; and $\varepsilon_{2_{ijkl}}$ is the sub-plot random error term assuming $NIID\ (0, \sigma_{\varepsilon_{2_{ijkl}}}^2)$.

Statistical analyses were conducted in SAS Edition 9.4 (SAS Institute, November 2018) using MIXED, UNIVARIATE, and GLM procedures. ANOVA was conducted in PROC MIXED. Residuals were obtained from the MIXED procedure and were then analyzed using the UNIVARIATE procedure to check for the assumptions of normality with Shapiro-Wilk and homoscedasticity. Original data was transformed using a log10 transformation with a qualifier to attain normality. A Brown–Forsythe Levene test was used to check the assumption of homogeneous variances within the experiment. Significant differences were calculated with a Tukey's adjustment and $\alpha = 0.05$. In the presence of a significant origin by level interaction, the slice option in LSMEANS of PROC MIXED was used to examine the significance of origin and level main effects.

Multivariate Analysis

Pearson correlation coefficients were calculated from the transformed aggressiveness data (T_AUDPC, T_FDK, and T_DON) using PROC CORR in SAS Edition 9.4 (SAS Institute, November 2018). Correlation coefficient threshold values ($|r|$) were modeled similar to Butts-Wilmsmeyer et al., 2019 to indicate weak, moderate, and strong relationships. Coefficients were utilized to calculate PCA variables in PROC PRINCOMP in SAS Edition 9.4 (SAS Institute, November 2018) along with means and standard deviations for each variable. PCAs with eigenvalues greater than one (Kaiser, 1970) were kept for further analysis as they explained the majority of variability between the variables. Selected eigenvalues were used to interpret eigenvectors for transformed AUDPC, FDK, and DON data sets. LSMEANS for selected PCA values were calculated by PROC MEANS in SAS Edition 9.4 (SAS Institute, November 2018). PROC CLUSTER in SAS 9.4 using Ward's Minimum Variance Approach produced a hierarchical dendrogram to determine a measurement of similarity or dissimilarity among the representative isolates. This in turn allowed for isolates to be binned into four groups based on their level of dissimilarity.

Results and Discussion

Univariate Analysis

Spore Quantification Assay

After statistical analysis (Table 3.1), the two-factor interaction of origin by level was significant ($p = 0.0033$). Of the single factors, origin was significant ($p < 0.001$), and level was significant ($p < 0.001$). Further study into slice statements can be found in Table 3.2. Viewing the analysis with origin being the main focus, isolates that originated from Carmi continuously produced

more spores than any other origin irrespective of the level of host resistance. Spore averages could be quantified between one hundred million and ten billion total spores. Isolates that originated from Brownstown produced the least number of spores across resistance levels especially in the moderately susceptible level with averages between ten and slightly over one hundred thousand total spores. Isolates that originated from Savoy produced more spores in the moderately resistant levels and less in the susceptible levels (Figure 3.3).

Given that the Carmi origin produced the most spores, it is assumed that isolates from this origin point would have a greater tendency to cause infection. For instance, the more spores a pathogen can produce, the more capabilities there are to cause infection. The opposite is depicted for isolates that originated from Brownstown since they consistently produced less spores. Isolates collected from the moderately resistant levels appeared to produce more spores than other resistance levels. This may be due to the higher the plant's resistance level is, the harder a pathogen must work to cause infection or the more specialized the pathogen must be in order to cause infection. By that standard, the trend dictates that isolates that originate from southern Illinois areas are higher spore producers and may have a higher capability of causing infection.

Field Assays

The goal of conducting field trials was to adequately define factors of aggressiveness given the environments and the amount of variability that wheat farmers experience. Environments are not equivalent from year-to-year as seen in this study. For instance, SRWW planted in Urbana, Illinois usually starts heading (Feekes 10.1) around the first week of May (May 3rd – 5th) and typically lasts for ten – twelve days until post-anthesis. In 2017, a mild winter was followed by a

cool spring and warm, wet weather. Urbana wheat started to head April 26th (Julian date 116) and ended May 9th (Julian date 129). In 2018, a late snow occurred in March followed by cool lingering temperatures that lead into hot and dry weather. Urbana wheat started heading May 12th (Julian date 132) and ended May 21st (Julian date 141). Wheat was roughly one to two weeks early in 2017 whereas in 2018, wheat was roughly ten days late for heading.

The field aggressiveness assay in 2017 for AUDPC values depicted lack of significance for the three-factor interaction between cultivar, origin, and resistance level ($p = 0.1578$) (Table 3.3). Of the two-factor interactions, cultivar by origin was non-significant ($p = 0.5655$), cultivar by level was non-significant ($p = 0.9813$), and origin by level was determined to be significant given the ANOVA analysis ($p < 0.001$). Of the single factors, cultivar was non-significant ($p = 0.1409$), level was significant ($p = 0.0140$), and level was significant ($p = 0.0020$). Further study into slice statements can be found in Table 3.4. ANOVA FDK values depicted lack of significance for the three-factor interaction between cultivar, origin, and level ($p = 0.6799$) (Table 3.3). Of the two-factor interactions, cultivar by origin was non-significant ($p = 0.6547$), cultivar by level was non-significant ($p = 0.2689$), and origin by level was determined to be significant given the ANOVA analysis ($p < 0.001$). Of the single factors, cultivar was non-significant ($p = 0.3803$), origin was significant ($p = 0.0033$), and level was significant ($p = 0.0001$). Further study into slice statements can be found in Table 3.4.

In 2017, isolates caused relatively low levels of infection and thus, had low FDK levels. A combined graph of AUDPC values and their corresponding FDKs (Figure 3.4) shows that the Brownstown moderately resistant isolate (BMR) and the Carmi moderately resistant isolate

(CMR) produced less infection and low FDKs as opposed to the other isolates which performed similar to one another.

The field aggressiveness assay in 2018 for AUDPC values depicted lack of significance for the three-factor interaction between cultivar, origin, and level ($p = 0.1529$) (Table 3.5). Of the two-factor interactions, cultivar by origin was significant ($p = 0.0171$), cultivar by level was significant ($p = 0.0511$), and origin by level was determined to be significant given the ANOVA analysis ($p < 0.001$). Of the single factors, cultivar was significant ($p = 0.0172$), origin was significant ($p = 0.0011$), and level was significant ($p < 0.001$). Further analysis from slice statements, (Table 3.6) suggested all factor levels provided high variance except for the highly susceptible level ($p = 0.4675$). ANOVA FDK values depicted lack of significance for the three-factor interaction between cultivar, origin, and level ($p = 0.1018$) (Table 3.5). Of the two-factor interactions, cultivar by origin was non-significant ($p = 0.1157$), cultivar by level was non-significant ($p = 0.5066$), and origin by level was determined to be significant given the ANOVA analysis ($p < 0.001$). Of the single factors, cultivar was significant ($p = 0.0501$), origin was significant ($p < 0.001$), and level was significant ($p < 0.001$). Further analysis from slice statements, (Table 3.6) suggested all factor levels provided high variance except for the highly susceptible level ($p = 0.7502$) and isolates that originated from Savoy ($p = 0.0733$).

In 2018, isolates caused relatively higher levels of infection and thus, higher FDK levels. A combined graph of AUDPC values and their corresponding FDKs (Figure 3.5) suggests statistically significant differences. The highly susceptible level generally had higher AUDPC and FDK values as opposed to the trend seen in the moderately susceptible and moderately

resistant levels. Carmi isolates appeared to produce high AUDPC and FDK values for the moderately susceptible and moderately resistant levels despite causing massive infection in the highly susceptible level.

Throughout both assays, AUDPC and FDKs responded similarly since diseased heads translate into damaged seeds. When AUDPC was high, FDKs followed suit and equivalent for low values. Given the weather differences between environments, AUDPC and FDK values were higher in 2018. Across both years, aggressiveness fluctuated given the level. For instance, resistance levels responded similarly in the moderately susceptible level and had slight rank changes in the highly susceptible and moderately resistant levels. It is to be expected that the more resistant a wheat cultivar is, the more specialized an isolate must be in order to cause infection. As described in the spore quantification assay, isolates that originated from Brownstown produced the least number of spores in comparison to other locations yet was highly aggressive in both AUDPC and FDKs in the highly susceptible and moderately resistant levels. Currently, this observation dispels the theory that the more spores a pathogen produces, the more aggressive it can be. Despite Carmi isolates producing the most spores, aggressiveness fluctuated given the level indicating that spore production does equate to high DS and FDKs. Given that isolates were collected in Savoy, a neighboring village to Champaign-Urbana, and utilized in Urbana field plots, the environment section of the disease triangle was similar. This equal environment suggests that isolates from a given area are specialized for aggressiveness and can be seen in the AUDPC and the corresponding FDK values from both years.

Greenhouse Assay

The greenhouse aggressiveness assay for AUDPC values suggested lack of significance for the three-factor interaction between cultivar, origin, and level ($p = 0.0593$) (Table 3.7). Of the two-factor interactions, cultivar by origin was non-significant ($p = 0.2123$), cultivar by level was non-significant ($p = 0.2422$), and origin by level was determined to be significant given the ANOVA analysis ($p < 0.001$). Of the single factors, cultivar was significant ($p = 0.0288$), origin was significant ($p = 0.0339$), and level was significant ($p < 0.001$). Further analysis from slice statements, (Table 3.8) suggested all factors provided high levels of variance except for the highly susceptible level ($p = 0.5242$) and isolates that originated from Savoy ($p = 0.1221$).

