Project FY22-BA-016 / PB-006: Contribution of Fusarium Diversity to Variability of FHB Resistance in Barley

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 on 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 second report for this project since receipt of FY23 funding. We first 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. To accomplish the **Objectives 1**, **2** and **3** in the last year, we had generated whole-genome sequencing (WGS) data for all FHB 97 isolates. After WGS for all 97 FHB isolates, we evaluated phylogenetic diversity and generated mycotoxin profiles in culture, completed FHB infection assays with selected 34 isolates following by metabolomic analysis (Table 1).

What were the major activities?

To meet the milestone of **Objective 1** in this year, we developed a genome annotation pipeline with the Funannotate program and successfully annotated all 97 genomes. In addition, using the Genome Analysis Toolkit (GATK) with best practice method, we also conducted the nucleotide polymorphism (SNPs) analysis for these 97 isolates (Table 1).

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	# of isolates of toxin produced in vitro	Genome Sequencing	Infection Assay	Funannotate 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

To confirm species identity and evaluate phylogenetic diversity of isolates, we have retrieved full-length sequences of three housekeeping (HK) genes (TEF1, RPB1 and RPB2) from all 97 genome sequences. In addition, to determine whether the 97 isolates belong to North American (NA), Southern Louisiana and Gulf Coast populations or not, we also retrieved 3 HK genes from each of 2-4 representative isolates from these known populations a aligned the gene sequences from these three marker loci using MEGA and performed maximum likelihood bootstrapping phylogenetic analyses using IQ-TREE (Figure 1) Phylogenetic inferred from 3HK genes in genomes of 97 isolates and three North American (NA 1, 2, 3), Gulf Coast and Southern Louisiana populations.

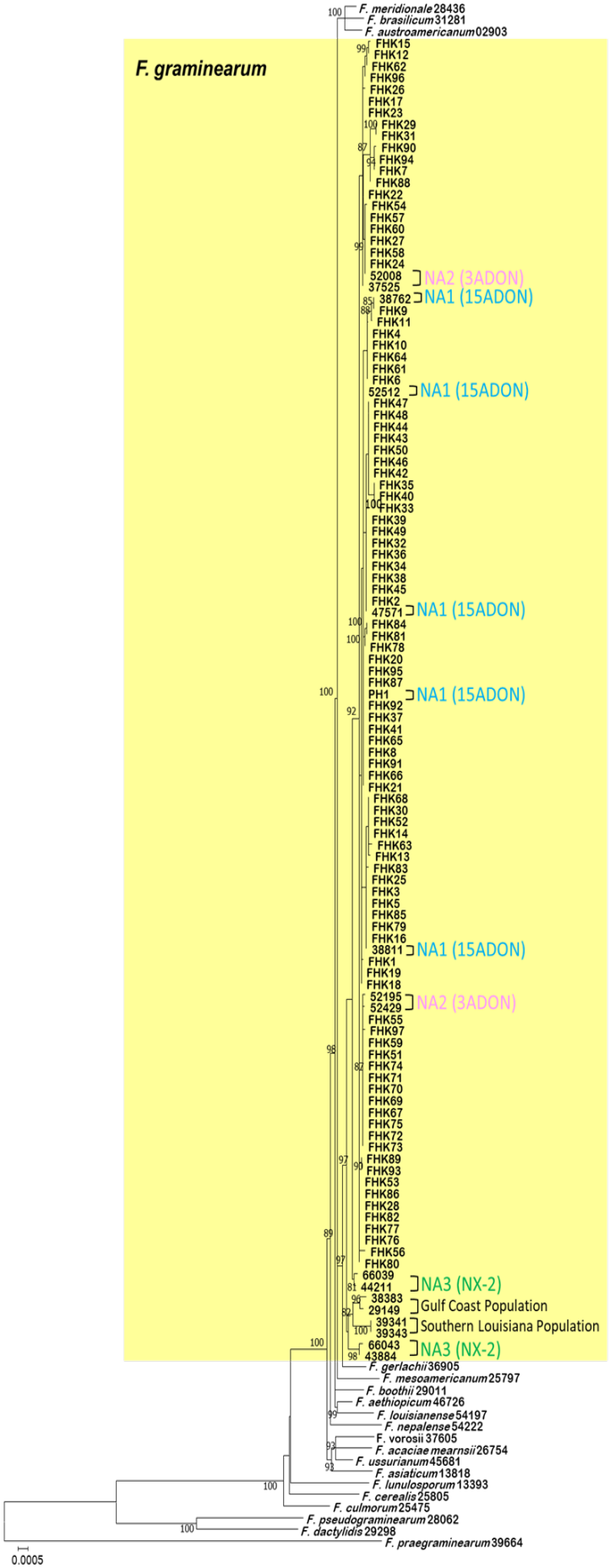

Figure 1. Phylogeny inferred from 3 HK genes (*RPB1*, *RPB2*, *TEF1*) in genomes of 97 FHB isolates and representative isolates from three North American, Gulf Coast, and Southern Louisiana populations.

