1. What are the major goals and objectives of the research project?

The goal of this project is to determine the contribution of *Fusarium* genome diversity to the variation in disease severity and mycotoxin contamination observed in barley genotype screening nurseries and facilitate incorporation of pathogen genotype data in variety screening programs to enhance the resilience of FHB resistance.

The specific objectives of this proposal are the following, **Objective 1**: Characterize genomic differences in FHB isolates within and among barley screening programs. **Objective 2**: Determine if standard susceptible and resistant barley cultivars exhibit the same level of disease and mycotoxin contamination in response to FHB isolates from different screening programs under controlled conditions. **Objective 3**: Determine if barley cultivars exhibit a differential metabolic response to genetically diverse FHB isolates.

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

This is the third report for this project since receipt of FY24 funding, (May 1, 2024 - April 30, 2025). We have assembled a collection of 97 FHB isolates from barley screening programs in six states to evaluate viability of strains within and among screening programs. Last year, we also received 65 isolates of *Fusarium* species from wild grass in collaboration with Dr. Milton Drott, USDA-ARS Cereal Disease Laboratory, MN to determine any genetic changes among this natural collection of isolates. In addition, we have successfully completed the Whole Genome Sequencing (WGS) for all 97 FHB and 65 wild-grass isolates (Table 1).

What were the major activities?

To accomplish the Objective 1,2,3, we have conducted the genome assemblies of all 144 isolates (97FHB plus 65 wild grass isolates), and they were subjected to the Funannotate genome annotation pipeline built on SCINet (a USDA High Performance Cluster computing system). We have successfully completed the functional annotation for all these isolates. In addition, we also conducted a nucleotide polymorphism (SNP) analysis with the software Genome Analysis Toolkit (GATK) for all genome sequences of 144 isolates (Table 1). In the coming year, we will begin the pan-genomics analysis and compare the genome-wide SNPs calling for all 144 isolates to understand the genetic change, identify genomic variations, evolutionary relationships and genotype-phenotype associations within a species or population. This comprehensive approach can reveal core genes common to all isolates; accessory genes present in subsets. We will utilize the pangenome graphs to detect structural variants (insertions, deletions and inversion) that may be missed by single reference approach. The Gene Presence-Absence variation (PAV) analysis in pan-genomic data can also determine the patterns of gene presence and absence across the isolates to gain insight into species evolution and adaptation.

Table 1. Number of FHB isolates that we have conducted for the toxin analysis in vitro, whole genome sequencing, infection assay and genome annotation, and SNP analysis.

State	# of FHB isolates received	Toxin production in culture	Genome Sequencing	FHB Assay	Genome Annotation	SNP analysis
Idaho	31	20 (15ADON), 11 (3ADON)	31	8	31	31
Maryland	10	10 (15ADON)	10	6	10	10
Minnesota	30	25 (15ADON), 4 (3ADON), 1 (No toxin)	30	7	30	30
New York	1	1 (15ADON)	1	1	1	1
North Carolina	19	19 (15ADON)	19	6	19	19
North Dakota	6	6 (15ADON)	6	6	6	6
Wild grasses	65	N/A	65	N/A	65	65

What were the significant results?

To confirm species identity, evaluate evolutionary relationship of these isolates and improve better resolution of phylogenetic tree construction, we have retrieved full-length sequences of 21 housekeeping

genes which are generally highly conserved across related species but often contain enough variation to differentiate closely related species of *F. graminearum*. For this analysis, we also included sequence data from 74 *Fusarium* isolates that have been previously examined with respect to confirm which *F. graminearum* population they belong to (i.e., NA1, NA2, NA3, Southern Louisiana, and Gulf Coast populations).

