Project FY22-MG-003 / FY22-PB-004: Fusarium Species Diversity within Spikes and Fields: Implications for FHB Management

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

Goal: To survey the occurrence of minority *Fusarium* pathogens in FHB-symptomatic wheat and barley; understand environmental factors driving higher frequencies of minority species, including emerging mycotoxin producers; and determine how interactions between *F. graminearum* and weaker *Fusarium* pathogens impact FHB progression and mycotoxins.

The MGMT (Management) program goal is being addressed through Objective 1: Conduct a broad geographic survey of emerging/traditional *Fusarium* spp. and mycotoxin diversity and assess environmental factors (e.g., weather/climate, crop management) driving *Fusarium* diversity in FHB-symptomatic wheat and barley spikes.

The PBG (Plant Biology & Genetics) program goal is being addressed through Objective 2: Identify whether less aggressive *Fusarium* spp. reduce FHB caused by the aggressive pathogen *F. graminearum* if inoculated first or co-inoculated.

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

What were the major activities?

1) In 2024, we sampled 25 farms of winter wheat from Illinois. We collected ~40-60 symptomatic heads per field and collected data where available on local agronomic practices for that field. 266 pure isolates from 4 of 25 fields were isolated from Nash-Snyder, single-spored, and stored as glycerol stocks in the Whitaker Lab.
    a. Sequence editing of the partial TEF-1α sequence and species identification is complete.
    b. The remainder of the heads were bulked by field and frozen. As with our 2022 sampling, the plan is to freeze-dry, hand-thresh, mill the developing seeds to a flour-like consistency, and then perform toxin analysis for Deoxynivalenol, Nivalenol, and Culmorin.
2) We completed sequence editing and genetic identification of the 2023 spring barley and winter wheat *Fusarium* isolates collected from 12 farms in North Dakota and performed species identification on 347 pure isolates.
3) We are working to chemotype a representative subset of *Fusarium* isolates collected during our 2022 sampling effort in Illinois using High-resolution melt curve analysis, with validation using *in vitro* growth in agmatine media. This effort will be made possible with assistance from Drs. Elmore (USDA-St. Paul) and McCormick (USDA-Peoria).
4) Completed phytohormone measurements and Improved statistical analysis of the in-head competition assays between *F. graminearum* (15-ADON/3-ADON) and *F. poae* isolates in Alsen and Norm and completed phytohormone analyses.
    a. Developed *F. graminearum*, *F. poae*, and *F. sporotrichoides*-specific qPCR primers for the PHO and Tri1 genes. Performed initial validation tests.

5) Published results on Fusarium crown rot etiological agents in Illinois based on sampling in 2022 in the journal *Plant Disease*.

What were the significant results?

Risk for FHB was high throughout large portions of southern IL, IN, and OH in 2024. As such, we were able to sample 25 fields in southern IL (Fig. 1) ranging from low/moderate risk at the time of flowering (primarily the southeastern counties) to high risk (southwest to central IL around the Springfield region). Our goal is to compare our previous results from 2022 (i.e., primarily *F. graminearum* isolates, unexpectedly high Nivalenol toxin in the grain) to the results from 2024.

