Project FY22-PB-003: Role of Chemotype in Aggressiveness and Toxigenicity of Fusarium graminearum to Wheat

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1. **What are the major goals and objectives of the research project?**
   The goal of our project is to test the hypothesis that the *F. graminearum* 3ADON chemotype confers higher levels of aggressiveness, toxicity, and competitiveness than the 15ADON chemotype, regardless of genetic background. The objectives are: **1)** Determine whether trichothecene toxin chemotype, mating type locus, and other genetic makers exhibit Mendelian segregation among progeny from crosses of strains from different *F. graminearum* populations; **2)** Determine whether individuals and mixtures of progeny from outcrosses differ in aggressiveness, toxicity, and competitiveness in susceptible and moderately resistant wheat in the presence and absence of fungicides; and **3)** Identify DNA markers associated with aggressiveness and high toxin production by analyzing whole genome sequence data from pools of progeny that differ in these traits.

2. **What was accomplished under these goals or objectives?** *(For each major goal/objective, address these three items below.)*

   a. For **Objective 1,** our goal was to test the hypothesis that chemotype, mating type, and other genetic makers will exhibit Mendelian segregation among progeny from crosses of strains from different *F. graminearum* populations (NA1, NA2, and NA3). The mapping populations we expected to generate as part of this objective would be stored as a permanent collection and made available to the community on request.
      i. **What were the major activities?**
         1. We obtained a collection of 37 North American *F. graminearum* strains from wheat, representing four different chemotypes (15ADON, 3ADON, NIV, NX2)**.**
         2. The chemotype of each strain from the U.S. was confirmed by PCR and/or by sequencing, and each was phenotyped for growth and sporulation *in vitro*, sexual fertility, and aggressiveness and toxicity on the susceptible spring wheat variety Wheaton to aid in selection of the most suitable parents for our crosses.
         3. One strain each of the 3ADON, NIV, and NX2 chemotypes was crossed with our 15ADON Δ*mat1-1-1* tester that was derived from PH-1.
         4. Approximately 230 progeny were collected from 20 perithecia (10-12 per perithecium) from each of three crosses (3ADON x 15ADON, NIV x 15ADON, and NX2 x 15ADON). A subset of 80-90 progeny from each cross was genotyped by PCR, and segregation patterns of the chemotype (TOX) and mating type (MAT) markers were evaluated**.**
      ii. **What were the significant results?**
         1. The strains varied markedly in the traits analyzed. **We selected the crossing parents for this objective based on their growth *in vitro* and their aggressiveness to Wheaton**. Strains that grew abnormally or sectored frequently, produced few conidia, exhibited low levels of fertility in crosses with the tester, or had very low levels of aggressiveness, were removed from consideration as parents in the crosses.
         2. Three strains (3ADON strain NRRL 46434; NIV strain NC016; and NX2 strain NRRL 66040) were chosen as the most suitable parents for the crosses with the 15ADON tester. The genome of each parent strain was sequenced to determine which population it belonged to. NRRL 46434 (3ADON) belonged to NA2, and NRRL 66040 (NX2) was NA3. NC016 (NIV) appeared to be a hybrid of NA1 and NA2. The 15ADON tester belonged to the NA1 population.

3. Each parent strain was evaluated for aggressiveness and toxicity on the susceptible spring wheat variety Wheaton. The 3ADON parent produced significantly higher levels of toxin than the NIV, NX2, or 15ADON parent strains. It was also more aggressive than the NIV and 15ADON strains, but similar to the NX2 parent.
4. Progeny from the 3ADON and NIV crosses produced expected Mendelian segregation patterns of the TOX and MAT markers (25% of each parental and recombinant type). **In contrast** the cross involving the NX2 strain showed an anomalous 3:1 segregation pattern (15ADON-NX2) for the TOX marker. The MAT marker segregated as expected (1:1).
5. The marker segregation patterns for individual perithecia revealed that between one and three in each cross (out of 20 total) had resulted from selfing by the WT parent. Progeny from selfed perithecia were not included in the final mapping populations.

iii. List key outcomes or other achievements.

1. Segregating progeny (~200) have been collected from each cross, single-spored, verified, and stored in a permanent collection.
2. **This objective is now completed.** The mapping populations and associated data can be viewed here: and will be made available to the community on request with appropriate permits.

b. For **Objective 2** our goal was to determine whether individuals and mixtures of progeny from the outcross of the 15ADON/MAT- tester strain and the 3ADON/MAT+ parent (NRRL 46434**)** differ in aggressiveness, toxicity, and competitiveness in susceptible and moderately resistant wheat in the presence and absence of fungicides. Recall that the 3ADON strain was more aggressive and produced more toxin than the 15ADON tester strain.

i. What were the major activities?