ANOVA FDK values suggested lack of significance for the three-factor interaction between cultivar, origin, and level ($p = 0.1207$) (Table 3.7). Of the two-factor interactions, cultivar by origin was non-significant ($p = 0.4217$), cultivar by level was non-significant ($p = 0.1933$), and origin by level was determined to be significant given the ANOVA analysis ($p = 0.0055$). Of the single factors, cultivar was significant ($p = 0.0346$), origin was non-significant ($p = 0.8268$), and level was non-significant ($p = 0.0631$). Analysis from slice statements, (Table 3.8) suggested most factors provided high levels of variance except for the highly susceptible level ($p = 0.2138$), the moderately resistant level ($p = 0.1343$), and isolates that originated from Savoy ($p = 0.1226$).

Isolates caused high levels of infection and thus, high FDK levels. A combined graph of AUDPC values and their corresponding FDKs (Figure 3.6) suggests statistically significant differences among origins and levels. The highly susceptible level generally had higher AUDPC and FDK

values as opposed to the trend seen in the moderately susceptible and moderately resistant levels. The moderately resistant level produced the lowest AUDPC and FDK values, denoting little infection occurred under lower FDKs. Origin-wise, isolates collected from Carmi and Savoy tended to be more aggressive.

AUDPC and FDKs were correlated with one another since diseased heads lead to damaged seeds. When AUDPC was high, FDKs followed suit and equivalent for low values with the exception of the isolate collected from a highly susceptible wheat line from Carmi. Given greenhouse conditions, AUDPC and FDK values were higher than those reported in the field assays, with AUDPC values as high as 11 and near 50% FDKs. Isolates from the moderately level tended to have lower levels of aggressiveness, suggesting that isolates from this level have a lack of specialization for infection. Isolates within the moderately susceptible level responded with low levels of aggressiveness except for isolates that originated from Carmi. Isolate aggressiveness was higher for AUDPC and FDKs in the greenhouse assay, but trends were similar to field assays with minor differences.

Mycotoxin Assay

DON extracted from threshed seed in the 2017 field aggressiveness assay suggested lack of significance for the three-factor interaction between cultivar, origin, and level ($p = 0.8643$) (Table 3.3). Of the two-factor interactions, cultivar by origin was non-significant ($p = 0.7890$), cultivar by level was non-significant ($p = 0.4509$), and origin by level was determined to be significant given the ANOVA analysis ($p < 0.001$). Of the single factors, cultivar was non-significant ($p = 0.3733$), origin was non-significant ($p = 0.3267$), and level was significant

($p = 0.0200$). Further analysis from slice statements, (Table 3.4) suggested all factors provided high levels of variance except for the highly susceptible level ($p = 0.1884$) and isolates that originated from Savoy ($p = 0.5121$). After mycotoxin extraction, isolates produced a range of DON levels. Isolates within the highly susceptible level tended to produce more DON than the other levels (Figure 3.7). Isolates that originated from Brownstown tended to produce more DON than other areas in respect to resistance level.

DON values for seed threshed from the 2018 field aggressiveness assay suggested lack of significance for the three-factor interaction between cultivar, origin, and level ($p = 0.9522$) (Table 3.5). Of the two-factor interactions, cultivar by origin was significant ($p = 0.0054$), cultivar by level was non-significant ($p = 0.2312$), and origin by level was determined to be significant given the ANOVA analysis ($p < 0.001$). Of the single factors, cultivar was significant ($p = 0.0256$), origin was significant ($p < 0.001$), and level was non-significant ($p < 0.001$). Analysis from slice statements, (Table 3.6) suggested all factors provided high levels of variance except for the highly susceptible level ($p = 0.1330$). Isolates produced a range of DON levels similar to the prior year's assay. Isolates within the highly susceptible resistance level tended to produce more DON than the other levels (Figure 3.8). Isolates that originated from Brownstown tended to produce more DON than isolates from other areas in respect to resistance levels. Isolates from Carmi and Savoy produced lower levels of DON in the moderately susceptible and moderately resistant levels.

DON extracted from threshed seed in the greenhouse aggressiveness assay suggested lack of significance for the three-factor interaction between cultivar, origin, and level ($p = 0.7123$)

(Table 3.7). Of the two-factor interactions, cultivar by origin was non-significant ($p = 0.4251$), cultivar by level was non-significant ($p = 0.4882$), and origin by level was determined to be significant given the ANOVA analysis ($p < 0.001$). Of the single factors, cultivar was significant ($p = 0.0132$), origin was non-significant ($p < 0.001$), and level was significant ($p < 0.001$). Further analysis from slice statements, (Table 3.8) suggested all factors provided high levels of variance except for the highly susceptible level ($p = 0.7075$) and isolates that originated from Savoy ($p = 0.4046$).

After mycotoxin extraction, isolates produced a range of DON levels that exceeded prior assays with DON levels reaching near 70ppm. Isolates within the highly susceptible level tended to produce more DON across all origins than the other levels (Figure 3.9). Highly and moderately susceptible isolates that originated from Carmi produced more DON than those collected from the moderately resistant level. Highly susceptible and moderately resistant isolates from Brownstown produced more DON than those collected from the moderately susceptible level which produced close to negligible amounts of DON. Savoy isolates consistently produced DON across levels but tended to produce less DON the more resistant the wheat line was.

Throughout all assays, AUDPC, FDKs, and DON modeled one another. This is to be expected since host infection causes FDKs, and one outcome of such damage is DON contaminated seed. Across all assays, FDKs and DON followed similar trends. DON analysis shows that isolates collected from the highly susceptible level produced higher DON levels, suggesting that wheat resistance level plays a large role in determining this measure of aggressiveness. While isolates in the moderately resistant level depict the opposite, isolates that originated from Brownstown

showed high DON irrespective of wheat's level of resistance. Current FDA standards dictate permissible amounts of DON for human and animal consumption through grain, grain byproduct, and finished wheat products. Throughout the field and greenhouse assays, including DON quantifications, isolate origin and level of host resistance both played a large role in dictating aggressiveness. When isolates collected from the surrounding areas were used to inoculate fields near the isolate's origin, most aggressiveness factors per isolate rose, including AUDPC, FDKs, and DON. Along with origin, aggressiveness levels based upon host resistance level changed from highly susceptible to moderately resistant. The hypothesis that a high number of spores equated to high levels of aggressiveness is dispelled as seen from isolates which mass produced spores yet lacked in aggressiveness. Overall, these findings could be of great addition to the arsenal that phytopathologists currently use to help develop cultivars with resistance to *F. graminearum*.

Multivariate Analysis

Pearson correlation coefficients denoted strong positive correlation between all three quantitative variables (Table 3.9). This is to be expected since progression of the pathogen causes diseased wheat spikelets with damaged kernels that translate into mycotoxin filled grain as seen in prior assays. Means and standard deviations for the transformed aggressiveness data can be seen in Table 3.10. Utilizing the PROC PRINCOMP output, PCA_1 accounted for 87.65% of the combined variability between the coefficients whereas PCA_2 explained 8.33% and PCA_3 described 4.02% of the combined variability (Table 3.11). Since only PCA_1 had an eigenvalue greater than one, it was utilized for the remainder of the analysis (Figure 3.10). Focusing on the PCA_1 vector loading, all variables held positive correlations (Table 3.12). Cluster analysis using

Ward's Minimum Variance Approach produced four clusters based on PCA_1 (Figure 3.11). Utilizing all prior assays and their findings, a quantifiable definition of cumulative aggressiveness was produced.

$$AGR = \frac{PCA1_{AUDPC_T} * \left(AUDPC_T - \mu_{AUDPC_T}\right)}{\sigma_{AUDPC_T}} + \frac{PCA1_{FDK_T} * \left(FDK_T - \mu_{FDK_T}\right)}{\sigma_{FDK_T}} + \frac{PCA1_{DON_T} + \left(DON_T - \mu_{DON_T}\right)}{\sigma_{DON_T}}$$

where AGR is aggressiveness defined by the additive effects between the respective PCA_1 variable, an individual value from a given transformed data set (T_AUDPC, T_FDK, and T_DON), the mean of the transformed data set, and the standard deviation of the transformed data set. Using the parameters of this research, the following equation can be used to quantify aggressiveness through the collective means of AUDPC utilizing DS, FDKs, and DON.

$$AGR = \frac{0.590793 * (AUDPC_T - 0.41258)}{0.51898} + \frac{0.5645 * (FDK_T + 0.76925)}{0.502221} + \frac{0.576 + (DON_T - 0.86011)}{0.79299}$$

High AGR values denote an isolate is highly aggressive to its host where low AGR values signify low quantitative aggressiveness. Utilizing this comprehensive quantitative measure of aggressiveness allows for a standard definition of aggressiveness across multiple disease measures that has yet to be applied to phytopathology and plant breeding.