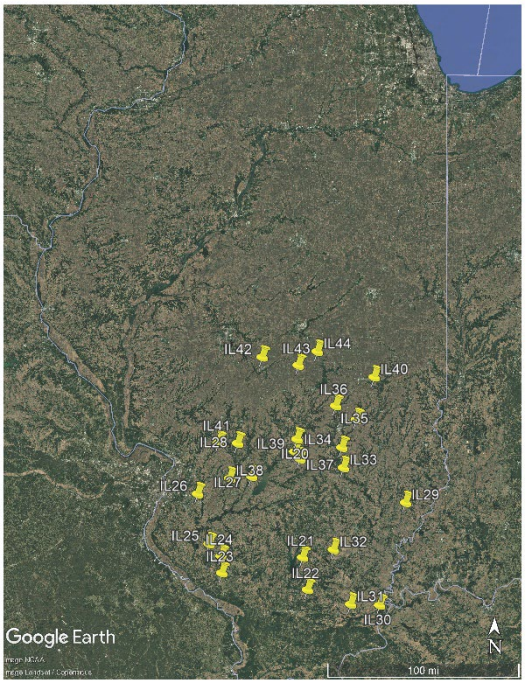
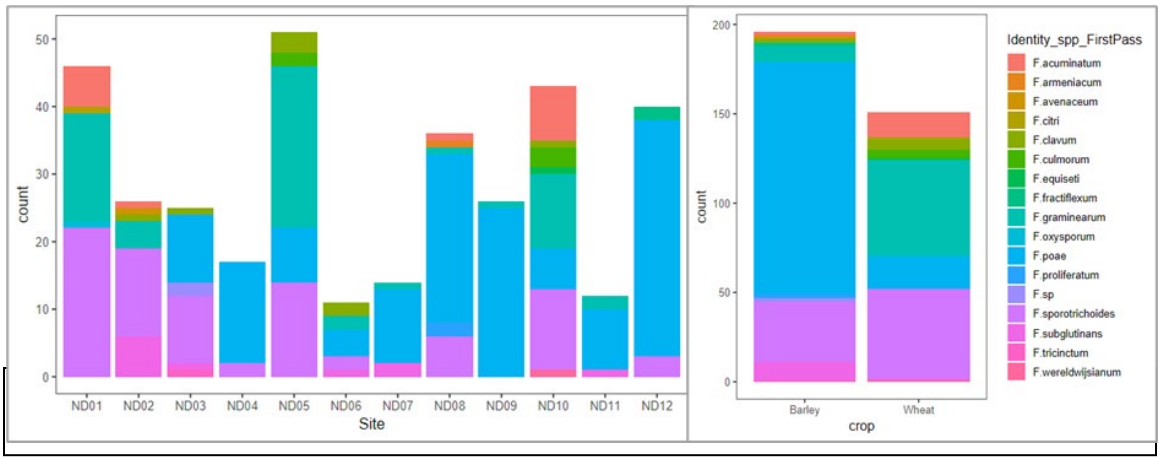


Fig. 1 – Map showing location of 25 Illinois winter wheat fields sampled for Fusarium spp. and toxin in 2024.

We used Nash-Snyder media to isolate *Fusarium* from 4 of 25 fields, with preliminary phylogenetic analysis of the partial TEF1 α complete for 266 isolates completed. Phylogenetic analysis indicated that 100% of the isolates were *F. graminearum*, which is similar to the predominance (>95%) of *F. graminearum* from IL wheat heads in 2022. Hand-threshing of grain collected across all 25 fields is ongoing, but once complete – samples will be extracted and toxin profiles (DON, NIV, CUL) quantified on a GCMS, as before.

Additionally, we completed sequence editing and preliminary phylogenetic identification from the 347 *Fusarium* strains isolated in 2023 from across 12 winter wheat and spring barley fields in ND. Risk was generally low for scab in ND in 2023; however, a much higher diversity of *Fusarium* species was noted in ND wheat and barley then seen in IL in either 2022 or 2024. Specifically, *F. poae*, *F. graminearum*, and *F. sporotrichoides* were the most abundant species overall, with variable abundances



across fields and crop type (Fig. 2). For example, while *F. poae* was more common in barley and *F. graminearum* more common in wheat, *F. sporotrichoides* was relatively abundant in both crops, and all three major pathogens were isolated from both crops (Fig. 2).

Interestingly, we also identified multiple co-infected isolates within the same wheat or barley head. Of the 24 successfully co-isolated pairs of *Fusarium* from a single barley head, 21% represented different species. Of the 37 successfully co-isolated pairs of Fusarium from a single wheat head, 41% represented different species. **We are hoping to use this information to design more useful co-inoculation experiments for Obj. 2.** For example, dual infections of *F. graminearum* and *F. sporotrichoides* were comparatively common in wheat, and a study of their co-infection's impact on toxin profiles could be warranted.