1. To evaluate aggressiveness and toxicity: 67 progeny from our 3ADON/MAT+ vs 15ADON/MAT- cross were selected at random for individual pathogenicity assays on susceptible Wheaton and moderately resistant Alsen wheat varieties. Wheat heads were collected, ground, and sent to Virginia Tech for mycotoxin analysis. Three experiments, including 5 replications for each progeny strain plus parental strains in a RCB design, were completed for Wheaton, and two experiments were completed for Alsen.
2. To evaluate competitiveness: 70 progeny from the 3ADON/MAT+ vs 15ADON/MAT- cross (the 67 above plus 3 more) were randomly assorted into seven pools, each containing 10 strains (Five 3ADON and five 15ADON) in equal amounts. The pools were inoculated onto Wheaton. Three experiments, including 5 replications of each pool plus parents and controls in an RCB design, were completed in the presence or absence of fungicide (Tubuconazole). Disease severity was evaluated, and wheat heads were collected and ground. Part of the ground material was sent for mycotoxin analysis, while the rest was used to isolate single-spored colonies (24 per sample). A random subset of these (~8 per sample) were chemotyped by PCR.

ii. What were the significant results?

1. There was significantly less disease overall on the Alsen variety versus the Wheaton variety, as expected. The heritability of disease severity on Wheaton was 0.458, suggesting a substantial genetic contribution. The Alsen variety did not perform as well in our greenhouse assays since the negative controls developed physiological symptoms similar to bleaching caused by FHB late in the experiment.
2. For both wheat varieties, there was **no significant difference** in the aggressiveness of the 3ADON versus 15ADON progeny, whether measured as disease severity at individual time points, rate of disease progress, or Area Under the Disease Progress

Curve (AUDPC). There was no significant association of chemotype with high versus low aggressiveness. There was also no significant effect of the MAT deletion.

3. The mycotoxin analysis revealed the mycotoxin profiles matched the PCR chemotype of the progeny with only one exception on both Wheaton and Alsen. Toxin levels were positively correlated with disease severity on both wheat varieties. There was **no significant difference** in the amount of toxin produced by the 3ADON versus 15ADON progeny in either wheat variety.
4. Nine progeny that were consistently more aggressive and toxigenic than the average (aka. "high" progeny), and 5 that were consistently less aggressive and toxigenic (aka. "low" progeny), were identified and confirmed in two additional greenhouse experiments on Wheaton with 15 replications per treatment. This confirmed the phenotypes of these high and low aggressive strains and verified that there was **no relationship** between the chemotype and high or low aggressiveness or toxicity.
5. For the competition studies: disease severity data are available for all three experiments, and mycotoxin and PCR data are complete for the first two experiments.
    a) Disease severity and mycotoxin levels were reduced in the presence of fungicide, as expected. The 3ADON parent strain was more tolerant of the fungicide than the 15ADON tester strain.
    b) Pools were less aggressive on average than the parent or individual progeny strains, indicating the presence of antagonism.
    c) In the two experiments that were analyzed, 3ADON and 15ADON generally co-occurred at similar levels in wheat heads that were inoculated with the pools, either in the presence or absence of fungicide. Furthermore, PCR results did not show consistent dominance of either chemotype overall, although within individual pools there were often significant differences in the numbers of 3ADON versus 15ADON strains that were recovered.

iii. List key outcomes or other achievements.

1. The completed part of this objective allows us to reject part of our original hypothesis, that the 3ADON chemotype is **directly** responsible for the higher level of aggressiveness and toxicity of the NRRL 46434 3ADON strain. Our data suggest instead that there are multiple other factors segregating in the cross that contribute to aggressiveness and toxicity. Extrapolating this result to the rest of the 3ADON population, we could conclude that monitoring chemotype alone may not provide sufficient information for risk assessments.
2. Although results on competitiveness are still incomplete, so far they suggest that other factors (genetic, environmental, or both) are more important than chemotype in determining dominance during co-inoculations.
3. In this objective we have identified segregating progeny that are highly aggressive, highly toxic, or highly competitive in the presence versus absence of fungicides. In future it may be possible to use them to identify genetic factors that play important roles in these traits, and associate those with markers allowing them to be tracked in the population as potential risk factors.

c. For **Objective 3**, our goal was to conduct genome-wide SNP (single nucleotide polymorphism) analysis of bulked progeny pools from the cross of the 3ADON and 15ADON chemotype. The aim was to identify new genetic markers associated with aggressiveness, competitiveness, and toxigenicity.

i. What were the major activities?

1. Genome sequence assemblies for the selected parental strains were generated in the Proctor laboratory at the USDA-ARS in Peoria. For genome sequencing, strains were first grown on a growth medium amended with antibiotics to remove any

potential bacterial contamination. Genomic DNA was isolated from liquid cultures using the Qiagen DNeasy Plant Mini Kit. Resulting DNA was then subjected to a 16S PCR screen to confirm the absence of bacterial DNA, and then used to prepare libraries for sequencing with an Illumina MiSeq instrument. Resulting sequence reads were processed with the computer program CLC Genomics Workbench. Adapter and low-quality sequences were removed from sequence reads, and then reads were screened against 73 bacterial genome sequences to remove low levels of bacterial sequence reads resulting from contaminated reagents and/or equipment. The unmapped sequences were then used to generate an assembled genome sequence using CLC Genomics Workbench.