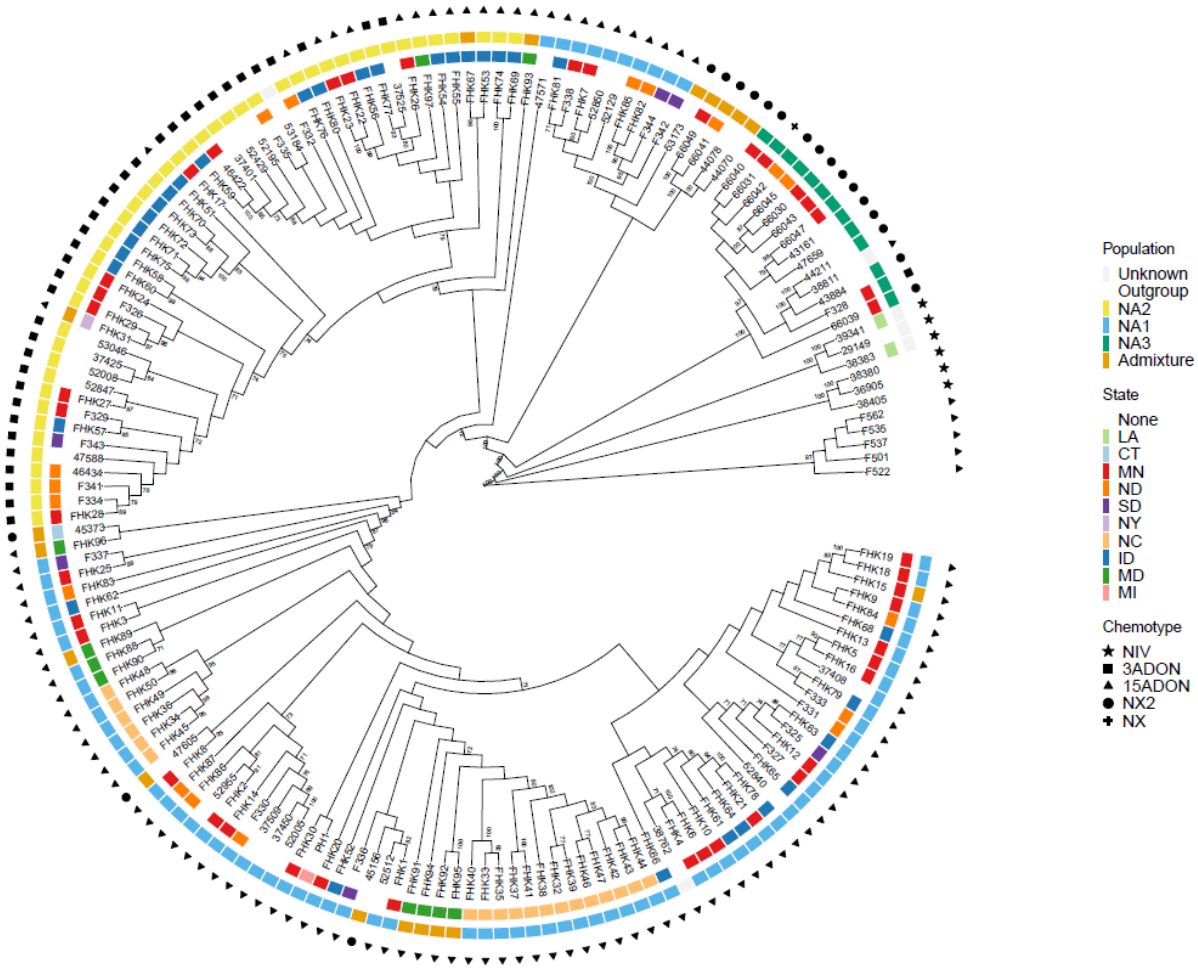

Figure 1. A maximum likelihood phylogeny tree inferred from 21 HK genes retrieved from the genome sequences of 171 *Fusarium* isolates. The first outer color-coded ring represents the location where these isolates were collected from. The second outer color-coded ring indicates the population structure for each isolate (Blue:NA1, Yellow: NA2, Green: NA3, Orange: admixed isolate). The last outer ring shows the chemotype designated by a symbol at the end of outlier.

This indicates that all 97 FHB isolates are members of either population NA1 or NA2. However, the NA1 and NA2 clades shown in Figure 1 include several reference strains (orange color-coded in the second outer ring) that were previously assigned to other populations. We hypothesized that these strains might represent admixture, i.e., the result of crosses between members of different populations. To address this hypothesis, we conducted a SplitsTree analysis (see below) in collaboration with Dr. Milton Drott in the USDA-ARS, MN. For this analysis, we included all 97 FHB isolates and 67 isolates that were previously assigned to *F. graminearum* populations (Kelly and Ward *et. al.*, 2018) to review their distance matrix and phylogenetic network relationship. None of the isolates that we examined group with strains in the NA3 population. A total of 60 isolates that belong to the NA1 population structure are 15ADON producers. Interestingly, 28 of 97 FHB isolates were grouped with the NA2 population (Figure 1 and 2); 12 of the isolates are 15ADON producers and 16 are 3ADON producers. A total of 10 isolates are admixed, and all of them are 15ADON producers.