In collaboration with Dr. Milton Drott in the USDA-ARS, Cereal Disease Laboratory, MN, we also generated SplitsTree with SNP analyses data of a total of 164 isolates that include 97 FHB isolates and 67 isolates from known population structures previously published (Kelly and Ward *et. al.*, 2018) to review their distance matrix and phylogenetic network relationship. This data has been filtered with GATK4 method to be biallelic. We also removed the missing data and mapped the 97 isolates with these four annotations such as three populations (NA1, 2, 3), admixture and outgroups.

To address the **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 on two barley varieties (Table 1). Depending on the availability of plant growth room space, we have conducted infection assays with different number of isolates, respectively (1st infection: 4 isolates, 2nd infection: 3 isolates and control with tween, 3rd infection: 5 isolates, 4th infection: 5 isolates, 5th infection: 5 isolates, 6th infection: 12 isolates). Additionally, each two strains from NA1, NA2, and NA3 population were also tested for the FHB virulence assays as reference strains (7th infection: 6 isolates). Spores of each *Fusarium* isolate were obtained from 4-day old mung bean liquid cultures. The macroconidia were collected and suspended in a 0.04% tween solution with a concentration of 105 conidia per milliliter. About sixty days after emergence, barley heads were inoculated by dip inoculation. Fifteen barley heads

were inoculated for each strain of Fusarium.

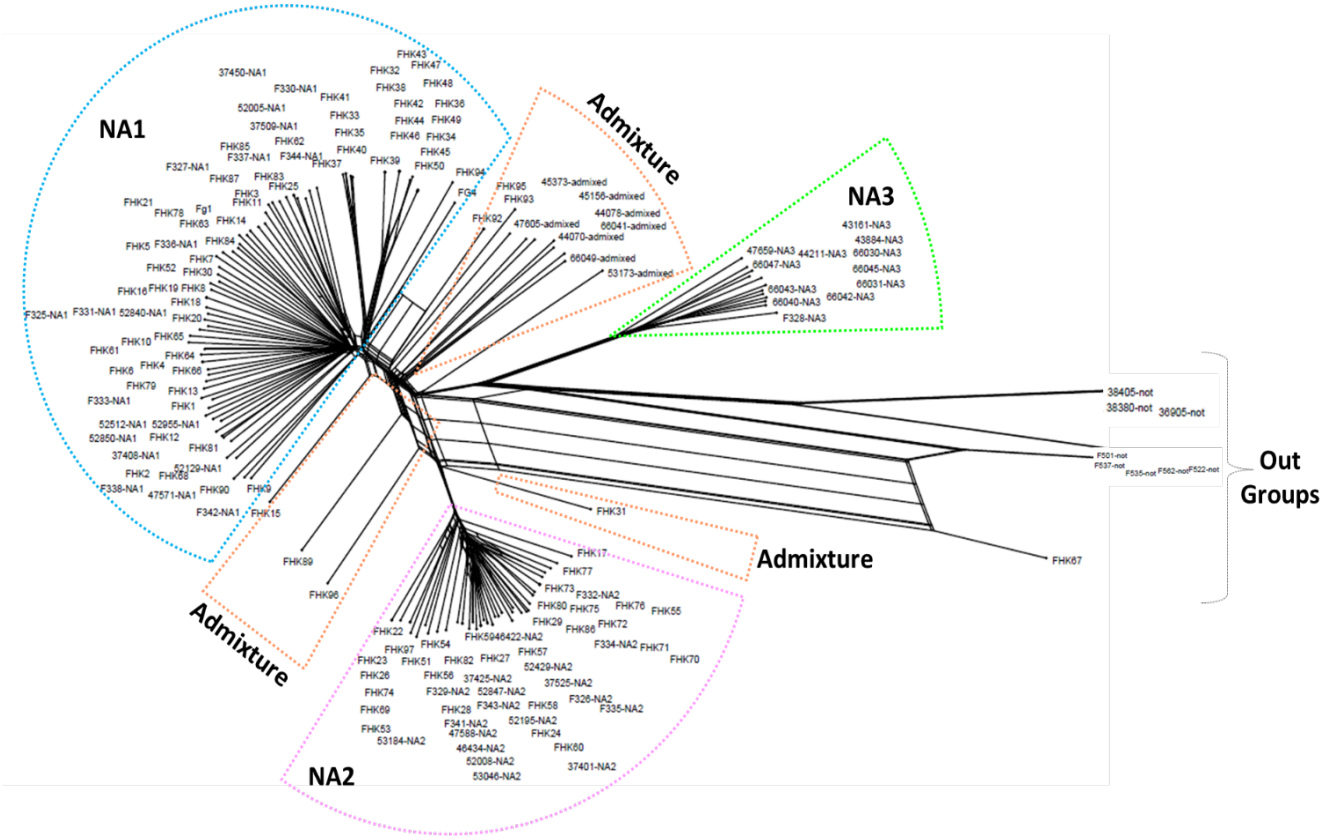

Figure 2. Results of SNP-based Splits Tree analysis

For the 7th infection assay including the NA1, NA2, and NX strains, twenty barley heads were used per strain. Inoculated barley heads were covered with plastic bags for high humidity, bags were removed after three days. FHB disease was scored at 4-, 7-, and 10-days post-inoculation. Disease severity scoring has been completed and calculated for the isolates used (Figure 3).