2. The genomes of the parental strains were aligned with each other and with a set of *F. graminearum* genome assemblies from strains representing the three major North American populations (NA1, NA2, NA3). SNPs were identified with "iSNPcaller" (https://github.com/drdna/iSNPcaller).
3. The high-low strains from the 3ADON vs 15ADON cross (see above) were sent to the Proctor laboratory for sequencing. Although we had originally planned to sequence bulked pools of the high and low progeny, a new method became available in the Proctor lab that allowed us to sequence the genomes of the strains individually.

ii. What were the significant results?

1. Genome assemblies for the parent strains ranged from 33 to 36.7 Mb in size and included 393-593 contigs. The number of SNPs differentiating the parent strains from the 15ADON *mat1-1-1* deletion strain ranged from 120,517 to 166,083. There were 144,116 SNPs that distinguished the 3ADON NRRL 46434 and 15ADON parents.
2. 14 progeny from the cross of 3ADON and the 15ADON tester were sequenced, and segregation of 1568 representative SNP markers that were evenly dispersed across all four chromosomes was evaluated. The vast majority (81%) of the SNP markers showed the expected 1:1 segregation (**Figure 1**).

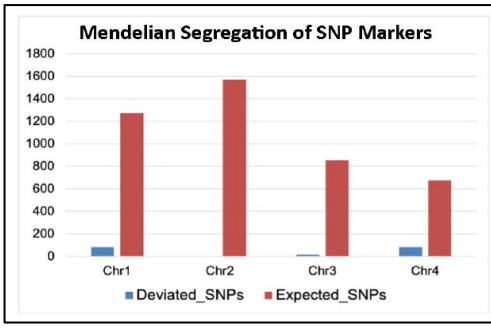

Figure 1: Number of SNP markers that conformed (red bars) or deviated (blue bars) from expected 1:1 Mendelian segregation patterns among 14 progeny of the 3ADONx15ADON cross.

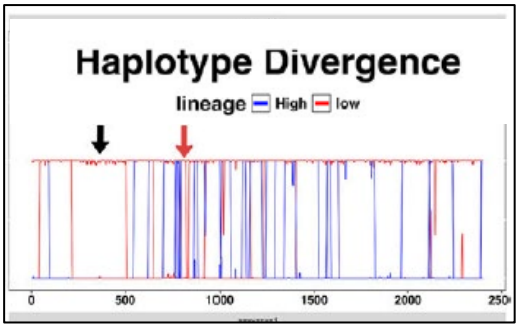

Figure 2: Haplotype divergence plot for chromosome 2, including high (blue) and low (red) progeny. The black arrow indicates a recombination cold spot; the red arrow is a recombination hot spot.

3. Haplotype divergence plots were generated by comparing haplotypes of the progeny to the 15ADON parent strain across sliding windows along each of the four chromosomes. In these plots, the x-axis shows the window position along the sequence while the y-axis shows divergence values (ranging from 0 to 0.5). Divergence values near 0.0 indicate regions where the progeny matched the 15ADON parent, suggesting inheritance from this parent. Divergence values of 0.5 indicate regions inherited from the alternate parent (the 3ADON strain). All the

progeny are plotted on the same image to compare patterns of recombination. Recombination hot spots were visualized on these plots as regions where there were many switches between 0.0 to 0.5 (**Figure 2**, red arrow). Recombination cold spots were seen as regions with very few switches (**Figure 2**, black arrow). All four chromosomes had frequent recombination hot and cold spots: the cold spots reveal blocks of genes that will tend to co-segregate more frequently.

4. Haplotype plots revealed one region of chromosome 2 that appeared to be associated with aggressiveness (**Figure 3**). All the high aggressive progeny (red) inherited this region from the more aggressive 3ADON parent, while all the low aggressive progeny (blue) inherited it from the 15ADON parent. This region was not linked to the TOX (chemotype) locus which is on the other arm of chromosome 2.