Lastly, we are working with the Elmore and McCormick labs to chemotype a representative subset of *Fusarium* isolates collected during our 2022 IL sampling effort. We have extracted DNA from 88 isolates and will perform a multiplex, high-resolution melt curve analysis of the *TRI1*, *TRI8*, and *TRI13* genes, which together can distinguish between the four potential chemotypes for *F. graminearum* (3-ADON, 15-ADON, NX-2, NIV; Singh et al. 2024 https://doi.org/10.1038/s41598-024-81131-5). Cultures will be tested for toxin production in both Agmatine and Rice media to validate the results of the DNA-based method.

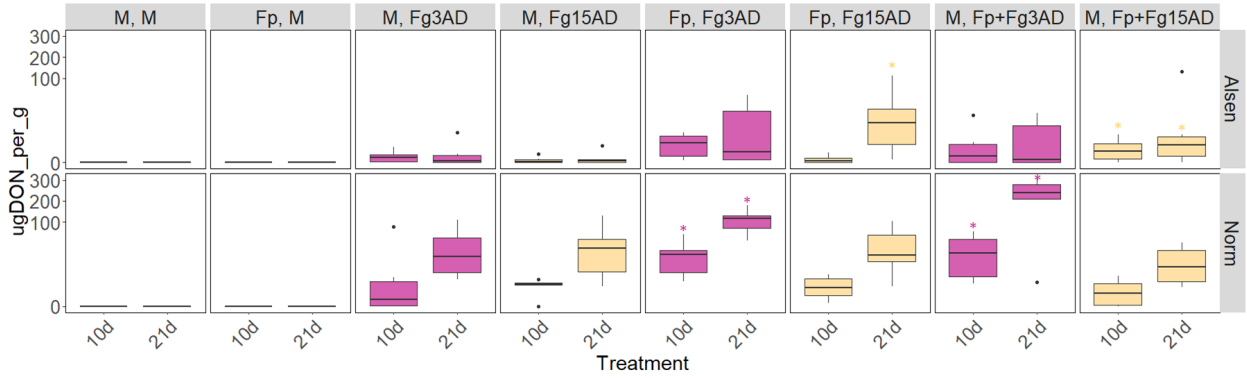


Fig. 3 – DON toxin accumulation across two wheat varieties and eight Fusarium competition treatments. M = mock, Fp = F. poae, Fg3AD = F. graminearum 3ADON, Fg15AD = F. graminearum 15ADON. Alsen (top panels) and Norm (bottom panels) varieties. Treatments where Fp was inoculated two days prior to a Fg strain are separated by a comma; where they were simultaneously inoculated a plus symbol is used.

For the PBG portion of the grant, we completed the GC-MS-derived phytohormone analyses for the experiments where an *F. poae* strain (Fp) was either pre- or co-inoculated with either a 3-ADON *F. graminearum* strain (Fg3) or a 15-ADON *F. graminearum* strain (Fg15) in two host varieties (Alsen and Norm, spring hard red wheat). All the results were derived from a paired set of experiments: in the first, we allowed the heads to mature and measured disease symptoms; in the second, heads were collected, 1,2,3, & 4 days post-infection and used for phytohormone and gene-expression measurements. **Ultimately, the impact on DON mycotoxin accumulation of**

competing *F. poae* and *F. graminearum* strains was cultivar and strain dependent (Fig. 3). Specifically, DON accumulation was greater in Alsen hosts when the Fp strain was either pre- or co-inoculated with the Fg15 strain, relative to infection by Fg15 alone (Fig. 3). Our analyses of DON accumulation and *F. graminearum* biomass showed consistent trends within cultivars but did not correlate well with disease severity (data not shown) – indicating mycotoxin accumulation may not correspond synonymously with visible symptoms.

Unfortunately, the jasmonic acid (JA) and salicylic acid (SA) quantification data did not reveal any statistically significant differences that could explain the noted differences in disease severity and DON accumulation (Fig. 4). This leads us to speculate that other virulence and defense factors are shaping the outcome of *F. graminearum-F. poae*-wheat interactions *in planta*.