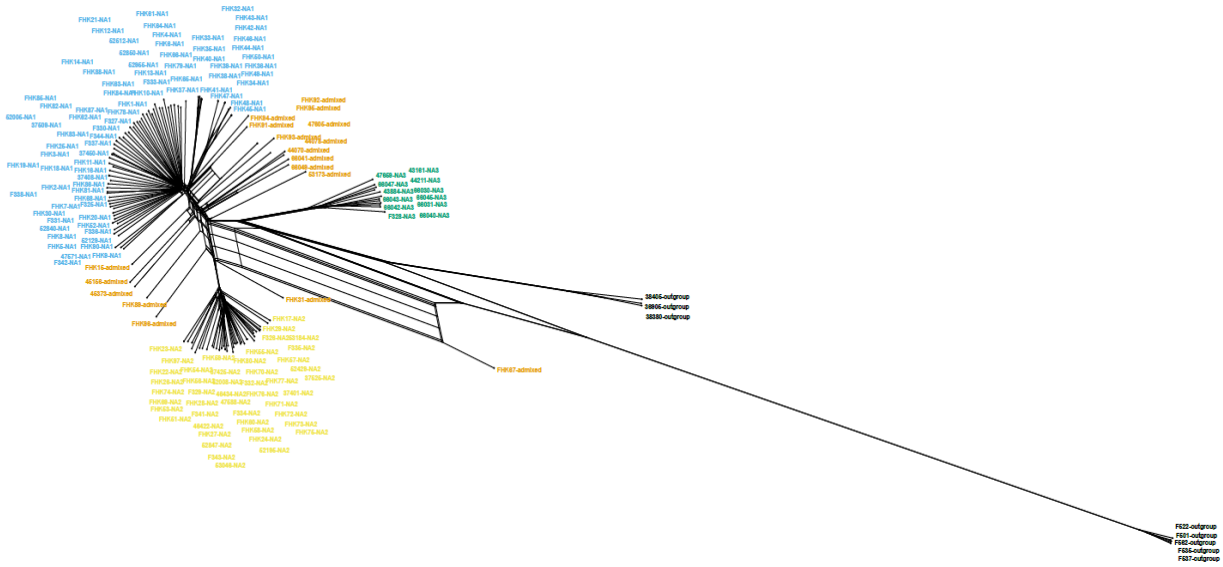

Figure 2. A phylogenetic network constructed with SplitsTree using whole genome SNP dataset from 164 *F. graminearum* isolates. Each isolate was mapped with different colors that belong to each population structure: The Blue color indicates the NA1 population, yellow color indicates the NA2 population, green color shows the NA3 population. The admixed isolates were color-coded with orange color in the SplitsTree.

To address **Objective 2**, we planted the check barley varieties, AAC Synergy (susceptible) and Pinnacle (resistant), for seed propagation. A total of 34 isolates representing 6 different states were tested for their ability to cause FHB in the two barley varieties (Table 1). We also included a representative strain from the NA1, NA2, and NA3 (also known as NX-2 strain) populations, bringing the total to 40 strains for the FHB assays on the two barley varieties. Fifteen barley heads were inoculated per FHB isolate, and 20 heads were used for each of the NA1, NA2, and NA3 reference strains. After inoculation, the barley heads were covered with plastic bags for three days to maintain high humidity. **FHB disease severity** was scored at 4-, 7-, and 10-days post-inoculation. Data has been collected for all 40 isolates (Figure 3).

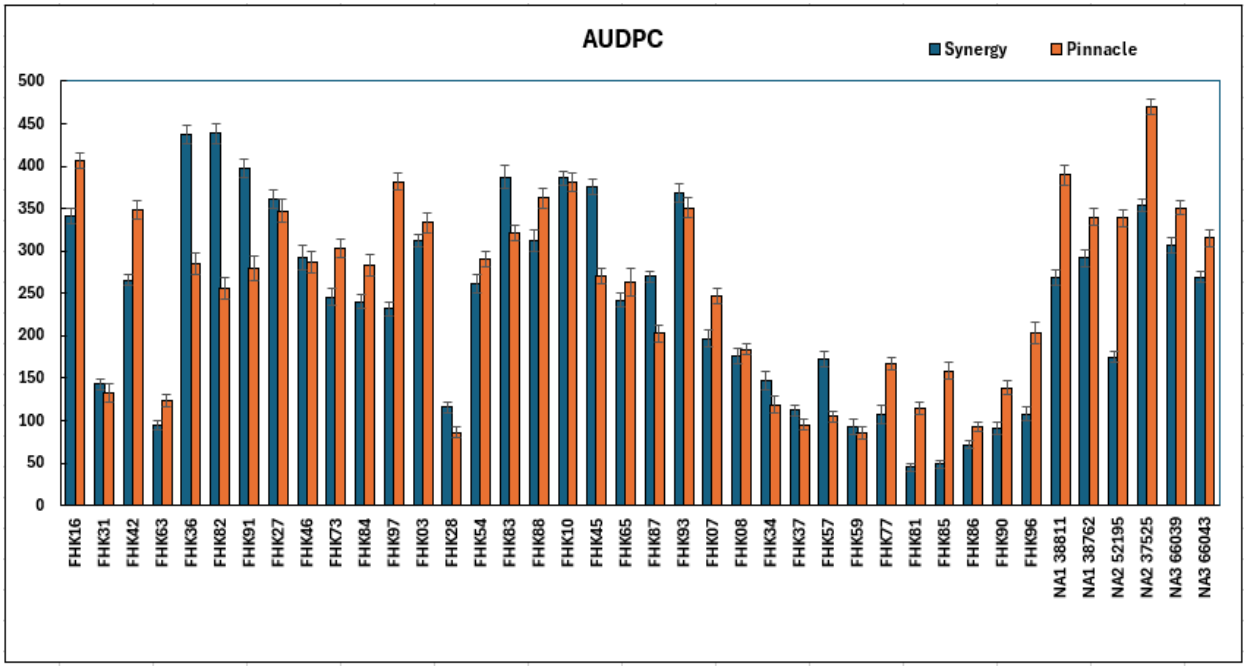

Figure 3. Average Area Under the Disease Progress Curve (AUDPC) for 40 individual isolates on Synergy and Pinnacle barley. Results from disease assay experiments 01-07 are shown in the graph.