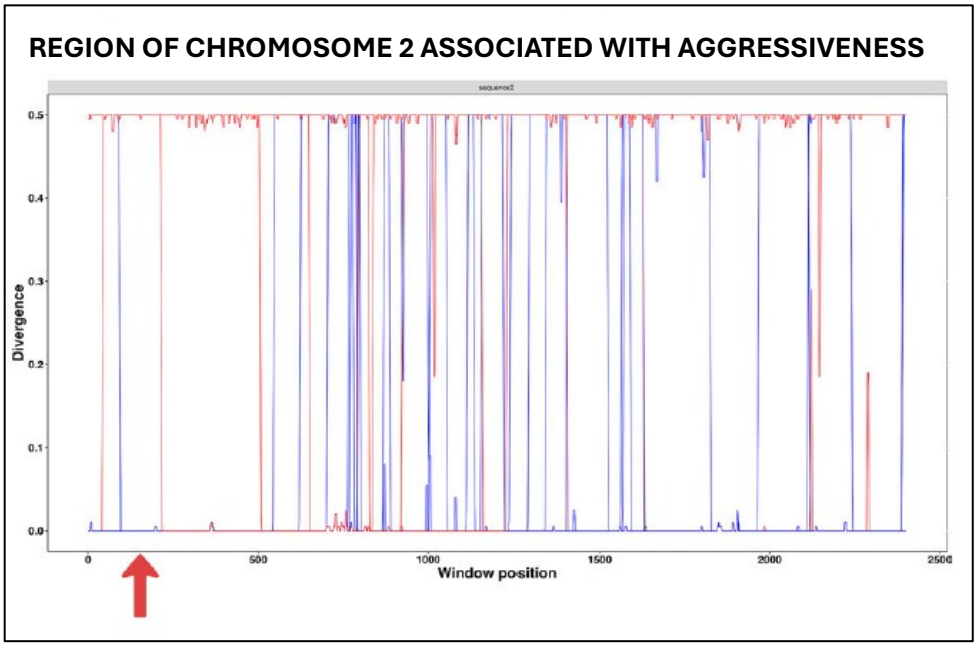

Figure 3: Haplotype divergence on chromosome 2 comparing high aggressive (red) and low aggressive (blue) progeny. The red arrow indicates a region that is associated with aggressiveness: high progeny have all inherited this region from the more aggressive 3ADON parent while low progeny have all inherited it from the less aggressive 15ADON parent.

iii. List key outcomes or other achievements.

1. Although the number of progeny we were able to analyze was limited due to cost of WGS, we have demonstrated the feasibility of this approach for association mapping and identified a novel region of chromosome 2 that may be associated with high aggressiveness/toxicity.
2. We have developed a representative set of SNPs that can be used for association mapping. Moreover, by comparing with additional genome sequences that are publicly available, we identified a core set of 500 of these SNPs that are flanked by conserved regions, amenable to PCR primer design. This would allow them to be screened simultaneously by amplification and sequencing in a multiplex approach, allowing very cost-effective genotyping of wild type and progeny strains. We intend to explore the feasibility of this genotyping tool in other on going work in our laboratory.

3. What opportunities for training and professional development has the project provided?

The M.S. student who began this project joined us in January 2023, with an excellent background in agronomy and plant breeding from her undergraduate studies. She made outstanding progress in her degree during the subsequent years, completing all her course requirements and mastering all the relevant molecular and pathological assays for our project, thus developing valuable new knowledge and skills in fungal genetics and phytopathology. She completed the Fusarium Laboratory Workshop in Manhattan Kansas which deepened her practical knowledge of the *Fusarium* genus and provided an opportunity for her to make new professional connections. She learned R and Linux programming so she could work with her genome data (Objective 3). After being recognized as the best M.S. student in our graduate program, she successfully defended her thesis last month (June 2025), having mastered a valuable combination of knowledge and skills relevant to crop improvement from both the host and the pathogen side. She will be moving on to Ph.D. studies with our colleague Dr. Nidhi Rawat at the University of Maryland, where she will continue working on the FHB pathosystem, but with a complementary focus on plant biotechnology and plant breeding. A new PhD student with an excellent background in agronomy (B.S.) and plant breeding (M.S.) will join us next month (August 2025) to complete the research objectives for this project and begin her PhD studies in plant pathology and fungal biology. She will be building on the work in this project, to continue to explore the genetic basis for pathogenicity, competitiveness, and toxicity in *F. graminearum* on wheat and alternate hosts across different environments.

4. How have the results been disseminated to communities of interest?

The student presented a research poster at the USWBSI forum in Austin in December 2024, and she also gave a talk on her research for the Plant Pathology Departmental Seminar in early June.

5. What do you plan to do during the next reporting period to accomplish the goals and objectives?

a. **The work for objective 1 is completed.**
b. **We have nearly finished work for objective 2.** During the next and final reporting period, we will complete this objective.
    i. We will repeat our analysis of the segregating progeny on at least one moderately resistant line, since Alsen didn't behave well in our greenhouse assays. We have already received seed of 10 candidate varieties from Dr. Jim Anderson. We will pre-screen these with our high-low progeny first and then, after choosing a suitable line, we will repeat our analyses of aggressiveness and toxicity with the 70 random progeny from the 3ADONx15ADON cross at least twice.
    ii. We will complete the work on the competition studies by finishing the PCR and mycotoxin evaluation of the third experiment. We will evaluate additional markers (using either telomere fingerprints or multiplex SNP analysis) to test whether there are highly competitive clones among these recovered strains. This will help us to understand the relative role of genetics versus environment in determining competitive outcomes.
    iii. We will complete and submit a publication describing the lack of association of 3ADON with aggressiveness and toxicity (this manuscript is currently in the advanced draft stage).
    iv. We will draft a manuscript to describe the results of the competition analysis.
c. **For Objective 3,** we hope to sequence at least ten additional high or low aggressive/toxicity strains to strengthen our conclusions regarding markers linked to these traits. This will also help to increase our understanding of the process of recombination in outcrosses of *F. graminearum*.
d. We will present our results as a poster at the Annual Forum of the USWBSI in Denver, Colorado, next December.