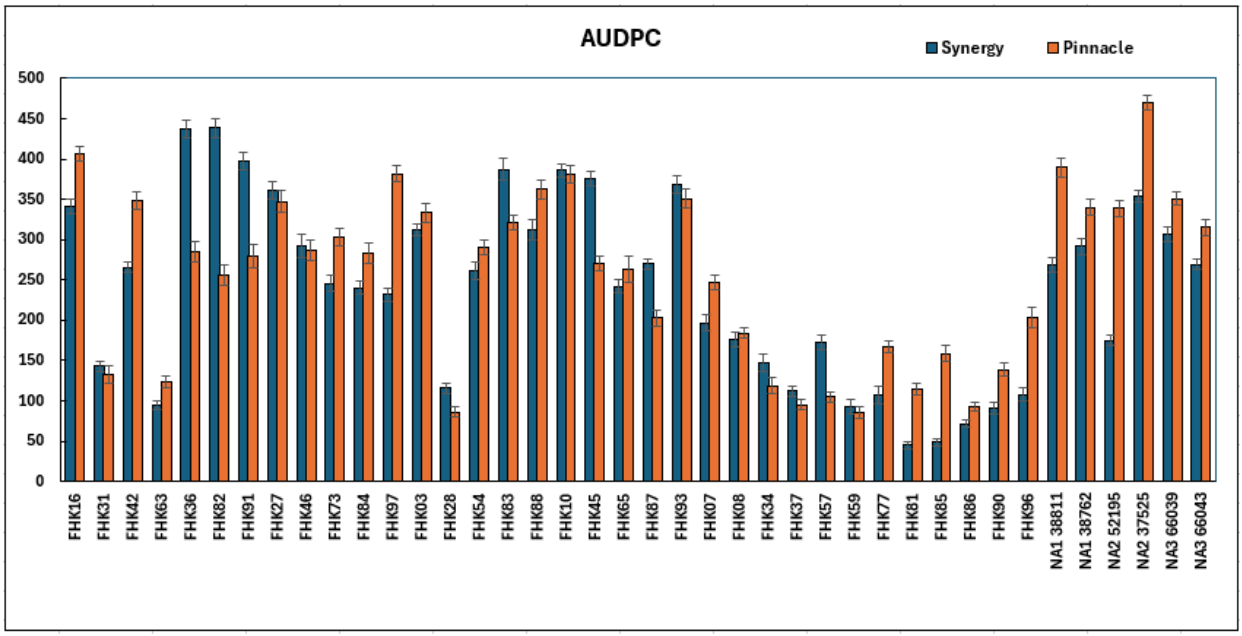

Figure 3. Average Area Under the Disease Progress Curve (AUDPC) for individual isolates on Synergy and Pinnacle barley. Results from disease assay experiments 1-7 are shown in graph.

The barley heads, immediately after excision from plants, were weighed, frozen in liquid nitrogen, lyophilized and pulverized (GenoGrinder 2010). Five biological replicates were used, each containing 0.5g of pulverized tissue. The tissue was extracted with acetonitrile water (86:14). Extracts were purified with Romer columns. Deoxynivalenol (DON) concentrations in ground tissue were determined using GC-MS. The DON content in the infected issues with all 34 isolates can be founded in Table 2.

	Synergy		Pinnacle	
State	Isolate	average ugDon/g	Isolate	average ugDon/g
MN	FHK16	94.77	FHK16	133.02
NY	FHK31	43.83	FHK31	53.92
NC	FHK42	66.71	FHK42	119.15
ID	FHK63	25.60	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	24.90	FHK85	81.46
MD	FHK86	42.78	FHK86	54.73
ID	FHK90	44.66	FHK90	64.00
ID	FHK96	51.85	FHK96	106.80
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	94.70
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	107.50	FHK65	137.65
ND	FHK87	274.83	FHK87	198.77
MD	FHK93	175.15	FHK93	127.57

Table 2. Isolates and the amount of DON produced per Isolate from the Infection Assays.

We also estimated pathogen biomass in the barley head by using a Fluidigm nanofluidic automated real-time PCR system. Quantification of fungal DNA in the barley heads at 10 days post inoculation were submitted, using DNA extracted from 40-60mg of pulverized tissue. Fusarium specific primers and barley GAPDH primers were used to obtain relative biomass of Fusarium in infected tissue by qPCR (Figure 4). The biomass quantification analyses by qPCR for the 4th-7th infection assay are underway.