The infection assays conducted on 34 FHB isolates demonstrated significant variation in disease severity between the two barley varieties, AAC Synergy and Pinnacle. The AUDPC values highlighted differences in pathogenicity, with some isolates showing stronger virulence on one variety over the other (Figure 3). Assay 7 was used as a control using isolates of known populations. NA1 38811, NA1 38762, NA2 52195, NA2 37525, NA3 66039, and NA3 66043 demonstrated higher virulence on Pinnacle compared to Synergy, with NA2 37525 showing the highest values for both Synergy and Pinnacle. Overall, virulence varied significantly across assays and cultivars. Some isolates such as FHK36 and FHK82 were consistently more virulent on Synergy, while others, such as FHK81 and FHK85 showed greater virulence on Pinnacle. This result contrasts with initial expectations and suggests that disease severity is strain-specific rather than solely dependent on the barley variety which matches fungal biomass results. We previously reported that isolate FHK28, which did not produce trichothecenes in culture, caused the lowest levels of disease symptoms on both barley varieties. However, after the 6th infection assay with 12 more isolates, isolates FHK81 and FHK85 caused the lowest levels of disease symptoms, especially on Synergy, while FHK28 caused the lowest level of disease symptoms on Pinnacle. These results are consistent with trichothecenes not being required for initial infection of barley. Nevertheless, FHK28, FHK81, and FHK85 produced the lowest levels of ADON in culture and caused the lowest levels of FHB symptoms on barley.

FHB strains typically produce acetylated trichothecenes in liquid culture: 3 acetyldeoxynivalenol (3ADON), 15-acetyldeoxynivalenol (15ADON), or 7-hydroxycalonectrin (NX-2). All but one of the 97 strains produced detectable trichothecenes. Results are summarized in Table 1. DON was detected in barley samples from inoculation with the 34 FHK strains as well as the four NA1 and NA2 reference strains (Table 2). Barley inoculated with NX-producing strains tested positive for NX3, which was detected at significant levels.

	Synergy		Pinnacle	
State	Isolate	Average µgDON/g	Isolate	Average µgDON/g
MN	FHK16	94.77	FHK16	133.02
NY	FHK31	43.83	FHK31	53.92
NC	FHK42	66.71	FHK42	119.15
ID	FHK63	25.6	FHK63	39.79
MN	FHK07	85.05	FHK07	92.04
MN	FHK08	64.13	FHK08	69.37
NC	FHK34	47.02	FHK34	52.77
NC	FHK37	26.32	FHK37	24.86
ID	FHK57	62.84	FHK57	59.75
ID	FHK59	53.83	FHK59	35.47
ND	FHK77	36.73	FHK77	75.26
ND	FHK81	17.87	FHK81	57.25
MD	FHK85	24.9	FHK85	81.46
MD	FHK86	42.78	FHK86	54.73
ID	FHK90	44.66	FHK90	64
ID	FHK96	51.85	FHK96	106.8
NC	FHK36	198.28	FHK36	99.37
ND	FHK82	144.88	FHK82	82.42
MD	FHK91	100.95	FHK91	83.47
MN	FHK27	132.54	FHK27	162.14
NC	FHK46	116.87	FHK46	113.56
ID	FHK73	128.69	FHK73	138.74
ND	FHK84	70.22	FHK84	94.7
MD	FHK97	78.03	FHK97	139.82
MN	FHK03	102.66	FHK03	109.61
MN	FHK28	39.75	FHK28	34.55
ID	FHK54	103.26	FHK54	112.48
ND	FHK83	181.83	FHK83	138.38
MD	FHK88	126.95	FHK88	147.13
MN	FHK10	149.63	FHK10	151.87
NC	FHK45	158.03	FHK45	98.38
ID	FHK65	107.5	FHK65	137.65
ND	FHK87	274.83	FHK87	198.77
MD	FHK93	175.15	FHK93	127.57
	NA1 38811	70.16	NA1 38811	91.59
	NA1 38762	81.48	NA1 38762	126.7
	NA2 52195	134.77	NA2 52195	249.76
	NA2 37525	252.7	NA2 37525	366.58
		Average µgNX3/g		Average µgNX3/g
	NA3 66039	343.92	NA3 66039	403.91
	NA3 66043	260.68	NA3 66043	257.64

Table 2. DON levels in barley tissue inoculated with *F. graminearum* isolates in Infection Assays 01 to 07.

Among the FHB isolates, North Dakota isolate FHK87 caused the highest levels of DON contamination in AAC Synergy, although it did not cause the most severe FHB. This finding highlights that toxin production does not always correlate directly with disease severity. On the other hand, NA2 strain 37525 caused the highest levels of DON in Pinnacle and had the most severe FHB. The lowest DON levels in AAC Synergy resulted from inoculation with isolate FHK81, which aligned with the low disease severity caused by the isolate. Conversely, strain FHK37, which caused the lowest DON levels in Pinnacle, did not align as well with disease severity data.

We also estimated pathogen biomass in the inoculated barley heads using a Fluidigm nanofluidic automated real-time PCR system. Analysis of fungal biomass in barley has been completed for 40 strains included in infection assays 01 - 07.