Project FY22-PB-003: Role of Chemotype in Aggressiveness and Toxigenicity of Fusarium graminearum to Wheat

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1. **What are the major goals and objectives of the research project?**
   The goal of our project is to test the hypothesis that the *F. graminearum* 3ADON chemotype confers higher levels of aggressiveness, toxicity, and competitiveness than the 15ADON chemotype, regardless of genetic background. The objectives are: **1)** Determine whether trichothecene toxin chemotype, mating type locus, and other genetic makers exhibit Mendelian segregation among progeny from crosses of strains from different *F. graminearum* populations; **2)** Determine whether individuals and mixtures of progeny from outcrosses differ in aggressiveness, toxicity, and competitiveness in susceptible and moderately resistant wheat in the presence and absence of fungicides; and **3)** Identify DNA markers associated with aggressiveness and high toxin production by analyzing whole genome sequence data from pools of progeny that differ in these traits.

2. **What was accomplished under these goals or objectives?** *(For each major goal/objective, address these three items below.)*
   a. For **Objective 1,** our goal was to test the hypothesis that chemotype, mating type, and other genetic makers exhibit Mendelian segregation among progeny from crosses of strains from different *F. graminearum* populations (NA1, NA2, and NA3). Note that the majority of work was done in the Vaillancourt laboratory at the University of Kentucky. Work done in the Proctor laboratory at the USDA ARS in Peoria focused on generation of genome sequence data and some analyses of those data. **(Note: most work towards this objective was conducted in the Vaillancourt Lab)**

   What were the major activities?
   1. We obtained a collection of 37 field isolates of *F. graminearum* recovered from wheat grown in the U.S. The isolates included representatives from the four North American chemotypes (15ADON, 3ADON, NIV, NX2)**.**
   2. The chemotype of each field isolate was confirmed by PCR and/or by Sanger sequencing. Each isolate was also examined for growth, conidiation, and sexual fertility under laboratory conditions, as well as for aggressiveness and toxicity on the susceptible spring wheat variety Wheaton under green house conditions. The resulting information was used to aid selection of the most suitable parents for sexual crosses of isolates.
   3. One field isolate each of the 3ADON, NIV, and NX2 chemotypes was crossed a 15ADON tester strain. This strain was derived from the standard 15ADON field isolate PH-1 by deleting the *MAT1-1-1* gene. The deletion rendered the 15ADON tester self-sterile, so that all perithecia it produces are a product of outcrossing with field isolates to which it is crossed.
   4. Approximately 230 progeny were collected from 20 perithecia (10-12 progeny per perithecium) from each of three crosses with the 15ADON tester (3ADON x 15ADON, NIV x 15ADON, and NX2 x 15ADON). For each cross, the genotype with respect to chemotype (TOX) and mating type (MAT) was determined by PCR for a subset of 80-90 progeny**.**

   What were the significant results?
   1. The field isolates varied markedly in the traits analyzed. **We selected parents for the crosses based on their growth, conidiation and sexual fertility in culture and their aggressiveness on Wheaton wheat**. Field isolates that grew abnormally, sectored

frequently, produced few conidia, exhibited low levels of sexual fertility, or had very low levels of aggressiveness, were not considered as parents in the crosses.

2. Three field isolates (3ADON strain NRRL 46434; NIV strain NC016; and NX2 strain NRRL 66040) were selected as the most suitable parents for the crosses with the 15ADON tester. Genome sequence of each parent was generated to determine to which North American (NA) population it belonged. NRRL 46434 (3ADON) belonged to NA2, and NRRL 66040 (NX2) was NA3. NC016 (NIV) appeared to be a hybrid of NA1 and NA2. The 15ADON tester belonged to the NA1 population.
3. When each parent isolate/strain was evaluated for aggressiveness and toxicity on the susceptible spring wheat variety Wheaton, the 3ADON parent produced significantly higher levels of toxin than the NIV, NX2, or 15ADON parent strains. The 3ADON and NX2 parents had similar levels of aggressiveness and both were more aggressive than the NIV and 15ADON parents.
4. Progeny from the 3ADON x 15ADON and NIV x 15ADON crosses produced expected Mendelian segregation patterns for the TOX and MAT markers (25% of each parental and recombinant type). **In contrast,** progeny from the NX2 x 15ADON cross exhibited an anomalous 3:1 15ADON to NX2 segregation pattern for the TOX markers. The MAT marker exhibited the expected 1:1 segregation pattern.
5. Analysis of the TOX and MAT segregation patterns for individual perithecia revealed that between one and three perithecia from each cross resulted from selfing by the 3ADON, NIV or NX-2 parent. Progeny from perithecia that resulted from selfing were excluded from subsequent analyses.

List key outcomes or other achievements.