Conclusion

Results from the spore quantification assay gave inclination as to which isolates could cause higher levels of aggressiveness. For instance, the more spores a pathogen can produce, the more capabilities there are to cause infection. The field and greenhouse assays suggested that the number of spores played little role in determining pathogen aggressiveness and is a mere factor of pathogen biology. An example can be seen as isolates that originated from Brownstown consistently produced less spores yet was highly aggressive for AUDPC, FDKs, and DON content in field and greenhouse assays. Currently, this observation dispels the theory that the more spores a pathogen produces, the more aggressive it can be. For this reason, spore production can indicate potential aggressiveness but should not be the only trait utilized to determine pathogen aggressiveness. Throughout all assays, the aggressiveness traits (AUDPC, FDKs, and DON) tended to model one another. This is to be expected since host infection causes damaged kernels, and one outcome of such damage is DON contaminated seed. Given the environmental differences between field experiments, traits were higher in 2018 indicating that pathogen infection increases the further anthesis is delayed. After multivariate analysis, aggressiveness traits (AUDPC, FDKs, and DON) were highly positively correlated and should be utilized when trying to determine potential aggressiveness. Results consistently displayed that isolates from the highly susceptible level outperformed other resistance levels in pathogen aggressiveness. This concept was reiterated through a hierarchical tree diagram that visualized the similarity and dissimilarity between the four clusters of isolates (highly aggressive, moderately aggressive, and two non-aggressive bins). This comprehensive quantitative measure of aggressiveness can be utilized to determine a standard definition of aggressiveness across multiple disease measures.

Tables and Figures

Table 3.1: ANOVA for spore quantification assay. If $p \leq 0.05$, factor is significant.

Type 3 Analysis of Variance				
Source	**DF**	**Error DF**	**F Value**	**P Value**
Rep	2	16	1.81	0.1949
Origin	2	16	59.84	<.0001
Level	2	16	22.38	<.0001
Origin*Level	4	16	6.19	0.0033
Error	16	.	.	.

Table 3.2: Slice statements for spore quantification assay between origin of isolate and resistance level. If $p \leq 0.05$, factor level is significant.

Tests of Effect Slices						
Effect	**Origin**	**Level**	**Num DF**	**Den DF**	**F Value**	**P Value**
Origin*Level	**Brownstown**		2	16	10.17	0.0014
Origin*Level	**Carmi**		2	16	1.18	0.3322
Origin*Level	**Savoy**		2	16	23.40	<.0001
Origin*Level		**Highly Susceptible**	2	16	16.33	0.0001
Origin*Level		**Moderately Resistant**	2	16	22.99	<.0001
Origin*Level		**Moderately Susceptible**	2	16	32.89	<.0001

Table 3.3: ANOVA for 2017 field aggressiveness assay across AUDPC, FDKs, and DON values. If $p \leq 0.05$, factor is significant.

Type 3 Analysis of Variance												
	AUDPC				FDK				DON			
Source	**DF**	**Error DF**	**F Value**	**P Value**	**DF**	**Error DF**	**F Value**	**P Value**	**DF**	**Error DF**	**F Value**	**P Value**
Cultivar	1	2.0539	5.47	0.1409	1	2.0247	1.24	0.3803	1	2.0088	1.29	0.3733
Rep	2	2	1.14	0.4677	2	2	0.92	0.5216	2	2	1.37	0.4218
Cultivar*Rep	2	70	2.53	0.0873	2	70	5.49	0.0061	2	32	0.90	0.4167
Origin	2	70	4.54	0.0140	2	70	6.20	0.0033	2	32	1.16	0.3267
Level	2	70	6.80	0.0020	2	70	10.48	0.0001	2	32	4.43	0.0200
Cultivar* Origin	2	70	0.57	0.5655	2	70	0.43	0.6547	2	32	0.24	0.7890
Cultivar* Level	2	70	0.02	0.9813	2	70	1.34	0.2689	2	32	0.82	0.4509
Origin * Level	4	70	11.88	<.0001	4	70	19.98	<.0001	4	32	8.94	<.0001
Cultivar* Origin * Level	4	70	1.71	0.1578	4	70	0.58	0.6799	4	32	0.32	0.8643
Error	70	.	.	.	70	.	.	.	32	.	.	.

Table 3.4: Slice statements for 2017 field aggressiveness assay across AUDPC, FDKs, and DON values between origin of isolate and resistance level. If $p \leq 0.05$, factor level is significant.

Tests of Effect Slices								
			AUDPC		FDK		DON	
Effect	**Origin**	**Level**	**F Value**	**P Value**	**F Value**	**P Value**	**F Value**	**P Value**
Origin*Level	**Brownstown**		27.80	<.0001	31.72	<.0001	14.07	<.0001
Origin*Level	**Carmi**		3.44	0.0374	17.89	<.0001	6.90	0.0032
Origin*Level	**Savoy**		0.01	0.9946	0.02	0.9804	0.68	0.5121
Origin*Level		**Highly Susceptible**	0.26	0.7704	0.74	0.4815	1.76	0.1884
Origin*Level		**Moderately Resistant**	4.61	0.0132	21.07	<.0001	7.72	0.0018
Origin*Level		**Moderately Susceptible**	22.10	<.0001	31.72	<.0001	9.20	0.0007

Table 3.5: ANOVA for 2018 field aggressiveness assay across AUDPC, FDKs, and DON values. If $p \leq 0.05$, factor is significant.

Type 3 Analysis of Variance												
	AUDPC				FDK				DON			
Source	**DF**	**Error DF**	**F Value**	**P Value**	**DF**	**Error DF**	**F Value**	**P Value**	**DF**	**Error DF**	**F Value**	**P Value**
Cultivar	1	4.0004	15.40	0.0172	1	4.0012	7.69	0.0501	1	4	12.03	0.0256
Rep	4	4	3.98	0.1048	4	4	7.26	0.0405	4	4	3.53	0.1247
Cultivar*Rep	4	152	3.39	0.0109	4	152	1.19	0.3186	4	64	1.92	0.1184
Origin	2	152	7.10	0.0011	2	152	22.83	<.0001	2	64	275.53	<.0001
Level	2	152	25.90	<.0001	2	152	30.86	<.0001	2	64	132.31	<.0001
Cultivar* Origin	2	152	4.18	0.0171	2	152	2.19	0.1157	2	64	5.66	0.0054
Cultivar* Level	2	152	3.03	0.0511	2	152	0.68	0.5066	2	64	1.50	0.2312
Origin * Level	4	152	13.70	<.0001	4	152	11.63	<.0001	4	64	78.73	<.0001
Cultivar* Origin * Level	4	152	1.70	0.1529	4	152	1.97	0.1018	4	64	0.17	0.9522
Error	152	.	.	.	152	.	.	.	64	.	.	.

Table 3.6: Slice statements for 2018 field aggressiveness assay across AUDPC, FDKs, and DON values between origin of isolate and resistance level. If $p \leq 0.05$, factor level is significant.

Tests of Effect Slices								
			AUDPC		FDK		DON	
Effect	**Origin**	**Level**	**F Value**	**P Value**	**F Value**	**P Value**	**F Value**	**P Value**
Origin*Level	**Brownstown**		21.13	<.0001	9.71	0.0001	14.44	<.0001
Origin*Level	**Carmi**		26.23	<.0001	41.75	<.0001	266.35	<.0001
Origin*Level	**Savoy**		5.44	0.0052	2.66	0.0733	8.99	0.0004
Origin*Level		**Highly Susceptible**	0.76	0.4675	0.29	0.7502	2.08	0.1330
Origin*Level		**Moderately Resistant**	20.82	<.0001	17.36	<.0001	232.59	<.0001
Origin*Level		**Moderately Susceptible**	12.83	<.0001	28.38	<.0001	198.32	<.0001

Table 3.7: ANOVA for greenhouse aggressiveness assay across AUDPC, FDKs, and DON values. If $p \leq 0.05$, factor is significant.

Type 3 Analysis of Variance												
	AUDPC				FDK				DON			
Source	**DF**	**Error DF**	**F Value**	**P Value**	**DF**	**Error DF**	**F Value**	**P Value**	**DF**	**Error DF**	**F Value**	**P Value**
Cultivar	1	3.0033	15.64	0.0288	1	3.0095	13.53	0.0346	1	3	27.97	0.0132
Rep	3	3	0.93	0.5216	3	3	1.04	0.4881	3	3	0.43	0.7484
Cultivar*Rep	3	47	1.47	0.2354	3	47	0.52	0.6721	3	48	0.42	0.7391
Origin	2	47	3.64	0.0339	2	47	0.19	0.8268	2	48	19.26	<.0001
Level	2	47	13.02	<.0001	2	47	2.93	0.0631	2	48	30.88	<.0001
Cultivar* Origin	2	47	1.60	0.2123	2	47	0.88	0.4217	2	48	0.87	0.4251
Cultivar* Level	2	47	1.46	0.2422	2	47	1.70	0.1933	2	48	0.73	0.4882
Origin * Level	4	47	16.09	<.0001	4	47	4.19	0.0055	4	48	63.50	<.0001
Cultivar* Origin * Level	4	47	2.45	0.0593	4	47	1.93	0.1207	4	48	0.53	0.7123
Error	47	.	.	.	47	.	.	.	48	.	.	.