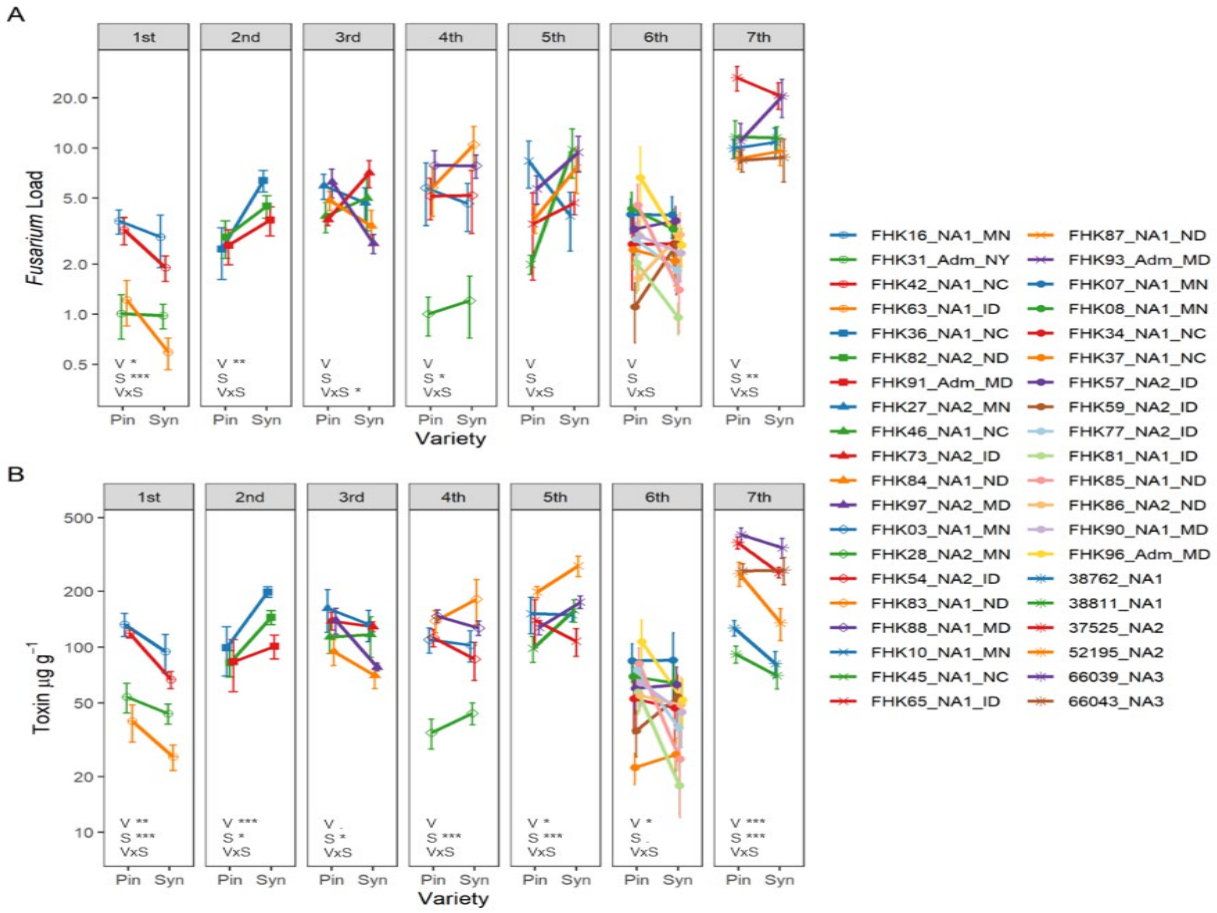

Figure 4. Assay (as a stand-in for environment) and strain identity were major determinants for the ability to detect varietal differences in disease resistance. Interaction plots for A) Fusarium biomass and B) Toxin concentrations per the seven assays – which are denoted by the horizontal gray strips at the top of the vertical panels. Toxin concentrations are expressed in units of micrograms per gram of plant material and represent Deoxynivalenol (assays 1st-6th) or NX-3 (assay 7th). Each point represents the average value across the entire Fusarium strain treatment (denoted by point color and shape), with error bars displaying the standard error of the mean. Lines connecting the points between Pinnacle (Pin) and AAC Synergy (Syn) illustrate differential response between the two host varieties for each Fusarium strain treatment. Nonparallel lines are indicative of statistical interactions, while parallel lines are indicative of significant main effects or nonsignificant interactions. The y-axes are presented on a log-transformed scale to aid interpretability. Results from ANOVAs are shown for each assay: variety (V), strain (S), and the interaction between variety and strain (VxS): P values '.' 0.05-0.10, '*' < 0.05, '**' < 0.01, '***' < 0.001.

High levels of fungal biomass were detected in both barley varieties inoculated with reference strains 38811 (NA1), 38762 (NA1), 37525 (NA2), and 66039 (NA3) as well as barley nursery isolates FHK83, FHK03, and FHK88. All biomass results were consistent with disease severity and DON production (see Figure 5). Low levels of fungal biomass were detected in both Synergy and Pinnacle tissues inoculated with FHK63, which also caused low levels of DON contamination in barley heads and caused low levels of disease.

To address the **Objective 3**, subsamples of frozen pulverized plant tissue (45-55mg) from the barley varieties mentioned above were extracted for LC-MS metabolomic analysis. Interpretation of raw metabolomic data was aided by processing with metabolomics software, Compound Discoverer 3.3. Due to growth chamber space constraints, all disease assays were not conducted simultaneously.