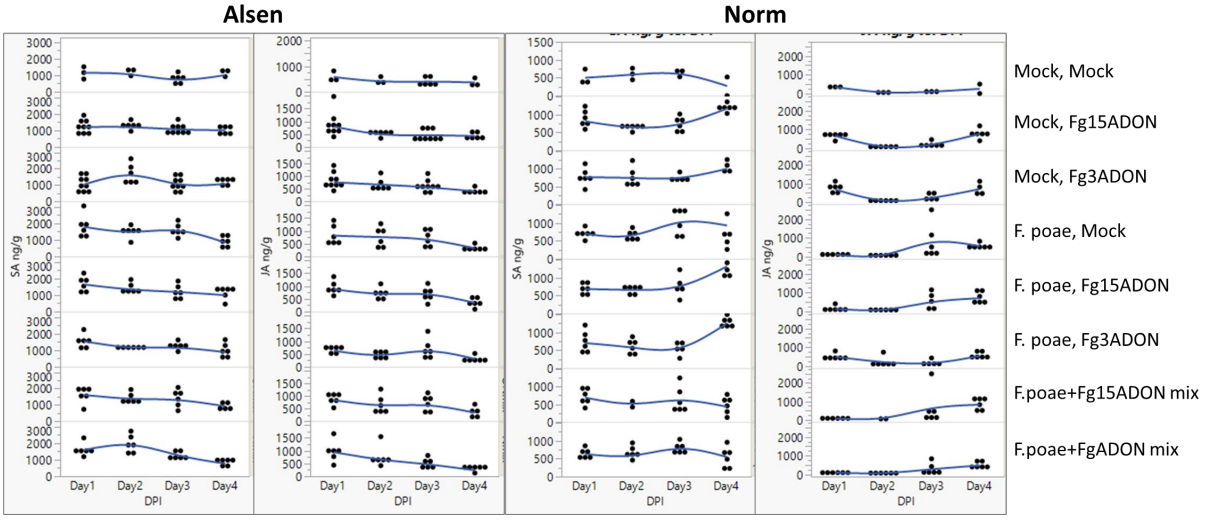


Fig. 4 – Phytohormone concentrations in the four days post infection (dpi), across two wheat varieties and eight Fusarium competition treatments. Alsen (two leftmost panels) and Norm (two rightmost panels) varieties. (abbreviations) SA = Salicylic acid, JA = Jasmonic Acid, Fg3AD = F. graminearum 3ADON, Fg15AD = F. graminearum 15ADON. Treatments where Fp was inoculated two days prior to a Fg strain are separated by a comma; where they were simultaneously inoculated a plus symbol is used.

List key outcomes or other achievements.

1) *F. graminearum* was the primary causal agent behind FHB in Illinois winter wheat, in a low-moderate and moderate-high risk year (2022 & 2024).
2) *F. sporotrichoides*, *F. poae*, and *F. graminearum* were the primary causal agents of FHB in North Dakota winter wheat and spring barley (2023), with noted variation across crops and fields. Importantly, *F. sporotrichoides* can produce T-2 and *F. poae* can produce NIV, which are emerging mycotoxins of concern.
3) Fusarium Tricinctum Species Complex (FTSC) species, including *F. avenaceum* and *F. acuminatum*, are known producers of emerging mycotoxins. However, FTSC contamination was minimal in IL Wheat (2022, 2024) and in ND small grains (2023).

4) Simultaneous dual infections of *Fusarium* strains are common in field grown wheat and barley. *F. graminearum* and *F. sporotrichoides* dual infections were comparatively more common in ND wheat and barley, yet the impacts of these co-infections on mycotoxin profiles and disease are unknown.
5) The impact on DON mycotoxin accumulation of competing *F. poae* and *F. graminearum* strains is cultivar and strain dependent.
6) Phytohormone analysis did not reveal notable changes over time, or across *Fusarium* species treatments, that could indicate mycotoxin accumulation profiles.