Table 3.8: Slice statements for greenhouse aggressiveness assay across AUDPC, FDKs, and DON values between origin of isolate and resistance level. If $p \leq 0.05$, factor level is significant.

Tests of Effect Slices								
			AUDPC		FDK		DON	
Effect	**Origin**	**Level**	**F Value**	**P Value**	**F Value**	**P Value**	**F Value**	**P Value**
Origin*Level	**Brownstown**		23.97	<.0001	5.37	0.0079	54.08	<.0001
Origin*Level	**Carmi**		17.29	<.0001	3.41	0.0414	102.88	<.0001
Origin*Level	**Savoy**		2.20	0.1221	2.20	0.1226	0.92	0.4046
Origin*Level		**Highly Susceptible**	0.66	0.5242	1.59	0.2138	0.35	0.7075
Origin*Level		**Moderately Resistant**	8.01	0.0010	2.09	0.1343	92.81	<.0001
Origin*Level		**Moderately Susceptible**	26.99	<.0001	4.66	0.0142	53.11	<.0001

Table 3.9: Pearson correlation coefficients between transformed AUDPC (T_AUDPC), transformed FDK (T_FDK), and transformed DON (T_DON). Coefficient values of $0.7 \leq |r| \leq 1.0$ signified a strong interaction relationship.

Pearson Correlation Coefficients Prob |r| under H_O: Rho = 0 Number of Observations			
	T_AUDPC	**T_FDK**	**T_DON**
T_AUDPC		0.84508 < 0.0001 54	0.86752 < 0.0001 48
T_FDK			0.75595 < 0.0001 48
T_DON			

Table 3.10: Mean and standard deviation calculations for transformed AUDPC (T_AUDPC), transformed FDK (T_FDK), and transformed DON (T_DON).

Simple Statistics			
	T_AUDPC	**T_FDK**	**T_DON**
Mean	0.41258	-0.76925	0.86011
Standard Deviation	0.51898	0.50221	0.79299

Table 3.11: Output eigenvalues denote PCA_1 accounts for 87.65% of the combined variability between coefficients.

Eigenvalues of the Correlation Matrix				
	Eigenvalue	**Difference**	**Proportion**	**Cumulative**
PCA_1	2.62950302	2.37954178	0.8765	0.8765
PCA_2	0.24996124	0.12942549	0.0833	0.9598
PCA_3	0.12053574	NA	0.0402	1.0000

Table 3.12: Vector loadings of each principal component. PCA_1 denotes positive correlations between all variables.

Eigenvectors			
	PCA_1	**PCA_2**	**PCA_3**
T_AUDPC	0.590793	-0.170036	-0.788702
T_FDK	0.564534	0.785509	0.253529
T_DON	0.576423	-0.595033	0.560064

Figure 3.1: Field inoculated wheat: (a-b) center spikelet inoculated with isolate, (c) freshly inoculated heads covered with shoot bags for 48hrs.

Figure 3.2: Greenhouse inoculated wheat: (a-b) center spikelet inoculated with isolate, (c) freshly inoculated heads were placed in humidity chamber that sprayed free-floating water droplets at given time intervals.

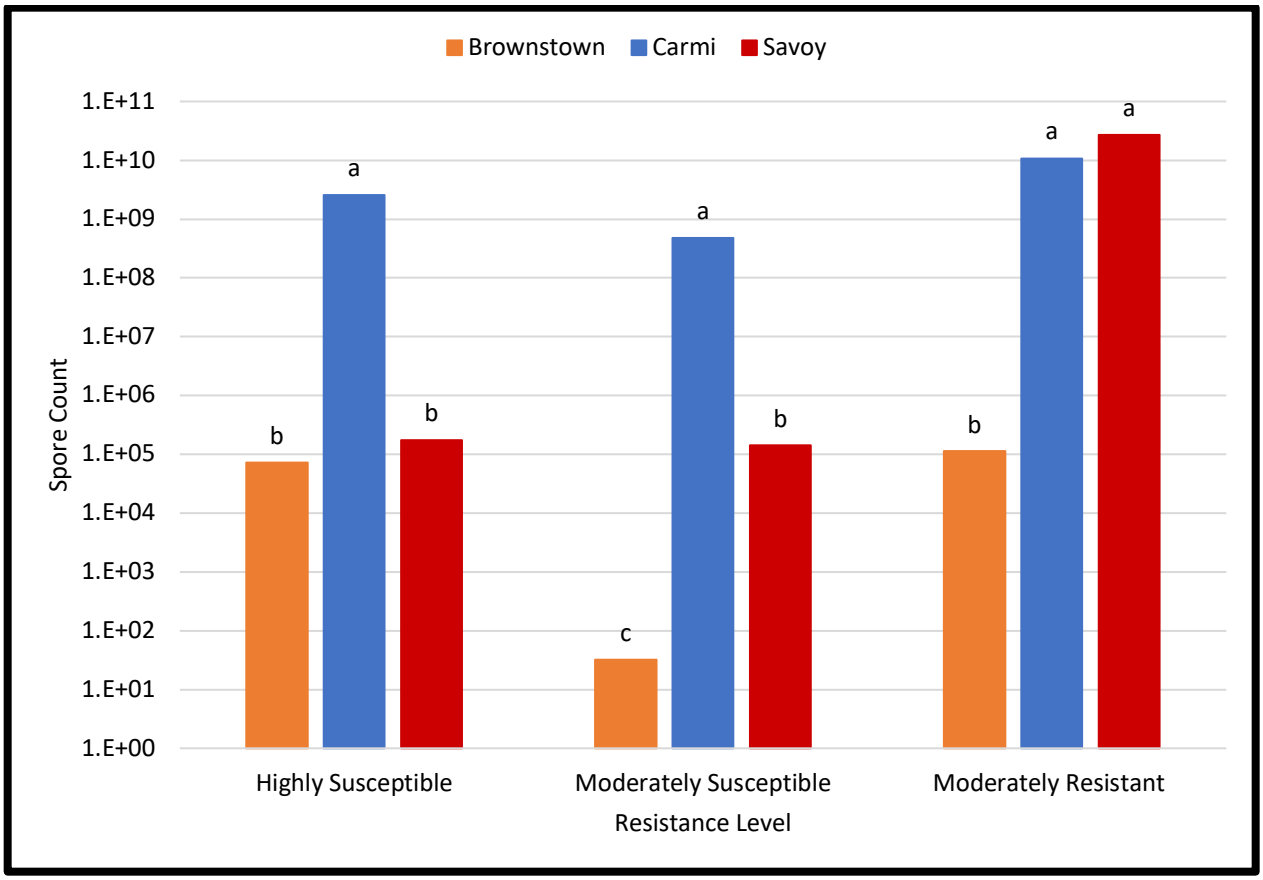


Figure 3.3: Back-transformed spore quantification assay for representative isolates denoted by origin of collection and resistance level. Different letters from a – c denote significant differences at $p \leq 0.05$.