Finally, the metabolomic data analyses for the 1st-7th infection assay with 34 FHK strains were completed in a different time frame than later assays (Figures 5-9). In the past report, the Principal Component Analysis (PCA) clearly showed distinct separations between the two barley varieties. Consequently, the goal was to consolidate all the disease assays into a single principal component analysis (PCA) plot to

compare the different FHK strains that we used for each experiment and barley varieties. However, after combining data from the first three experiments, it is evident that we cannot merge these experiments as planned. Other factors, such as small differences in growth chamber environment, are influencing the observed metabolomic differences (Figure 5).

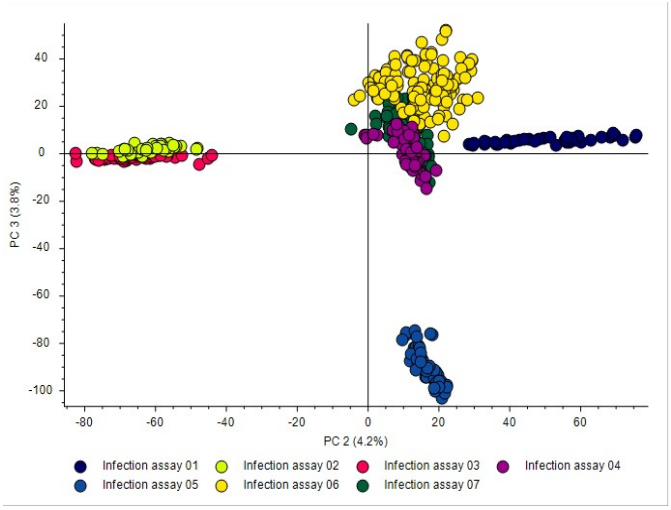

Figure 5. PCA plot of metabolomic data from infection assays 01 – 07.

The samples from the two different barley cultivars cluster and the controls also separate for these assays. We are currently combining the metabolomic data that were generated from the 6th (12 more FHB isolates) and 7th infection assays (reference strains from NA populations) to see if clustering can be observed with specific fungal isolates in the different barley varieties. Although more metabolomic data needs to be incorporated before we can evaluate the importance of the different compounds found in this analysis, we see some promising data from the 1st-5th infection assays (Figures 6-9). Metabolomic results show a distinct separation between the two barley varieties seen in Figure 6. AAC Synergy is seen in bright green, and Pinnacle is depicted in dark blue. Figure 6 highlights differences in metabolic responses between cultivars, with host genetics being the most significant factor, leading to a clear separation between cultivars. To ensure that the separation was not driven by DON we removed consideration of DON, but we can still see separation of isolates. Metabolomic box plots showing the amount of DON for each isolate are seen in Figure 10.

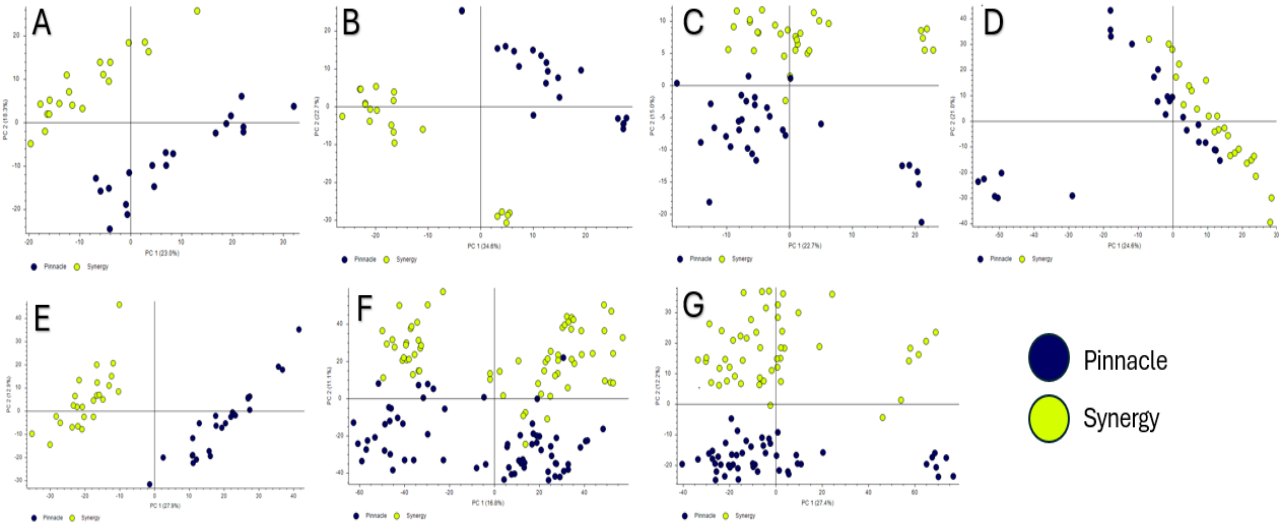

Figure 6. PCA plot of metabolomic data from infection assays 01 – 07. Plots A, B, C, D, E, F and G correspond to data from infection assays 01, 02, 03, 04, 05, 06 and 07 respectively.