1. Segregating progeny (~200) have been collected from each cross, single-spored, verified, and stored in a permanent collection at the University of Kentucky.
2. **This objective is now completed.** The mapping populations and associated data can be viewed here: and will be made available to the community on request with appropriate permits.

b. For **Objective 2** our goal was to determine whether individuals and mixtures of progeny from the outcross of the 15ADON/MAT- tester strain and the 3ADON/MAT+ parent (NRRL 46434**)** differ in aggressiveness, toxicity, and competitiveness in susceptible and moderately resistant wheat in the presence and absence of fungicides. Recall that the 3ADON strain was more aggressive and produced more toxin than the 15ADON tester strain. **(Note: most work towards this objective was conducted in the Vaillancourt Lab)**

i. What were the major activities?

1. To evaluate aggressiveness and toxicity: 67 progeny from our 3ADON/MAT+ vs 15ADON/MAT- cross were selected at random for individual pathogenicity assays on susceptible Wheaton and moderately resistant Alsen wheat varieties. Wheat heads were collected, ground, and sent to Virginia Tech for mycotoxin analysis. Three experiments, including 5 replications for each progeny strain plus parental strains in a RCB design, were completed for Wheaton, and two experiments were completed for Alsen.
2. To evaluate competitiveness: 70 progeny from the 3ADON/MAT+ vs 15ADON/MAT- cross (the 67 above plus 3 more) were randomly assorted into seven pools, each containing 10 strains (Five 3ADON and five 15ADON) in equal amounts. The pools were inoculated onto Wheaton. Three experiments, including 5 replications of each pool plus parents and controls in an RCB design, were completed in the presence or absence of fungicide (Tubuconazole). Disease severity was evaluated, and wheat heads were collected and ground. Part of the ground material was sent for mycotoxin

analysis, while the rest was used to isolate single-spored colonies (24 per sample). A random subset of these (~8 per sample) were chemotyped by PCR.

ii. What were the significant results?

1. There was significantly less disease overall on the Alsen variety versus the Wheaton variety, as expected. The heritability of disease severity on Wheaton was 0.458, suggesting a substantial genetic contribution. The Alsen variety did not perform as well in our greenhouse assays since the negative controls developed physiological symptoms similar to bleaching caused by FHB late in the experiment.
2. For both wheat varieties, there was **no significant difference** in the aggressiveness of the 3ADON versus 15ADON progeny, whether measured as disease severity at individual time points, rate of disease progress, or Area Under the Disease Progress Curve (AUDPC). There was no significant association of chemotype with high versus low aggressiveness. There was also no significant effect of the MAT deletion.
3. The mycotoxin analysis revealed the mycotoxin profiles matched the PCR chemotype of the progeny with only one exception on both Wheaton and Alsen. Toxin levels were positively correlated with disease severity on both wheat varieties. There was **no significant difference** in the amount of toxin produced by the 3ADON versus 15ADON progeny in either wheat variety.
4. Nine progeny that were consistently more aggressive and toxigenic than the average (aka. "high" progeny), and 5 that were consistently less aggressive and toxigenic (aka. "low" progeny), were identified and confirmed in two additional greenhouse experiments on Wheaton with 15 replications per treatment. This confirmed the phenotypes of these high and low aggressive strains and verified that there was **no relationship** between the chemotype and high or low aggressiveness or toxicity.
5. For the competition studies: disease severity data are available for all three experiments, and mycotoxin and PCR data are complete for the first two experiments.
    a) Disease severity and mycotoxin levels were reduced in the presence of fungicide, as expected. The 3ADON parent strain was more tolerant of the fungicide than the 15ADON tester strain.
    b) Pools were less aggressive on average than the parent or individual progeny strains, indicating the presence of antagonism.
    c) In the two experiments that were analyzed, 3ADON and 15ADON generally co-occurred at similar levels in wheat heads that were inoculated with the pools, either in the presence or absence of fungicide. Furthermore, PCR results did not show consistent dominance of either chemotype overall, although within individual pools there were often significant differences in the numbers of 3ADON versus 15ADON strains that were recovered.

iii. List key outcomes or other achievements.

1. The completed part of this objective allows us to reject part of our original hypothesis, that the 3ADON chemotype is **directly** responsible for the higher level of aggressiveness and toxicity of the NRRL 46434 3ADON strain. Our data suggest instead that there are multiple other factors segregating in the cross that contribute to aggressiveness and toxicity. Extrapolating this result to the rest of the 3ADON population, we could conclude that monitoring chemotype alone may not provide sufficient information for risk assessments.
2. Although results on competitiveness are still incomplete, so far they suggest that other factors (genetic, environmental, or both) are more important than chemotype in determining dominance during co-inoculations.
3. In this objective we have identified segregating progeny that are highly aggressive, highly toxic, or highly competitive in the presence versus absence of fungicides. In future it may be possible to use them to identify genetic factors that play important roles in

these traits, and associate those with markers allowing them to be tracked in the population as potential risk factors.

c. For **Objective 3**, our goal was to conduct genome-wide SNP (single nucleotide polymorphism) analysis of bulked progeny pools from the cross of the 3ADON and 15ADON chemotype. The aim was to identify new genetic markers associated with aggressiveness, competitiveness, and toxigenicity. **(Note: most work towards this objective was conducted in the Vaillancourt Lab)**

i. What were the major activities?