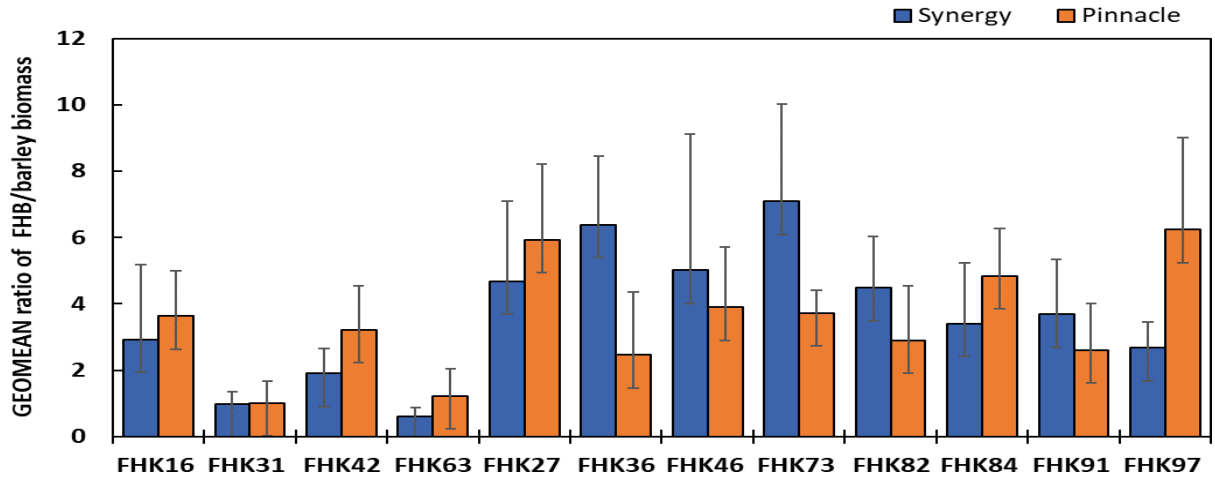

Figure 4. Quantitative PCR for 1st-3rd infection assay. qPCR results from the fungal DNA of 12 FHB isolates in barley heads at 10 days post inoculation and fungal biomass was estimated in barley heads by using nanofluidic Fluidigm.

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. The metabolomic data analyses for the 1st-5th infection assay with 22 FHK strains were completed in a different time frame than later assays. 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).

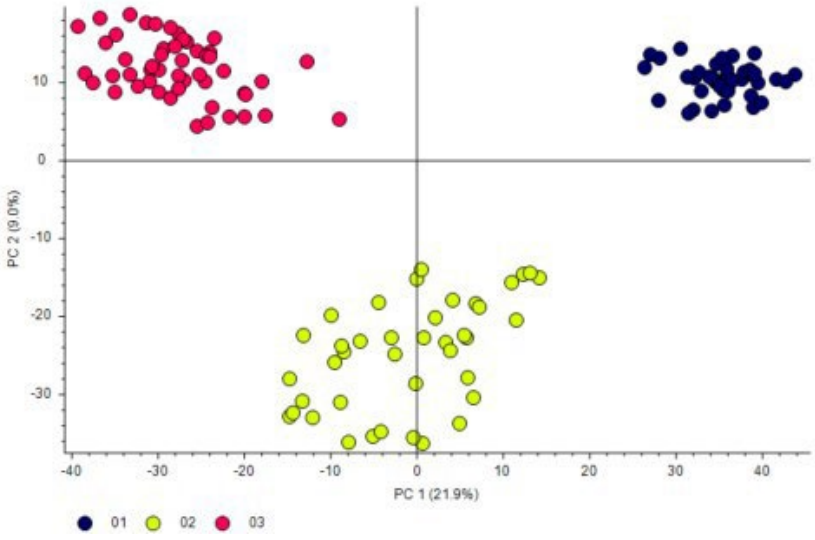

Figure 5. PCA Metabolomic plot combining disease assays 1-3.

What were the significant results?

Genome annotation, phylogenetic and SNPs analysis (Objective 1): We generated whole-genome sequence data for all 97 FHB isolates that we acquired for this project. All 97 genomes have been fully assembled and annotated by using the Funannotate pipeline that we set up on the USDA-SCINet (high performance cluster, Ceres). Phylogenetic analysis of three housekeeping genes retrieved from all 97 of the genome sequences confirmed that all 97 isolates were resolved with the reference *F. graminearum* strain, and it confirms that they are *F. graminearum* (Figure 1). In addition, we also included 2-4 reference isolates from known population structures for the phylogenetic analysis to investigate their population genetic relationship. As expected, most of FHB isolates from this project were grouped together with previously published population's structure data, mainly two North American (NA 1 and NA2). None of them are grouped with NA3 (NX-2), the gulf Coast and Southern and Louisiana populations which is consistent with SNP based Splits tree (Figure 2).

For SplitTree analysis based on SNPs calling, the majority number of isolates, a total number of 57 FHB isolates that produced 15 ADON in liquid media or solid rice substrate, grouped together as NA1 population. None of isolates that we examined belong to NA3 population. Interestingly, the 30 isolates out of 97 FHB isolates are grouped together into NA2 population (Figure 2). The FHK28 isolate which did not produce any trichothecene in a culture also belongs to NA2 population and 14 out of 30 isolates from the NA2 population are 15DON producing isolates and 16 of them are 3ADON producing isolates.

Infection assay (Objective 2): The results of the FHB assays with 34 isolates still varied depending on the isolate being used, and the two barley varieties did not have consistent relative FHB resistance. Inoculations with 18 of the isolates tested indicated that Synergy was slightly more resistant than Pinnacle. Most of the isolates caused similar levels of disease on the two varieties, and isolate FHK16 and FHK 97 caused significantly more disease on Pinnacle. This pattern was also seen in the reference strains that we used for the assay. (Figure 3). For example, all 6 isolates from each of populations (NA1, 2, 3) showed more disease severity. We reported that FHK28 that did not produce trichothecenes in cultures had the lowest levels of disease symptoms on both barley varieties last time. However, after the 6th infection assay with 12 more isolates, the isolate FHK81 and FHK 85 had the lowest level of disease symptoms especially on Synergy, while FHK28 had the lowest level of disease symptom on Pinnacle. This result is consistent with trichothecenes not being required for initial infection of barley but the lowest production of DON content in liquid or solid rice cultures (FHK81:17.87 ppm in Synergy and FHK 28: 34.55 ppm in Pinnacle) matches with their lowest disease severity. As expected, AAC Synergy was not as susceptible as Pinnacle. This suggests that the severity of the disease is fungal strain specific.