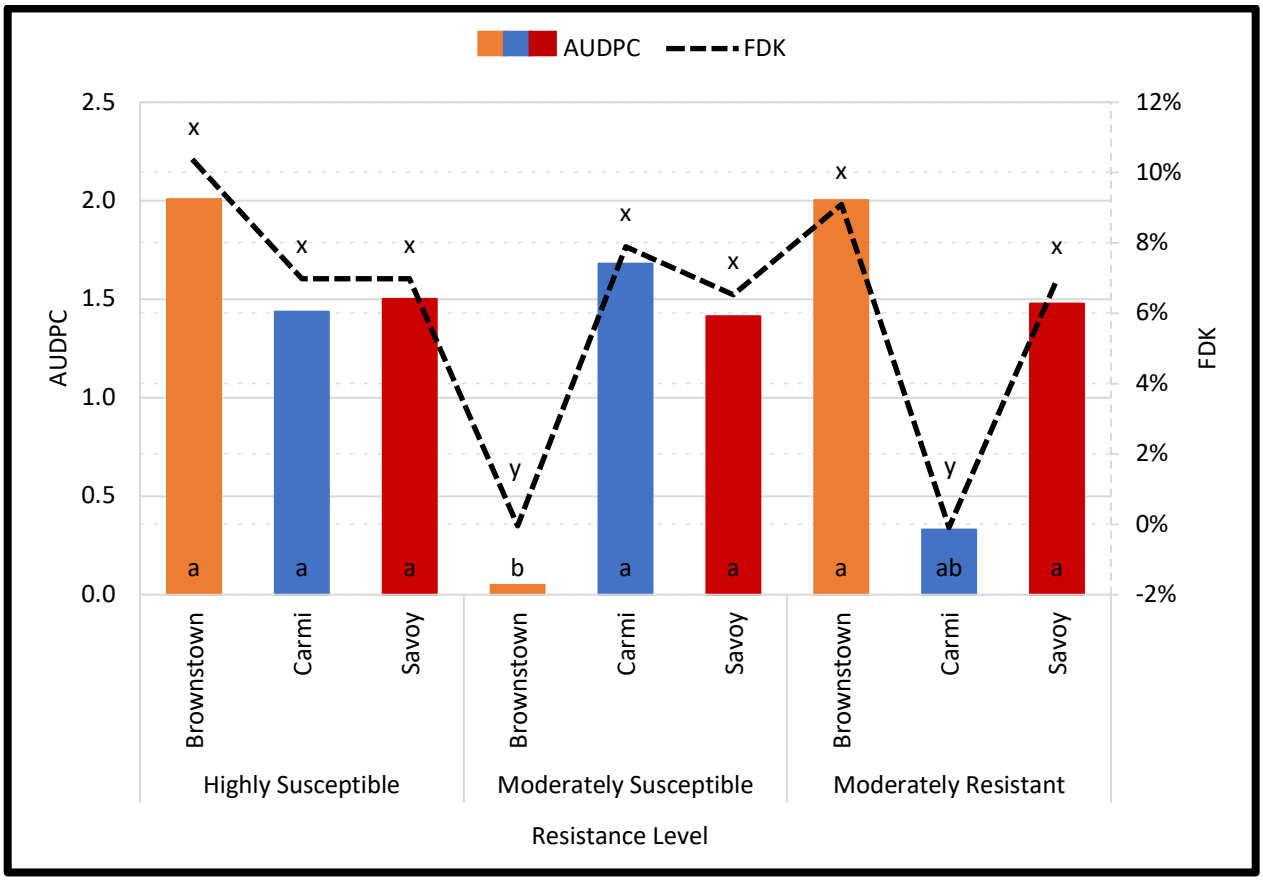


Figure 3.4: Back-transformed 2017 field aggressiveness assay for representative isolates denoted by origin of collection and resistance level. Bar graph describes AUDPC where different letters from a – b denote significant differences within AUDPC at $p \leq 0.05$. Line graph describes FDKs where different letters from x – y denote significant differences within FDKs at $p \leq 0.05$.

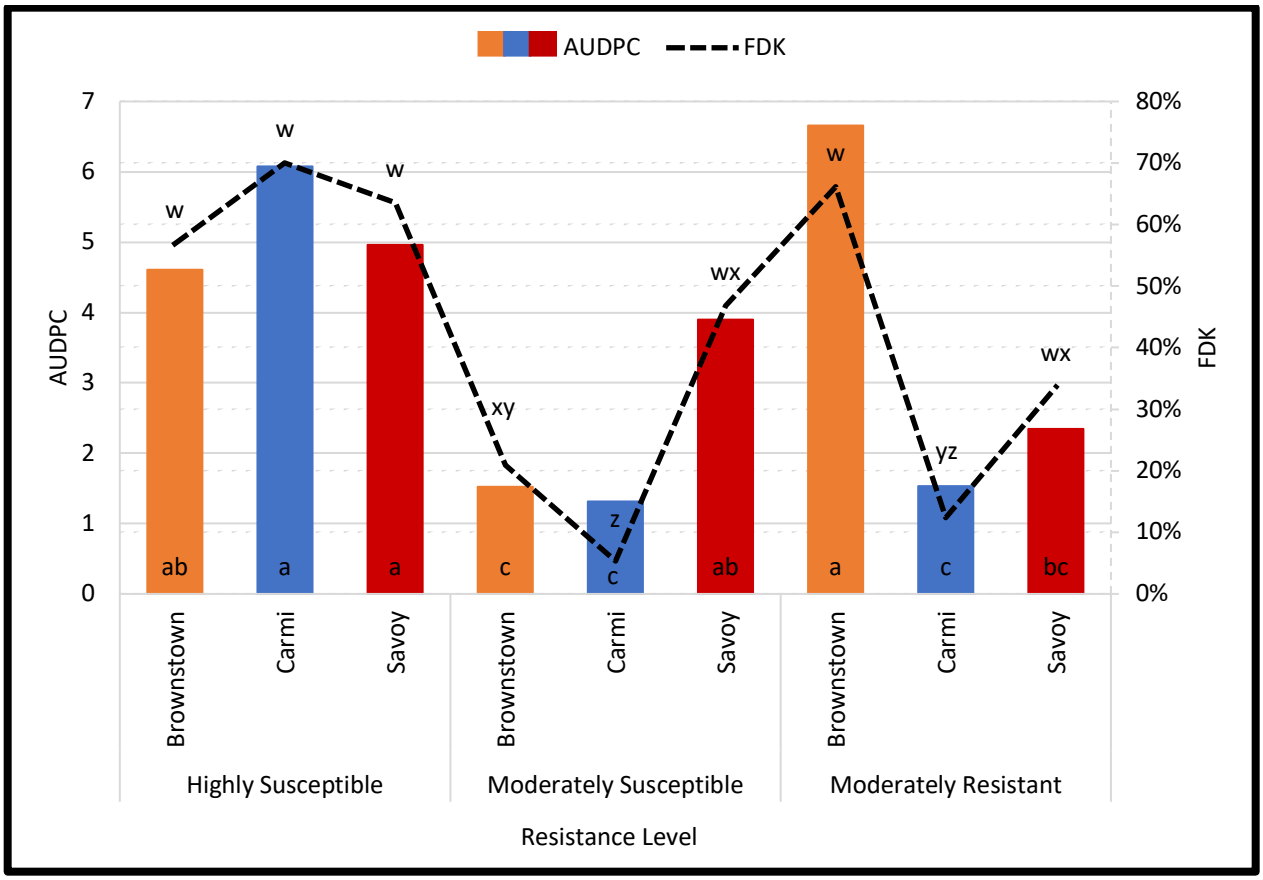


Figure 3.5: Back-transformed 2018 field aggressiveness assay for representative isolates denoted by origin of collection and resistance level. Bar graph describes AUDPC where different letters from a – c denote significant differences within AUDPC at $p \leq 0.05$. Line graph describes FDKs where different letters from w – z denote significant differences within FDKs at $p \leq 0.05$.

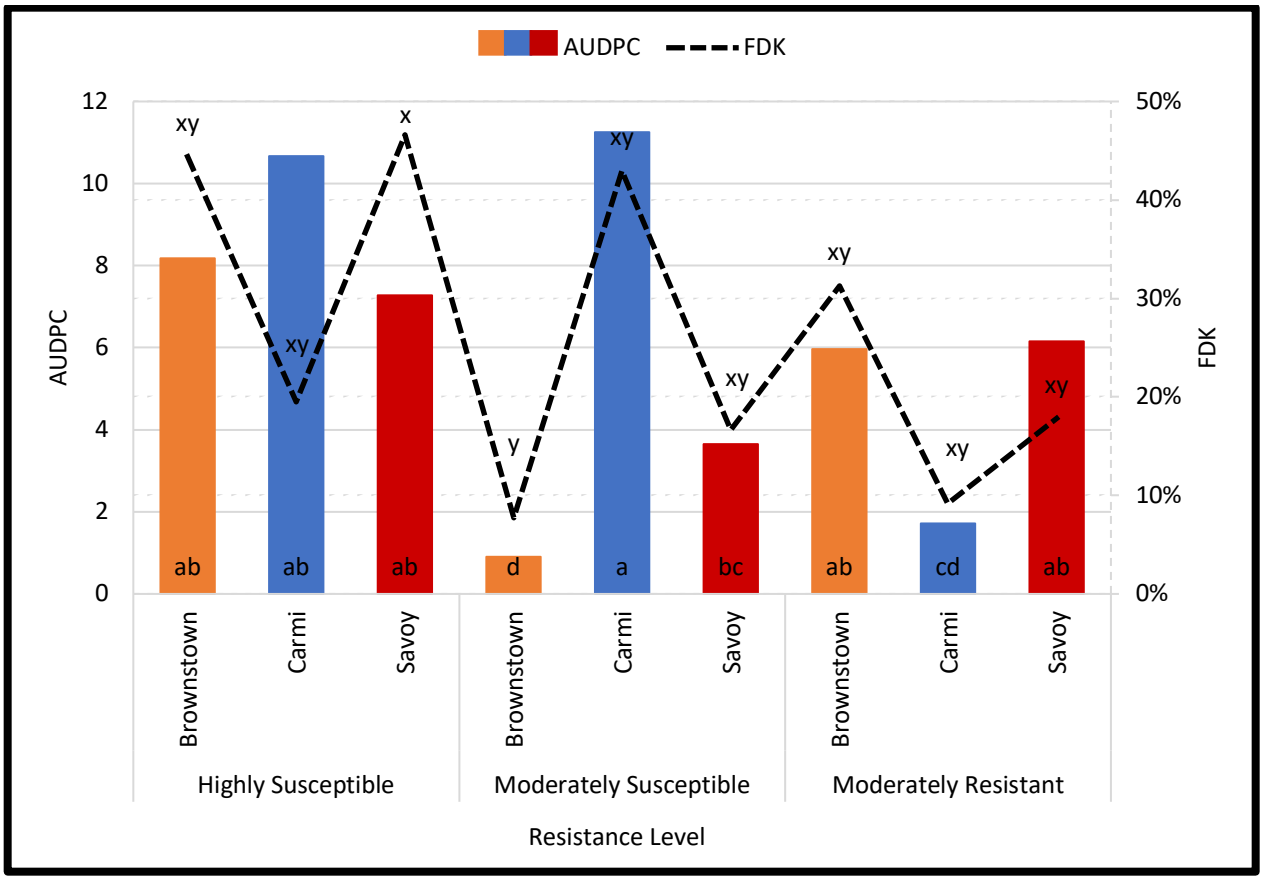


Figure 3.6: Back-transformed greenhouse aggressiveness assay for representative isolates denoted by origin of collection and resistance level. Bar graph describes AUDPC where different letters from a – d denote significant differences within AUDPC at $p \leq 0.05$. Line graph describes FDKs where different letters from x – y denote significant differences within FDKs at $p \leq 0.05$.