Key Outcomes: 1) We identified high *Fusarium* spp. diversity across 12 fields of winter wheat and spring barley in ND in 2023, with implications for emerging mycotoxin risk in this Great Plains state. 2) In addition, our *Fusarium* competition study indicates that predicting mycotoxin risk from competing *Fusarium* strain infections *in planta* is complicated by strain and cultivar-dependent effects.

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

This project has already provided training and professional development to 5 individuals at various career stages. Specifically, Imane Laraba served as a co-investigator while an ORISE funded postdoctoral scientist. She received training in mentorship of a graduate student and intern, as well as developed skillsets in plant hormone analysis. Dr. Laraba also participated in an outreach activity, by providing expertise in wheat pathology to local farmers. Pete Oppenheimer is a PhD student in the Cowger lab at NCSU, who received training in field sampling, fungal isolation, inoculation projects, and has developed methods in high-throughput *Fusarium* detection. Pete presented his research at the Scab 2024 conference in lightning talk and poster formats. This project also partially funded two post-degree interns Karly Cazzato and Nate Tyler, who received training in field sampling, fungal isolation, and molecular detection. Karly used the skillsets to pursue a career in statistical analysis, while Nate was recently accepted into a master's program in plant pathology at Michigan State University with Dr. Marty Chilvers. Lastly, Odalis Curzio was an undergraduate student intern from NEIU, who assisted and received training in the laboratory isolations for this project.

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

Imane Laraba and coauthors submitted and had accepted a manuscript on Fusarium crown rot etiological agents in Illinois in 2022 (Plant Disease https://doi.org/10.1094/PDIS-09-24-2034-RE). Pete Oppenheimer and coauthors submitted a manuscript on Fusarium metabarcoding to ISME Communications.

Briana Whitaker presented the project goals and previous results from the 2022 Illinois Wheat sampling to the NC1183 Mycotoxins in a Changing World extension meeting (May 2024), NCERA-184 Small Grain Diseases extension meeting (Feb 2025), and to the Illinois Wheat Association Annual Southern Plot Tours (May 2024).

Pete Oppenheimer presented a portion of this grant's results as a talk at the NC1183 Mycotoxins in a Changing World extension meeting (May 2025).

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

To continue Objective 1, we repeated sampling of wheat farms in southern Illinois in 2024 and 2025. With the spring showers in early to May 2024 and in specific southwestern counties in May 2025, we expect a wider range of FHB risk to provide a fuller picture of Nivalenol or 15 ADON contamination during high-risk years/regions. In addition, we are planning to expand the synthetic spike-in method for *Fusarium* metabarcoding to perform simultaneous diversity surveys and quantification of Fusarium species in mixed grain samples. This method has higher-throughput than culture-dependent methods and could provide more rapid identification of emerging etiological agents of concern in FHB.

To continue Objective 2, we will complete the design of qPCR primers that can successfully distinguish strains within the F. sambucinum species complex (including *F. poae*, *F. graminearum*, and *F. sporotrichoides*). It is possible that additional information on *F. poae* biomass could inform the differential toxin and disease severity patterns between the Alsen and Norm wheat varieties, as well as between the 15-ADON and 3-ADON producing strains of *F. graminearum*. Because the hormone data did not reveal informative patterns for the mycotoxin accumulation in the Fp-Fg3AD/Fg15AD experiments, we will instead consider experiments examining patterns of co-infection revealed by the ND survey. In particular, the higher relative occurrence of mixed *F. sporotrichoides* and *F. graminearum* could lead to higher risk for emerging mycotoxin and DON co-accumulation within heads and is a relationship that has been less explored in the literature. Finally, the ongoing chemotyping of collected strains from Obj. 1 is in progress and will reveal which isolates from ND produce emerging mycotoxins.

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

Fusarial diversity. This project aims to further illuminate the degree of species diversity among *Fusarium* strains that are causing FHB epidemics in small grains in the eastern US. A major goal is to get a better understanding of which *Fusarium* mycotoxins may be present in FHB-symptomatic wheat in this eastern US region. The importance of this is underscored by recent findings of substantial nivalenol-producing *Fusarium* infections in Illinois wheat.