Toxin analysis from infection assay (Objective 2): As shown in the Table 2, two isolates coming from North Dakota (FHK81) and North Carolina (FHK37) produced the lowest levels of DON in Synergy, whereas one isolate from North Dakota (FHK87) produced the highest levels of DON in both varieties of barley, Synergy and Pinnacle, respectively (Table 2). This did not align with the strains exhibiting the most FHB, suggesting the severity of disease was not correlated with amount of DON production. However, the least amount of DON produced in AAC synergy was seen in strain FHK81 and it is consistent with lowest disease severity scoring data, especially in Synergy. In addition, the FHK 28 from Minnesota that did not produce the trichothecene in the laboratory media had smaller amounts of DON in both varieties of barley (e.g. FHK28:39.75 ppm in Synergy and FHK 28: 34.55 ppm in Pinnacle) but also it showed a lower level of disease severity scoring data. However, the isolate that produced the lowest levels of DON for Pinnacle (FHK37) did not always align with disease severity scoring data because the isolates FHK59 produced higher amounts DON than that of FHK37 but FHK59 showed lower disease severity score data that that of FHK37. In conclusion, severity of disease cannot be correlated with amount of DON production in planta and laboratory cultures.

Fungal biomass results only from 1st -3rd infection assay (Objective 2): In the last report, the quantification of fungal biomass had been completed only for the 12 strains used in infection assays 1 – 3. The highest levels of fungal biomass were detected in both barley varieties inoculated with FHK16, FSK27, FHK 84, and FHK97, which was consistent with disease severity and DON production. The lowest levels of fungal biomass were detected in both Synergy and Pinnacle tissues inoculated with FHK63, which also produced the lowest levels of DON in planta and caused the lowest levels of disease. So far, we have completed the DNA extractions for 4th-6th infection assays and these samples are ready for downstream analyses. However, it will not be processed until the DNA extractions for the 7th infection assay are done because we are saving space on the Fluidigm chip by filling up the whole 96 samples. The DNA extraction of 7th infection assay is currently being prepared, so all samples from the 4th-7th infection assays will soon be processed for fungal biomass quantification.

Metabolomic results only from 1st-5th infection assay (Objective 3): We recently completed metabolomic analyses from the 1st through 5th infection assays (Figures 6-10). 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 6-10).

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. We were also able to see some separation based on isolate and cultivar as seen in Figure 7. To ensure that the separation was not driven by DON we removed consideration of DON levels from newly generated PCA plots. After the contribution of DON is removed, we can still see separation of isolates (Figure 8). Metabolomic box plots showing the amount of DON for each isolate is seen in Figure 9.

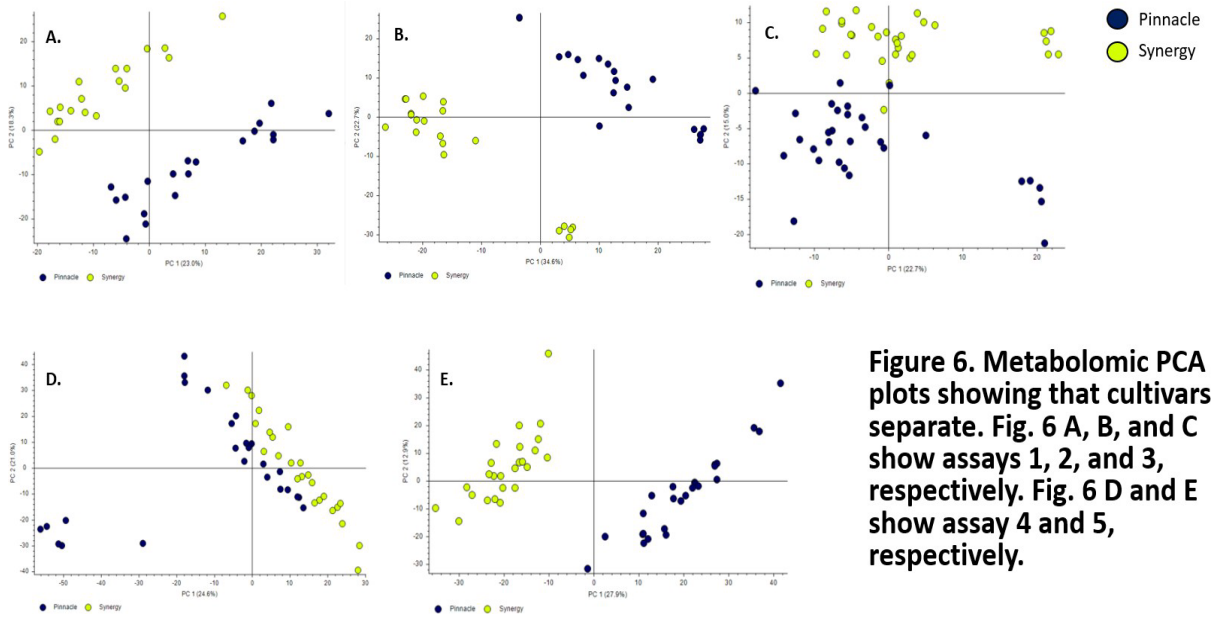

Figure 6. Metabolomic PCA plots showing that cultivars separate. Fig. 6 A, B, and C show assays 1, 2, and 3, respectively. Fig. 6 D and E show assay 4 and 5, respectively.