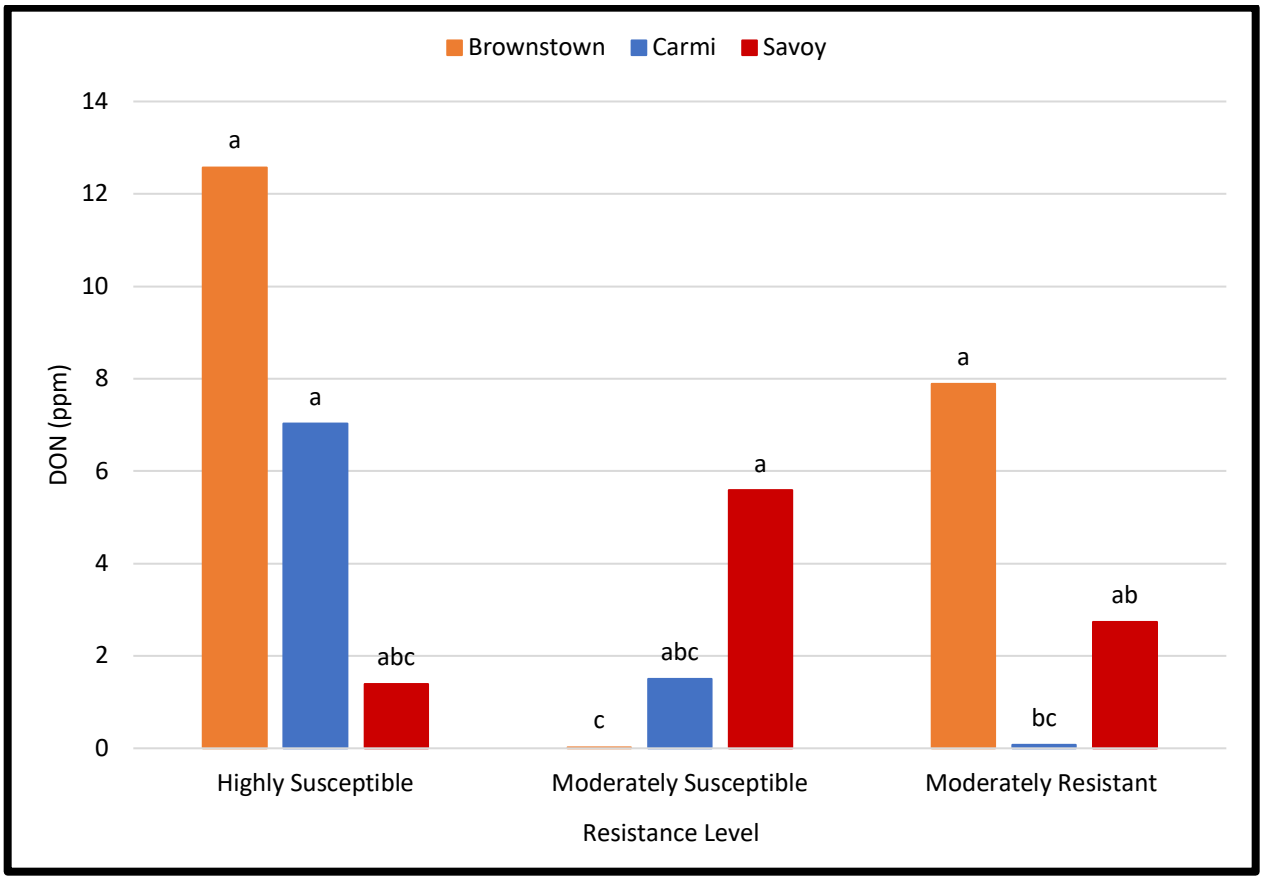


Figure 3.7: Back-transformed 2017 field aggressiveness assay for DON values with representative isolates denoted by origin of collection and resistance level. Different letters from a – c denote significant differences at $p \leq 0.05$.

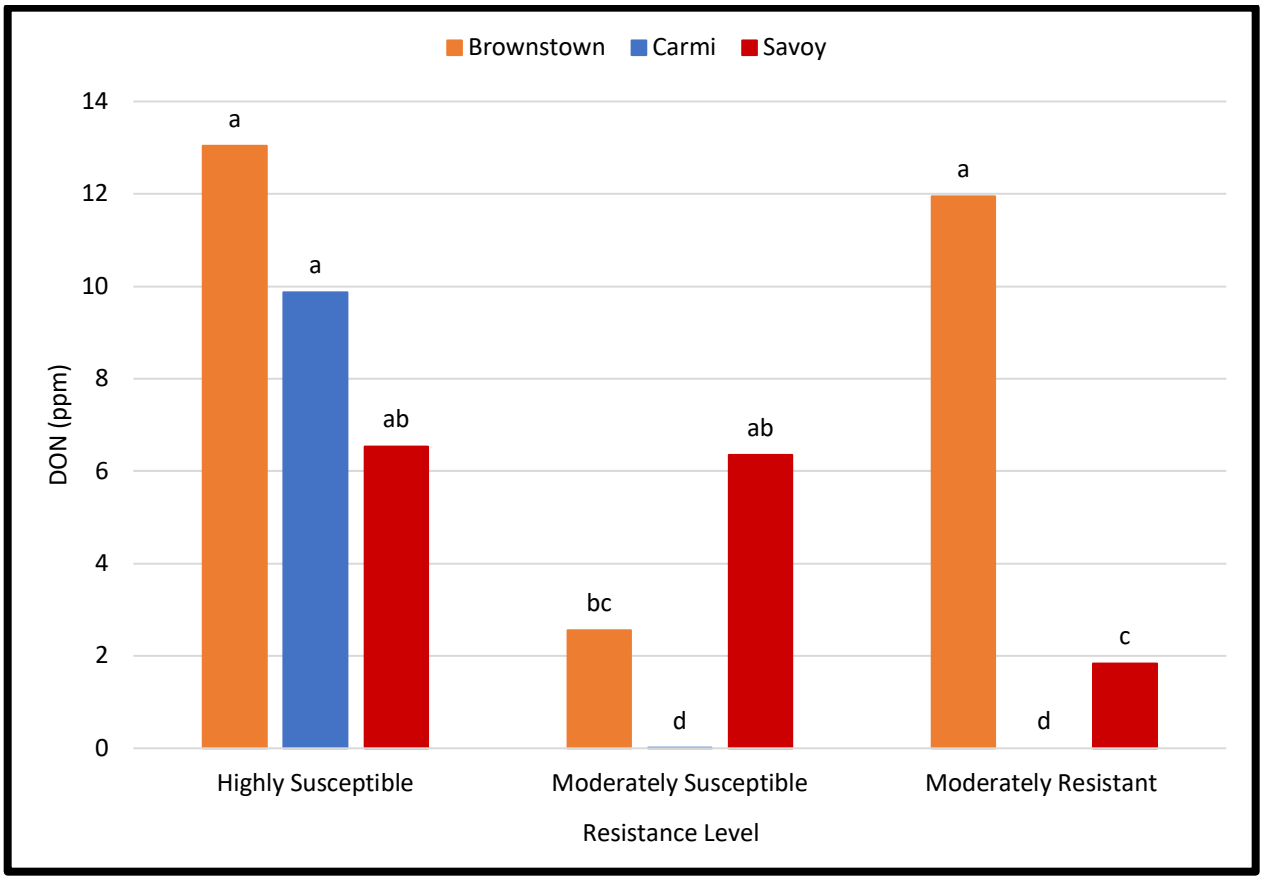


Figure 3.8: Back-transformed 2018 field aggressiveness assay for DON values with representative isolates denoted by origin of collection and resistance level. Different letters from a – d denote significant differences at $p \leq 0.05$.