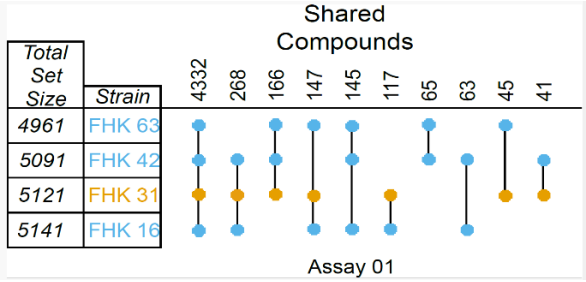

Figure 7. Upset plot showing shared unique compounds between isolates in Assay 01. Yellow indicates NA1 chemotype, blue represents NA2, orange denotes admixture between chemotypes, and green corresponds to NA3.

Overall, the infection assays 4th–7th clustered together, assays 2nd-3rd were somewhat separated, and assay 1st remained distinct from all others. Figures 7-9 represent the upset plots illustrating the shared and unique compounds across strains. Notably, 4,332 metabolites were shared among isolates FHK16, FHK31, FHK42, and FHK63, all of which were part of assay 1. Figure 11 further examines metabolite profiles by isolates, revealing distinct clustering patterns among isolate identity.

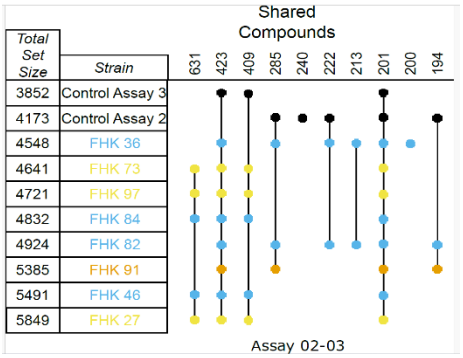

Figure 8. Upset plot showing shared unique compounds between isolates in Assays 02 and 03. Yellow indicates NA1 chemotype, blue represents NA2, orange denotes admixture between chemotypes, and green corresponds to NA3.

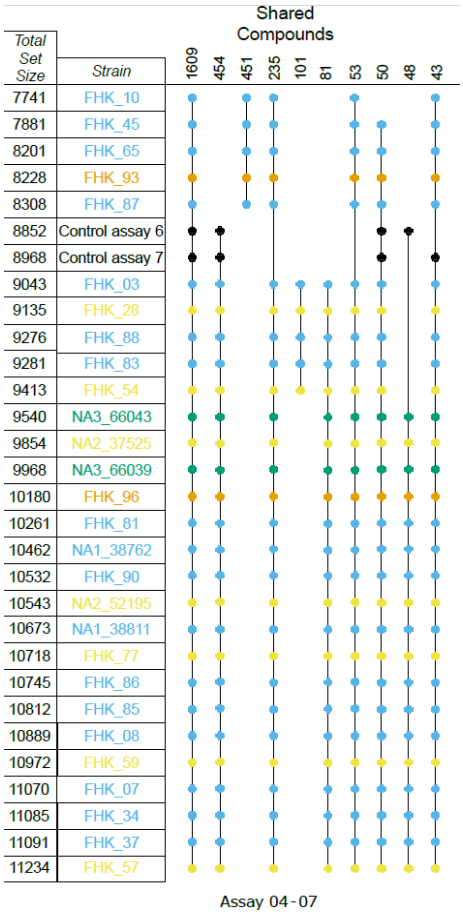

Figure 9. Upset plot showing shared unique compounds between isolates in Assays 04-07. Yellow indicates NA1 chemotype, blue represents NA2, orange denotes admixture between chemotypes, and green corresponds to NA3.

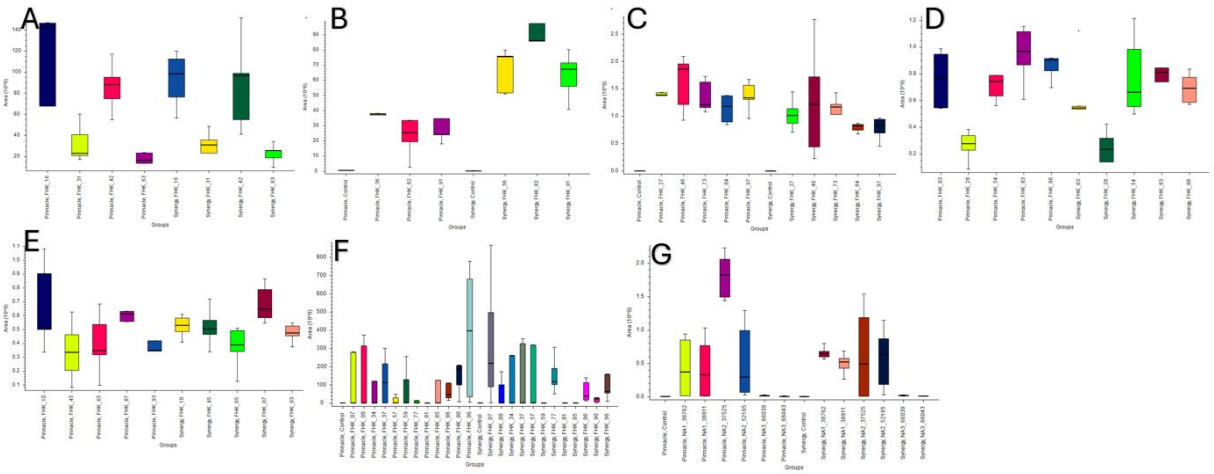

Figure 10. Box and whisker plots for DON levels in barley head tissue. Plots A, B, C, D, E , F and G correspond to data from infection assays 01, 02, 03, 04, 05, 06 and 07 respectively.