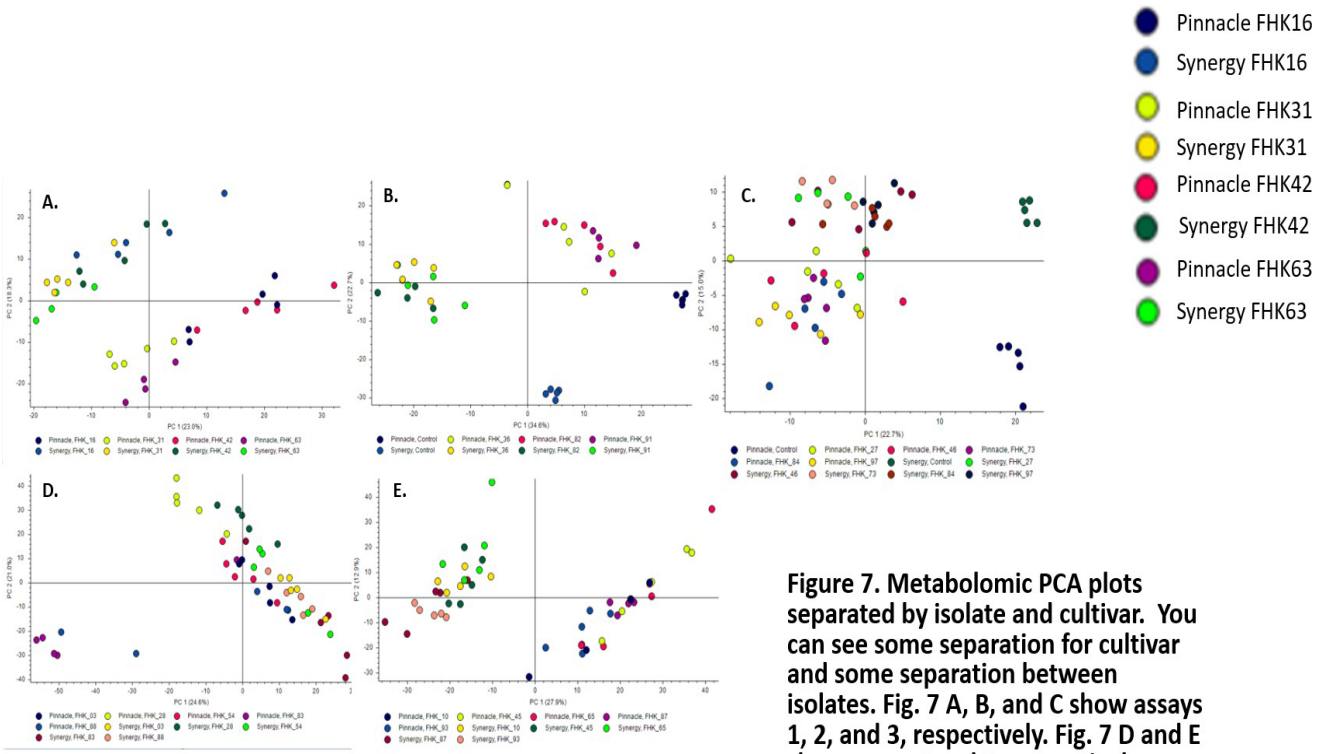

Figure 7. Metabolomic PCA plots separated by isolate and cultivar. You can see some separation for cultivar and some separation between isolates. Fig. 7 A, B, and C show assays 1, 2, and 3, respectively. Fig. 7 D and E show assay 4 and 5, respectively.