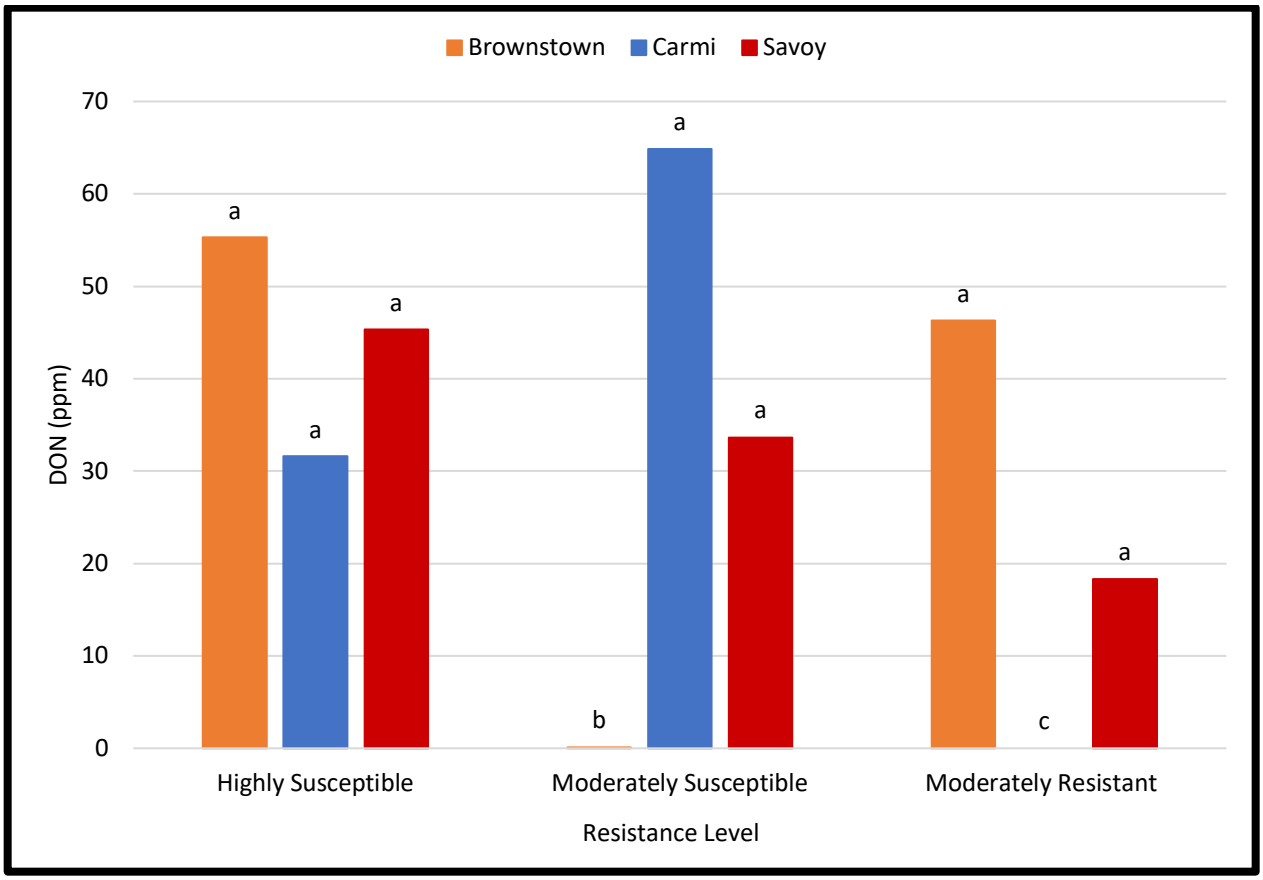


Figure 3.9: Back-transformed greenhouse aggressiveness assay for DON values with representative isolates denoted by origin of collection and resistance level. Different letters from a – c denote significant differences at $p \leq 0.05$.

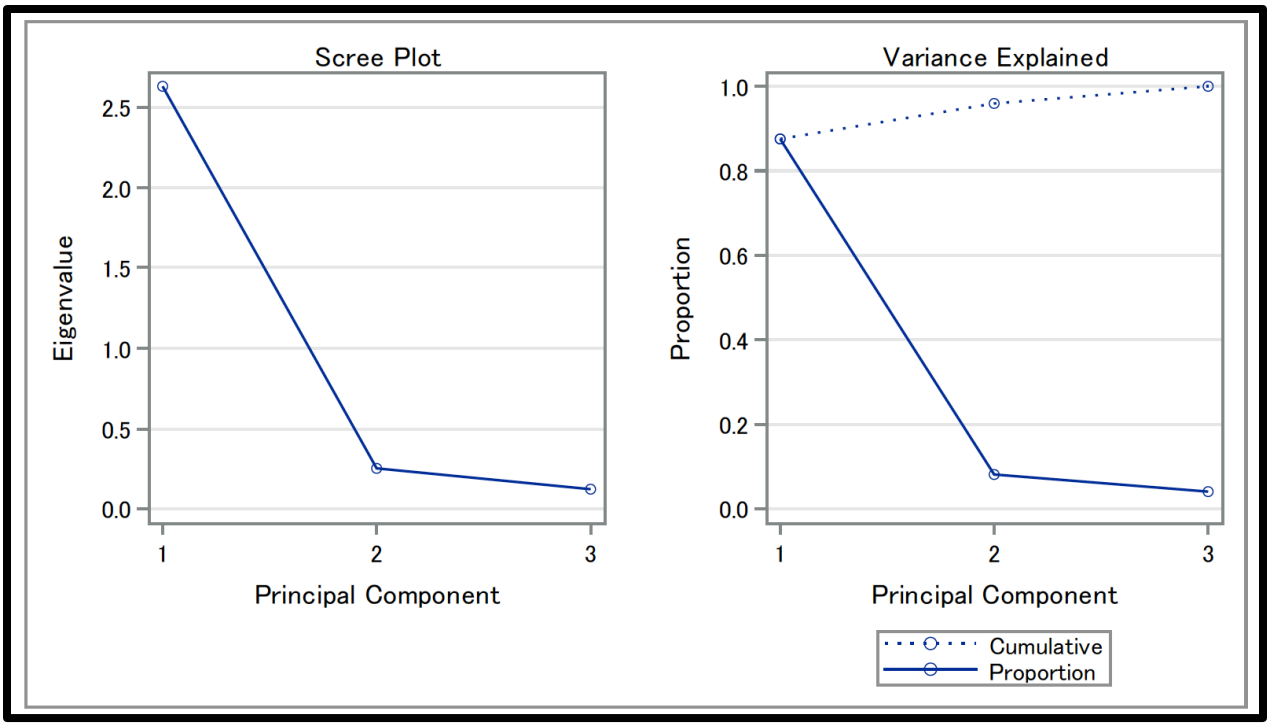


Figure 3.10: Output eigenvalues denote the proportion of variance explained per principal component. PCA_1 accounts for the highest amount of variability between the combined coefficients.

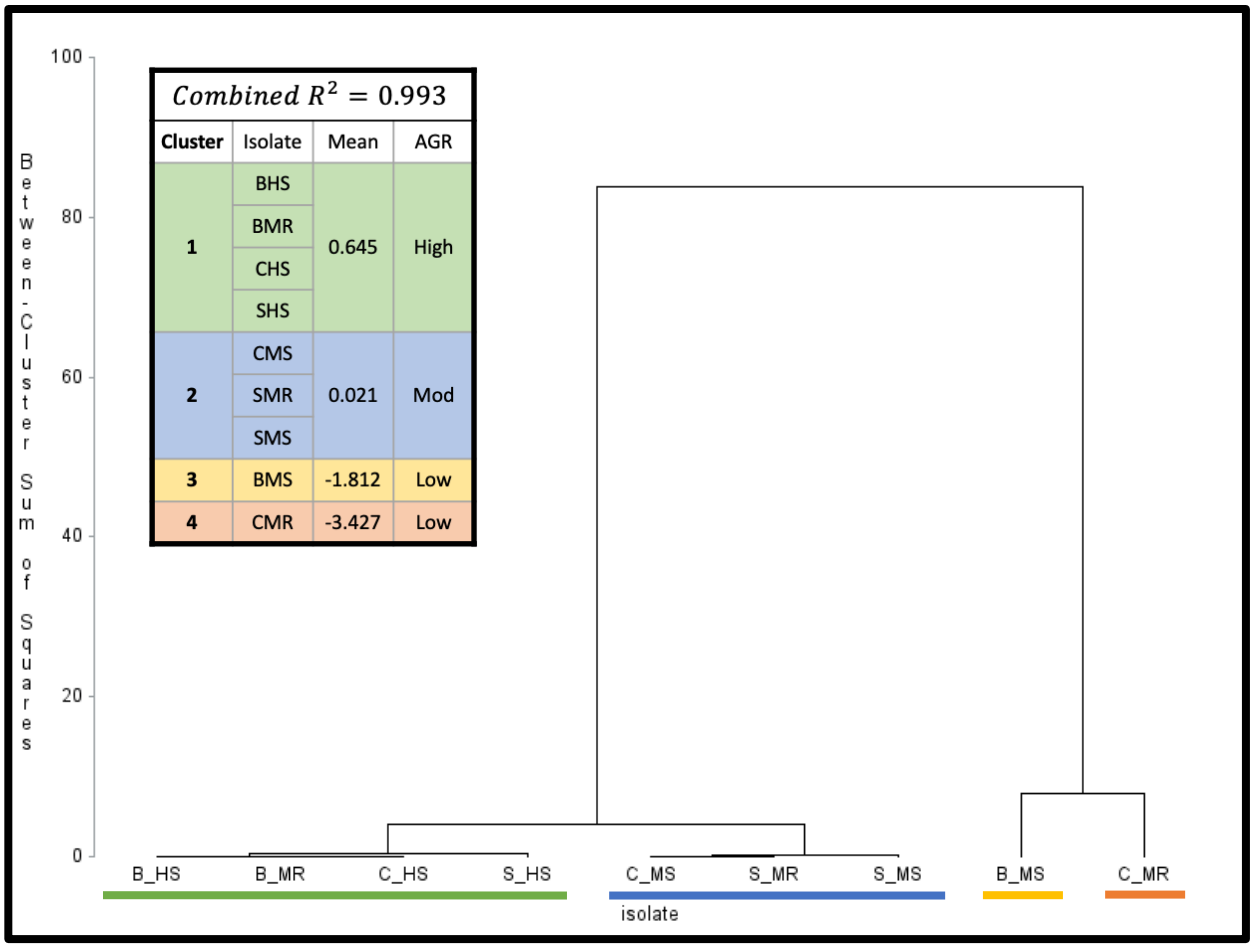


Figure 3.11: Dendrogram displays principal cluster analysis for representative isolates bifurcated into four bins based on the index value.