1. Genome sequence assemblies for the selected parental strains were generated in the Proctor laboratory at the USDA-ARS in Peoria. For genome sequencing, strains were first grown on a growth medium amended with antibiotics to remove any potential bacterial contamination. Genomic DNA was isolated from liquid cultures using the Qiagen DNeasy Plant Mini Kit. Resulting DNA was then subjected to a 16S PCR screen to confirm the absence of bacterial DNA, and then used to prepare libraries for sequencing with an Illumina MiSeq instrument. Resulting sequence reads were processed with the computer program CLC Genomics Workbench. Adapter and low-quality sequences were removed from sequence reads, and then reads were screened against 73 bacterial genome sequences to remove low levels of bacterial sequence reads resulting from contaminated reagents and/or equipment. The unmapped sequences were then used to generate an assembled genome sequence using CLC Genomics Workbench.
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3. The high-low strains from the 3ADON vs 15ADON cross (see above) were sent to the Proctor laboratory for sequencing. Although we had originally planned to sequence bulked pools of the high and low progeny, a new method became available in the Proctor lab that allowed us to sequence the genomes of the strains individually.

ii. What were the significant results?

1. Genome assemblies for the parent strains ranged from 33 to 36.7 Mb in size and included 393-593 contigs. The number of SNPs differentiating the parent strains from the 15ADON *mat1-1-1* deletion strain ranged from 120,517 to 166,083. There were 144,116 SNPs that distinguished the 3ADON NRRL 46434 and 15ADON parents.

2. 14 progeny from the cross of 3ADON and the 15ADON tester were sequenced, and segregation of 1568 representative SNP markers that were evenly dispersed across all four chromosomes was evaluated. The vast majority (81%) of the SNP markers showed the expected 1:1 segregation (**Figure 1**).

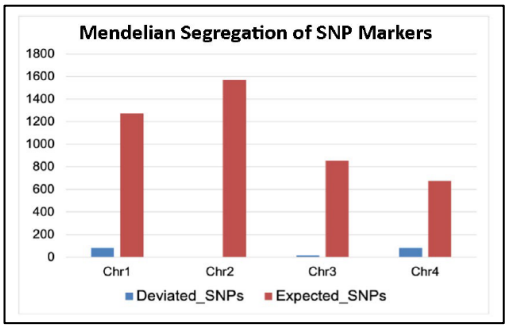

Figure 1: Number of SNP markers that conformed (red bars) or deviated (blue bars) from expected 1:1 Mendelian segregation patterns among 14 progeny of the 3ADONx15ADON cross.

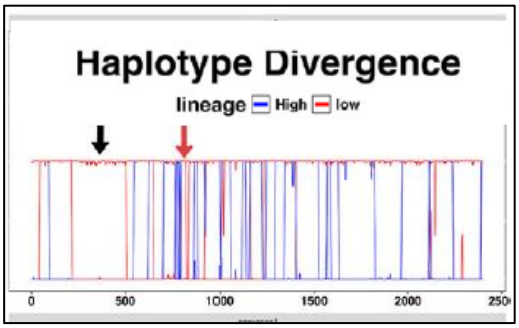

Figure 2: Haplotype divergence plot for chromosome 2, including high (blue) and low (red) progeny. The black arrow indicates a recombination cold spot; the red arrow is a recombination hot spot.

3. Haplotype divergence plots were generated by comparing haplotypes of the progeny to the 15ADON parent strain across sliding windows along each of the four chromosomes. In these plots, the x-axis shows the window position along the sequence while the y-axis shows divergence values (ranging from 0 to 0.5). Divergence values near 0.0 indicate regions where the progeny matched the 15ADON parent, suggesting inheritance from this parent. Divergence values of 0.5 indicate regions inherited from the alternate parent (the 3ADON strain). All the progeny are plotted on the same image to compare patterns of recombination. Recombination hot spots were visualized on these plots as regions where there were many switches between 0.0 to 0.5 (**Figure 2**, red arrow). Recombination cold spots were seen as regions with very few switches (**Figure 2**, black arrow). All four chromosomes had frequent recombination hot and cold spots: the cold spots reveal blocks of genes that will tend to co-segregate more frequently.

4. Haplotype plots revealed one region of chromosome 2 that appeared to be associated with aggressiveness (**Figure 3**). All the high aggressive progeny (red) inherited this region from the more aggressive 3ADON parent, while all the low aggressive progeny (blue) inherited it from the 15ADON parent. This region was not linked to the TOX (chemotype) locus which is on the other arm of chromosome 2.