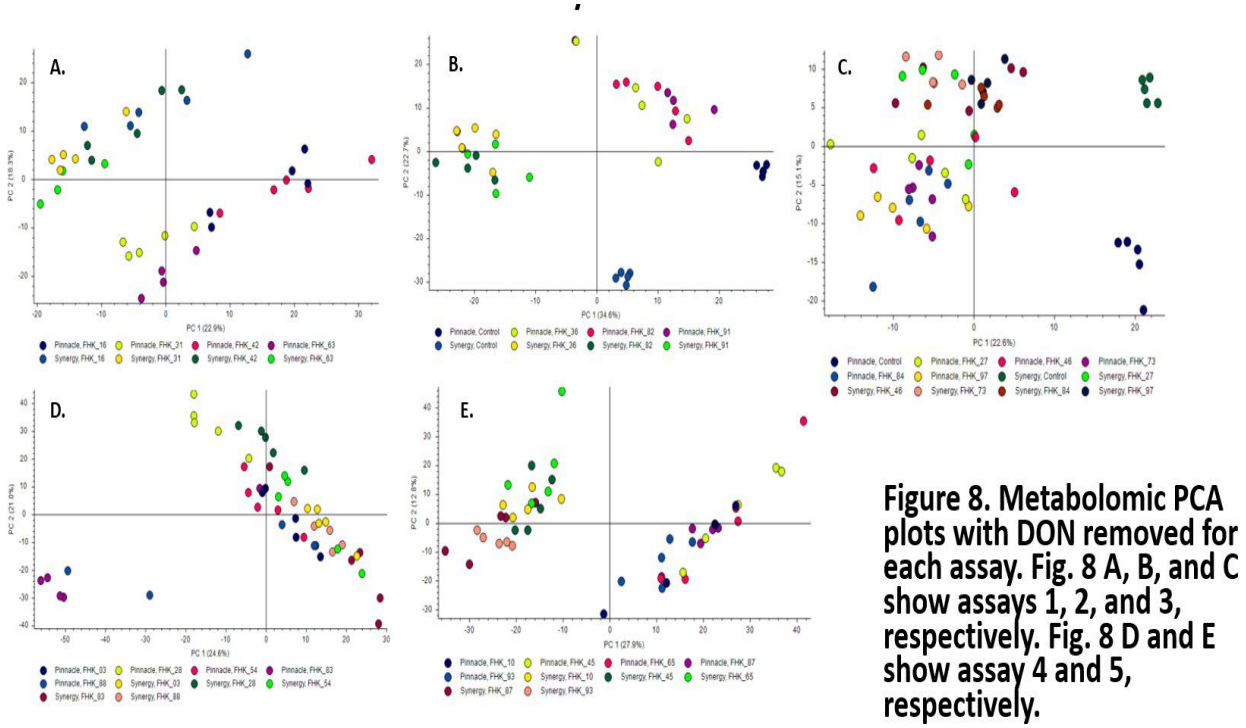

Figure 8. Metabolomic PCA plots with DON removed for each assay. Fig. 8 A, B, and C show assays 1, 2, and 3, respectively. Fig. 8 D and E show assay 4 and 5, respectively.

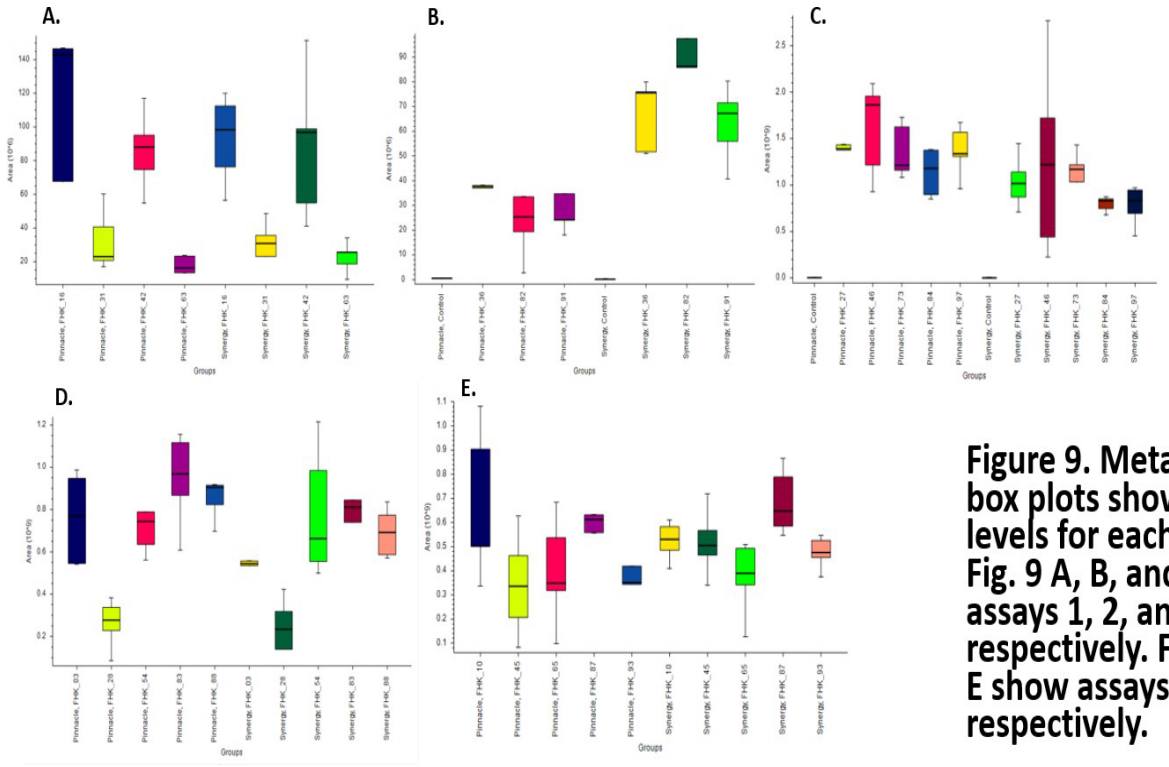

Figure 9. Metabolomic box plots showing DON levels for each isolate. Fig. 9 A, B, and C show assays 1, 2, and 3, respectively. Fig. 9 D and E show assays 4 and 5, respectively.