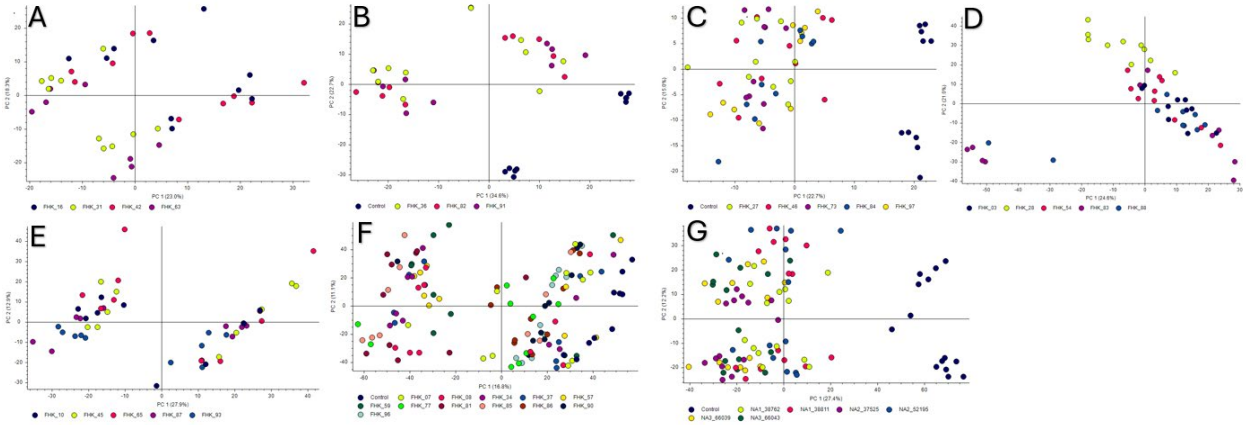

Figure 11. PCA plot of metabolomic data from infection assays 01 – 07 showing separation by isolates. Plots A, B, C, D and E correspond to data from infection assays 01, 02, 03, 04, 05, 06 and 07 respectively.

List key outcomes or other achievements. Less than 1 year after getting the FY24 funding, we have successfully generated all these results through the team's hard work (Please see the above major activities and significant result section). These achievements include the generation of whole genome sequences of 144 isolates (including 97 FHB and 65 wild-grass isolates), complete genome assembly and functional annotation of all genomes, SNPs analysis and infection assay with all 34 isolates and reference strains from known population structures, downstream analysis including toxin, fungal biomass and metabolomics analysis from infected tissues of assay 1st-7th. These accomplishments by employing a combination of genomic, molecular, and biochemical techniques have allowed us to meet all milestones this year for each of the three objectives and effectively study complex disease of FHB and work towards developing solutions that benefit agriculture. We continue to complete the remaining aims of the project to meet the multiple objectives and milestones for the following year.

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

Other than biological science lab work (e.g. DNA extractions, infection assays, fungal biomass and metabolomic data) that we trained ORISE fellow, we also gave an opportunity to fellow to learn how to build computational biology pipelines that include genome assembly, genome annotation, phylogenetic tree with IQ program, GATK SNP analysis workflow step. The fellow often troubleshoots the pipeline and workflow by modifying, updating the script and running different parallel batch jobs to both High Performance Clusters (HPC), Ceres and Atlas in SCINet. The fellow also learned how to optimize the pipeline and script that are compatible in HPC. The fellow also learned how to create MAFFT alignment that are required for generating phylogenetic trees for evolutionary aspects, conduct infection assays, and

analyze the fungal biomass and metabolomic data. In the coming year, the fellow will learn how to conduct pan-genome analysis.

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

We did attend the 2024 National FHB forum that was held in Austin, Texas (December 8-10, 2024) and presented this work in the SCAB meeting. As you can see from the above accomplishments, we have generated significant results and are now transitioning to the manuscript writing phase for submission this calendar year. This is a crucial step towards sharing your findings with the broader scientific community.

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

We had no major difficulties or problems meeting all our planned objectives. Since we have completed genome annotation and SNP analysis for all 141 isolates that included ~65 isolates of *Fusarium* species from wild grasses from Dr. Milton Drott's Lab, we will generate phylogenetic tree/Split trees analysis for all 141 isolates and then, select the best candidate isolates for the infection assay with same barley varieties and generate toxin or metabolomic data from these infection assays. If needed, we plan to perform some perithecia formation assays with selected isolates. Meanwhile, we will start the pangenome analysis with all this genomic data.