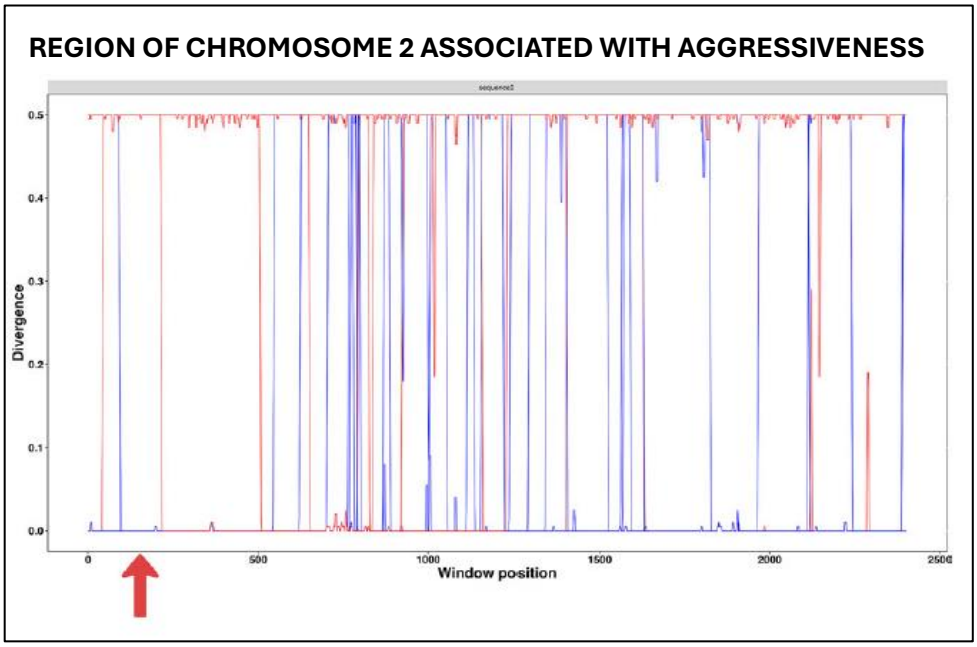

Figure 3: Haplotype divergence on chromosome 2 comparing high aggressive (red) and low aggressive (blue) progeny. The red arrow indicates a region that is associated with aggressiveness: high progeny have all inherited this region from the more aggressive 3ADON parent while low progeny have all inherited it from the less aggressive 15ADON parent.

iii. List key outcomes or other achievements.

1. Although the number of progeny we analyzed was limited due to cost of WGS, we have demonstrated the feasibility of this approach for association mapping and identified a novel region of chromosome 2 that may be associated with high aggressiveness/toxicity.
2. We have developed a representative set of SNPs that can be used for association mapping. Moreover, by comparing with additional genome sequences that are publicly available, we identified a core set of 500 of these SNPs that are flanked by conserved regions, amenable to PCR primer design. This would allow them to be screened simultaneously by amplification and sequencing in a multiplex approach, allowing very cost-effective genotyping of wild type and progeny strains. We intend to explore the feasibility of this genotyping tool in other on going work in our laboratory.

3. What opportunities for training and professional development has the project provided?

An M.S. Student was trained in the Vaillancourt Laboratory at the University of Kentucky. Personnel in the Proctor Laboratory interacted with the student on a regular basis to provide genome sequence data and the results of analyses assigned to the Proctor Laboratory. Additional information on the student can be found in the Vaillancourt progress report.

4. How have the results been disseminated to communities of interest?

The student presented a research poster at the USWBSI forum in Austin in December 2024, and she also gave a talk on her research for the Plant Pathology Departmental Seminar in early June.

5. What do you plan to do during the next reporting period to accomplish the goals and objectives?

a. **The work for objective 1 is completed.**
b. **We have nearly finished work for objective 2.** During the next and final reporting period, we will complete this objective.
    i. We will repeat our analysis of the segregating progeny on at least one moderately resistant line, since Alsen didn't behave well in our greenhouse assays. We have already received seed of 10 candidate varieties from Dr. Jim Anderson. We will pre-screen these with our high-low progeny first and then, after choosing a suitable line, we will repeat our analyses of aggressiveness and toxicity with the 70 random progeny from the 3ADONx15ADON cross at least twice.
    ii. We will complete the work on the competition studies by finishing the PCR and mycotoxin evaluation of the third experiment. We will evaluate additional markers (using either telomere fingerprints or multiplex SNP analysis) to test whether there are highly competitive clones among these recovered strains. This will help us to understand the relative role of genetics versus environment in determining competitive outcomes.
    iii. We will complete and submit a publication describing the lack of association of 3ADON with aggressiveness and toxicity (this manuscript is currently in the advanced draft stage).
    iv. We will draft a manuscript to describe the results of the competition analysis.
c. **For Objective 3,** we hope to generate genome sequence data for at least ten additional high or low aggressive/toxicity strains to strengthen our conclusions regarding markers linked to these traits. This will also help to increase our understanding of the process of recombination in outcrosses of *F. graminearum*.
d. We will present our results as a poster at the Annual Forum of the USWBSI in Denver, Colorado, next December.