Co- and pre-inoculation. In this experiment, the Raleigh team is testing *F. poae* and *F. acuminatum* as pre- and co-inoculants with *F. graminearum* on wheat heads. The goal is to determine whether pre-or co-inoculation with a less aggressive *Fusarium* species reduces the disease and toxin damage caused by *F. graminearum*. Inoculation is carried out with sprayed suspensions of spores.

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

What were the major activities?

Fusarial diversity. The process of sequencing and analysis for the synthetic spike-in metabarcoding (SSIM) assay was completed. This is a method for detecting and identifying *Fusarium* species in grain samples that relies on quantitative next-generation sequencing (NGS) methodology. The single-copy *TEF1* gene is amplified, so that species-specific primers are not needed and any *Fusarium* species can thus be detected in a sample. A spike-in is added to each sample, which allows the absolute abundance of each *Fusarium* species in the sample to be determined, such that the abundance of *Fusarium* species in samples can be accurately compared. For example, the new method enables us for the first time to detect multi-species co-infection of individual spikes.

The SSIM method was verified on 24 wheat samples from Italy, each representing a collection of symptomatic heads in a different field. Mycotoxins were predicted using the new method, and their presence and concentration were compared to quantification using LC-MS/MS.

Co- and pre-inoculation. Inoculation is carried out with sprayed suspensions of spores. In total, , the Raleigh team has performed six greenhouse inoculation trials that have generated data on disease severity and DON concentration. The first four of these trials have generated data on disease severity, DON, and plant defense hormones (jasmonic and salicylic acid, JA and SA). The final two greenhouse trials, conducted in December-March 2025, have served to generate samples for JA and SA analysis of this same co-inoculation pairing in order to strengthen the hormone component of the investigation. Samples are currently being analyzed by USDA-ARS staff in Peoria, IL.

At the same time, the experiment has so far included three completed field trials. The 4th and final field trial was planted in October 2024, rated in spring 2025, and harvested in spring 2025. Toxin data should be available in early fall 2025, and this will give us all the data from the field trials needed to finalize that analysis.

What were the significant results? List key outcomes or other achievements.

Fusarial diversity. The SSIM method is a novel and useful way of determining Fusarium species identity in wheat spikes, and could be adapted to other small grains. A manuscript about the new SSIM method was submitted to ISME Communications and reviewed; it is currently being revised. The method is being used for a survey of FHB-prone states in the soft winter wheat region (more information below).

Co- and pre-inoculation. Preliminary results show the *F. acuminatum* co-inoculation with *F. graminearum* reduced FHB symptoms and DON by 16% and 23%, respectively. In the greenhouse, preliminary results indicate that under conditions simulating low disease pressure, co-inoculation with *F. poae* can lower *F. graminearum* disease symptoms by ~27% and DON by ~28%. It should be emphasized that these results are preliminary; further data needs to be analyzed before final conclusions can be reached.

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

Graduate student Peter Oppenheimer is being trained in molecular analysis, field plot technique, inoculation and rating methodology, statistical analysis methods, population genetics, manuscript preparation, and public presentation.

Technicians are being trained in field plot technique, preparation and application of Fusarium inoculum, harvesting methods and sample processing.

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

- Peter spoke at the NC1183 "Mycotoxins in a Changing World" meeting at the Corn Dry Millers Conference. This occurred at the Donald Danforth Plant Science Center on May 28-29, 2025, St. Louis, Missouri.
- Briana Whitaker presented some of Peter's preliminary results along with her own work on fusarial diversity to the NCERA-184 meeting in February 2025 in Pensacola, FL.

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

The SSIM method is being used for a second study of *Fusarium* species diversity in 1,357 individual symptomatic wheat heads from 45 fields in six states. The fields were sampled in 2020, 2021, 2022 or 2023. The states are Kentucky, Pennsylvania, North Carolina, Virginia, Maryland and Georgia. This study will give us a comprehensive picture of FHB-causing species in wheat across a broad geographic area, and we will be able to look for trends based on previous crop and tillage.