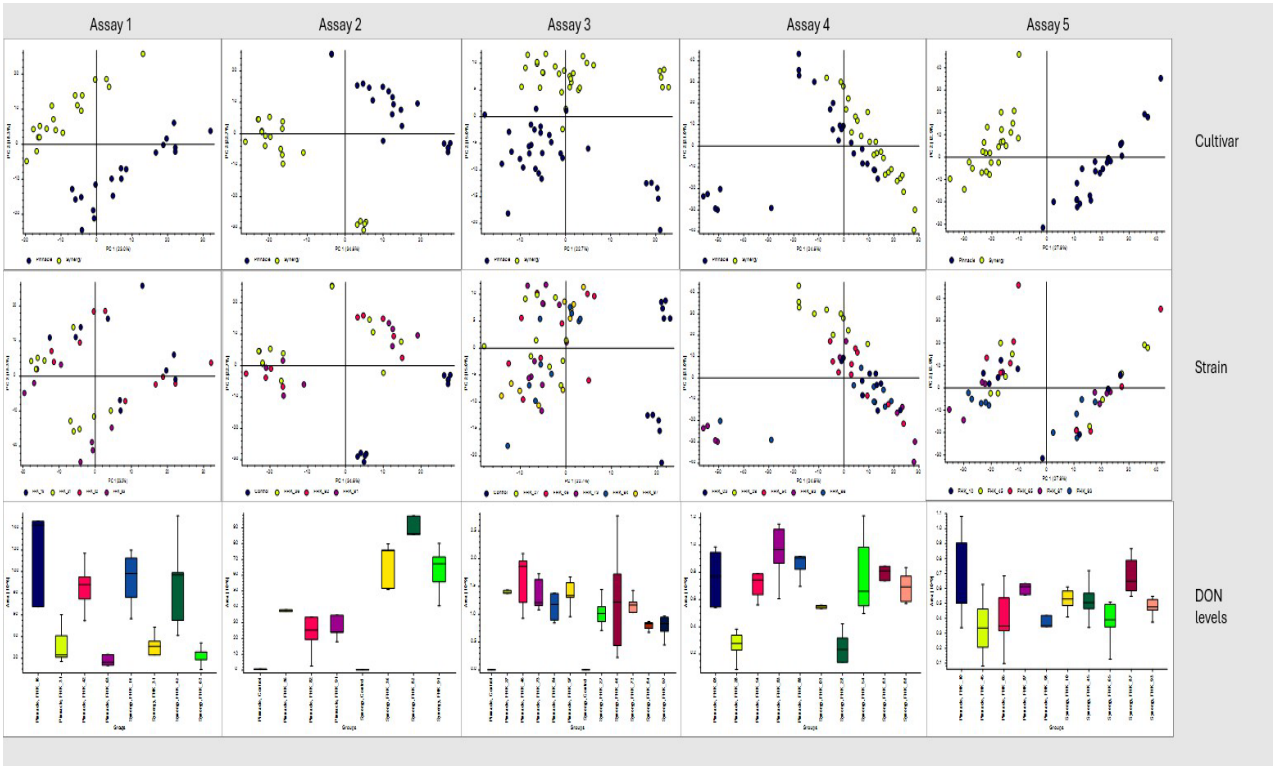

Figure 10. Metabolomic Data of Assays 01-05. Composite figures courtesy of Ethan Roberts.

List key outcomes or other achievements: Less than 1.5 year from the December 2022 hiring of an Oak Ridge Institute for Science and Education (ORISE) fellow, we have successfully generated these results through the team's hard work (Please see the above major activities and significant result section). These achievements include generation of whole genome sequences of all 97 FHB isolates, genome assembly and annotation for all genomes, SNPs analysis and infection assay with all 97 isolates and

reference strains from known population structures, downstream analysis including toxin, fungal biomass and metabolomics analysis from infected tissues. These accomplishments and achievements have allowed us to meet all milestones this year for each of the three objectives. We continue to complete remaining aims of the project in order to meet the multiple objectives and milestones for the following year.

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

I have trained the ORISE fellow on CLC Genomics Workbench for genome assembly, genome annotation, phylogenetic tree with IQ program, running different and parallel batch job scrips to SCINet (high performance cluster, Ceres) to annotate genomes by using Funannotate and run python script for modifying and adjusting input data compatible to the pipeline. The ORISE fellow also learned how to generate phylogenetic trees for evolutionary aspects, conduct infection assays, and analyze the fungal biomass and metabolomic data. The ORISE fellow will learn how to run Pangloss for the pan-genome analysis.

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

We did attend the 2023 National FHB forum that held in Cincinnati, Ohio (December 3-5, 2023) and presented this work in the SCAB meeting. We are currently working on first manuscript based on these new results and will plan to submit the paper soon.

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 to meet all our planned objectives. In collaboration with a researcher, Dr. Milton Drott in USDA-ARS, Cereal Disease Laboratory, MN, we recently received ~65 isolates of *Fusarium* species from wild grasses, and we are currently generating whole genome sequencing (WGS) for these isolates. With WGS data of these new wild grass isolates, we will continue to do the genome analysis that includes genome annotation, phylogenetic analysis, SNP analysis for underlying natural diversity of *Fusarium*. After that, we will select the best candidate isolates for the infection assay with same barley varieties and generate toxin data from these infection assays. Once annotation is completed for these new isolates, we will also start the pangenome analysis with all of these genomic data.