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APPENDIX A: TRIMMING FOR BLAST ANALYSIS

Representative isolate sequences were trimmed in the following manner: 1) all uncalled nucleotides (N) were removed after a 5N repeating sequence (***NNNNN***), 2: 160bps were trimmed from the 5' area, 3) 160bps were trimmed from the 3' area, and 4) all remaining bps were used for analysis (area marked in yellow).

Example of trimmed DNA sequence output for a given isolate.

5':GATGATANATCGGNGCGGATATGCAATAGCNGACCTNGGNGCTTNAGGCGCTCA
TNTNGGTCNCCTNAGNCTGCGGGGNCGGACTATTTTCTGATCTGCTGCGCGAANTTT
GNTTCCAATTNNCNCGACTNGTCTTGTCCTCCTTAANCATAGAGCGAACCATCGAGA
AGTTCGAGAAGGTTGGTCTCATTTTCCTCGATCGCGCGCCCTTTCCCTTTCGAAATAT
CATTCGAATCGCCCTCACACGACGACTCGATACGCGCCTGTTACCCCGCTCGAGGTC
AAAAATTTTGCGGCTTTGTCGTAATTTTTTTCCCGGTGGGGCTCATACCCCGCCACTC
GAGCGACAGGCGTCTGCCCTCTTCCCACAAACCATTCCCTGGGCGCTCATCATCACG
TGTCAACCAGTCACTAACCACCTGTCNATAGGAAGCCGCCGAGCTCGGTAAGGGTT
CCTTCNAGTACGCCTGGGTTCTTGACAAGCTCAAAGCCGAGCGTGAGCGTGGNATCN
CCATTGATATCGCCCTCTGGAAGTTCGAGACTCCNCGCTACNATGNCACCGNCNTTG
GNANGNNGNCNCCNCNGCNGNCNNCNNNTTCNCNNANNAANNNGG***NNNNN***NNANN
CNCCCGGNCNCCGNGNNTTCNNNNNNAANNNGANCNCTGGNNNCNNCNAANNNNN
NNNNNNNNNN:3'

APPENDIX B: R CODE FOR AUDPC STATISTICAL ANALYSIS

R code and packages used to determine AUDPC values from DS data points for field and greenhouse aggressiveness assays.

```
# install and load package to run AUDPC
install.packages("agricolae")
library(agricolae)

# rename input csv file
f17sev <- read.csv("field2017 DS.csv")

# manually change factors into numerical data
str(f17sev)

# manually change factors into numbers
f17sev$DS14 <- as.numeric(as.character(f17sev$DS14))
f17sev[1,9]

# disease severity measurements taken at 14, 21, & 28dpi
t0<-14
t1<-21
t2<-28

# dpi placed into a vector
time.period<-c(t0,t1,t2)

# place each row value into x
x<-(1:487)

# for all 3 DS values in x per row, calculate absolute AUDPC by dpi as a
number and print and insert back into sev(original csv)
for (val in x) {f17sev$AUDPC[val]<-print(audpc(as.numeric(f17sev[val,10:12]),
time.period)) }

# export file back to working directory
write.csv(f17sev, file = "field17_AUDPCvalues.csv")
```

APPENDIX C: SAS CODE FOR UNIVARIATE ANALYSIS

SAS code used to for univariate analysis of aggressiveness traits (AUDPC, FDKs, and DON) across field and greenhouse assays. Thank you to Dr. Carrie Butts-Wilmsmeyer for statistical guidance. An example is provided here for field 2017 AUDPC values.

```
data field2017;
infile "C:/Users/kolblab/Desktop/Melissa Salazar\Aggressiveness\2017 Field
Assay\field17_AUDPCvalues.csv" dlm="," firstobs=2;
input year exp$ rep origin$ level$ plot cultivar$ isolate$ head$ DS14 DS21
DS28 AUDPC FDK DON;

AUDPC_Tlog=log10(AUDPC+0.01);
FDK_Tlog=log10(FDK+0.01);
DON_Tlog=log10(DON+0.01);
run;

ods graphics on;

proc mixed data=field2017 method=type3;
class rep origin level cultivar;
model AUDPC_Tlog = cultivar origin level level*cultivar origin*cultivar
origin*level origin*level*cultivar / ddfm=kr outpred=AUDPC_Tresids;
random rep rep*cultivar;
lsmeans origin*level / slice=origin slice=level
run;

proc unnivariate data=AUDPC_Tresids normal plot; var resid; run;

proc glm data=AUDPC_Tresids;
class rep origin level cultivar;
model resid = cultivar origin level level*cultivar origin*cultivar
origin*level origin*level*cultivar;
means origin*level / hovtest=bf;
run;

proc glimmix data=field2017;
class rep origin level cultivar;
model AUDPC_Tlog = cultivar origin level level*cultivar origin*cultivar
origin*level origin*group*cultivar;
random rep rep*cultivar;
lsmeans origin*level / lines adjust=tukey;
run;

ods graphics off;
```

APPENDIX D: SAS CODE FOR MULTIVARIATE ANALYSIS

SAS code used to for multivariate analysis of aggressiveness traits (AUDPC, FDKs, and DON) across field and greenhouse assays. Thank you to Dr. Carrie Butts-Wilmsmeyer for statistical guidance.

```
data don;
infile "C:/Users/cjbutts2/Documents/All Don Values.csv" dlm="," firstobs=2;
length Exp$5 Cultivar$20 Loc$5 Group$5 Isolate$15 Plot$30;
input Exp$ Year Rep Cultivar$ Origin$ Level$ Isolate$ AUDPC T_AUDPC FDK T_FDK
Plot$ DON T_DON;
run;

proc corr data=don;
var t_audpc t_fdk t_don;
run;

proc princomp data=don out=scores;
var t_audpc t_fdk t_don;
run;

proc sort data=don;
by exp year cultivar origin level;
run;

proc means data=don noprint;
var t_audpc t_fdk t_don;
by exp year cultivar origin level;
output out=agrmeans;
run;

data agrmeans;
set agrmeans;
if _STAT_~="MEAN" then delete;
run;

proc corr data=agrmeans;
var t_audpc t_fdk t_don;
run;

proc princomp data=agrmeans out=scores;
var t_audpc t_fdk t_don;
run;

symbol1 v=dot c=blue;
symbol2 v=dot c=red;
symbol3 v=dot c=green;
symbol4 v=dot c=magenta;
symbol5 v=dot c=orange;
symbol6 v=dot c=cyan;
symbol7 v=dot c=gold;
symbol8 v=dot c=black;
symbol9 v=dot c=purple;
```

```
proc gplot data=scores;
plot prin1*level=origin;
plot prin1*origin= level;
run;

data scores;
set scores;
isolate=catx("_",origin,level);
run;

proc print data=scores (obs=10);
run;

proc sort data=scores;
by isolate;
run;

proc means data=scores noprint;
var prin1 t_audpc t_fdk t_don;
by isolate;
output out=pcameans;
run;

data pcameans;
set pcameans;
if _STAT_~= "MEAN" then delete;
run;

proc cluster data=pcameans method=ward simple noeigen nonorm rmsstd rsquare
out=clust;
var prin1;
id isolate;
run;

proc tree data=clust nclusters=4 out=shorttree;
id isolate;
run;

proc sort data=clust;
by isolate;
run;

proc sort data=shorttree;
by isolate;
run;

data merged_cluster;
merge clust shorttree;
by isolate;
run;

data merged_cluster;
set merged_cluster;
if isolate="" then delete;
run;
```

```
proc sort data=pcameans;
by isolate;
run;

proc sort data=merged_cluster;
by isolate;
run;

data merged_cluster;
merge merged_cluster pcameans;
by isolate;
run;

proc print data=merged_cluster;
run;

proc sort data=merged_cluster;
by cluster;
run;

proc print data=merged_cluster;
var isolate cluster;
run;

proc means data=merged_cluster;
by cluster;
var prin1;
run;

/*proc cluster data=pcameans method=ward simple noeigen nonorm rmsstd rsquare
out=clust;
var t_audpc t_fdk t_don;
id isolate;
run;*/

proc print data=pcameans;
run;
```