MYCOTOXINS: BIOSECURITY, FOOD SAFETY AND BIOFUELS BYPRODUCTS (NC129, NC1025)

NON-TECHNICAL SUMMARY: Mycotoxins associated with crops contamination can adversely affect animal and human health and result in economic losses to multiple sectors of the agriculture community including the producer, handler, processor and consumer. The most important relevant issues facing grain and livestock producers are preventing mycotoxin contamination of food and feed, and reducing the deleterious effects of mycotoxins on livestock. Mycotoxins in grains processed for biofuels becomes concentrated in the solid byproduct known as distillers grain (DG), thus mycotoxin contamination is a major concern. For grain buyers and food processors, the primary need is a reliable method for rapid assessment of grain quality pertaining to mycotoxins and mycotoxigenic fungi. These stakeholders need cost-effective methods to predict, monitor, and minimize mycotoxin production in the field, and to detoxify mycotoxins. The lowering of tolerance limits for mycotoxins in overseas markets has increased the burden for grain buyers and food processors; currently, levels of mycotoxins that are acceptable for some US products are unacceptable in European and Asian markets, resulting in non-tariff trade barriers. Mycotoxins play a global role in human and animal health; their effects are well documented such as Fusarium mycotoxins include fumonisins (neurotoxicity, hepatotoxicity, cardiotoxicity, and carcinogenesis), T-2 toxin (hemorrhage, enteritis, and immune suppression), deoxynivalenol (DON; decreased weight gain), zearalenone (estrogenic), and fusarochromanone (bone deformation). Thus, the adverse effects of mycotoxins are usually related to the mycotoxin type. Aflatoxins, fumonisins, and ochratoxins are considered human/animal carcinogens. Our research focuses on several mycotoxins and fungi that produce them. Fusarium graminearum causes a devastating disease in wheat, barley and corn (Fusarium Head Blight-FHB). The impact of this disease on human and animal health is primarily due to production of several mycotoxins by the fungus which then contaminate grain. Our work focuses on understanding how and why the fungus infects these grains, how it survives through the winter and how it reinfects in the spring. Barley malt is crucial for the beer industry, so we are studying FHB toxin development especially in barley. We use studies of gene expression and genetic manipulation to determine what factors are important to the fungal life cycle. Knowing what structures are used to survive year to year and to produce inoculum allows us to develop novel methods to reduce the impact of the disease cycle. An assay system using a model plant species has helped define genes that enhance resistance to FHB, which have been selected for introduction into wheat and barley and which are then tested for improved FHB resistance in the field. In genetically modifiable animal model systems, we are working to define which processes mycotoxins interfere with; this approach can screen for compounds that protect against FHB-related mycotoxins. Aflatoxins are produced by Aspergillus flavus. In the southeastern region of the United States, the infection of Aspergillus flavus in maize is a chronic problem. Commercial maize hybrids are generally susceptible to Aspergillus flavus. Significant levels of corn host plant resistance to the fungus have been bred into some inbred maize lines such as Mp313E and Mp715. However, the resistance, controlled by many genes, must be integrated into commercial maize lines to improve corn production. Our research focus on identification of as many corn resistance genes

as possible will speed up molecular marker aided resistance breeding. We are also involved in the identification of corn metabolites inhibiting A. flavus propagation in the rachis (corn cob). Our research studies fungal dispersal and AF production, with a focus on a fungus-specific regulator VelB. This project will test its central role by identifying genes that it controls and defining associated gene networks related to fungal growth and AF production. VelB may be an excellent broad-spectrum anti-fungal target. Another fungal virulence factor we study is LaeA; its signaling pathways important in fungal pathogenicity and A. flavus receptor targets that impede aflatoxin synthesis are being investigated. Our project's cooperative efforts are intended to accelerate progress in understanding mycotoxigenic fungal biology to facilitate diminishing fungal hazards to our food supply.

OBJECTIVES: 1. Develop data for use in risk assessment of mycotoxins in human and animal health 2. Establish integrated strategies to manage and to reduce mycotoxin contamination in cereal and distiller's grains. 3.Define the regulation of mycotoxin biosynthesis and the molecular relationships among mycotoxigenic fungi.

APPROACH:

Objective 1: Research methodologies and approaches will include: dose-response studies in animal models; analysis of mycotoxin structure-function relationships, and mechanistic studies of mycotoxin effects on mammalian cellular activity and gene expression. Dose response assessments will be used by IA to determine and model mycotoxin toxicity including acute toxicity, carcinogenesis, and immunomodulation. Exposure assessments for mycotoxins must take into account route of exposure duration of exposure to the mycotoxin. Dose response data for DON and DON-glucoside in mice will be obtained by IA. Data will be generated for risk assessment of the use of mycotoxin contaminated grains, focusing on fumonisins, with both conventional and insect-resistant transgenic maize, used in the biofuels industry and its subsequent byproducts. We will continue to assess acute toxicity of fumonisins and DON in mouse and other animal models in oral feeding studies with naturally contaminated foods or purified toxin.

Objective 2: Methodologies and approaches will include: development and application of molecular surveillance tools for detection of mycotoxins and mycotoxigenic fungi in grains and grain products; and analysis of methods for detoxification of grains and grain products. Committee members from MO, IA, and ND will collaborate on research on technology that will eliminate the mycotoxins or reduce their toxicity, such as use of binders in animal models. Yearly field surveys for mycotoxins will be performed on grain crops in various areas of KS. This data will give a precise idea about the frequency of mycotoxin occurrence and should help determine factors critical to prevent contamination in the future. This will assist to accesses risks associated with mycotoxins and prepare the US agricultural governmental agencies to deal effectively with a mycotoxin outbreak. Survey of current operations will be initiated to better

understand the extent of raw grain contamination and how that relates to the finished distiller's grains products (KS, IA). We will explore new sample preparation approaches for analysis of different mycotoxins simultaneously in various types of grain products. Mass spectrometry analytical methods, particularly using liquid chromatography for mycotoxins, will be developed to provide a wider application, easy of sample preparation, and higher sensitivity.

Barley samples will be obtained from commercial fields, regional grain elevators, and inoculated nurseries (ND). Samples will be micro-malted immediately after harvest, and then following several months of storage. The impact of malting process conditions on Fuasium growth and mycotoxin production will be evaluated. Trichothecene mycotoxins on barley and malts will be determined by GC-ECD and LC-QTOFMS. Fusarium biomass on the barley and malt samples will be estimated by real-time PCR. The overall objectives of these studies are to demonstrate the mycotoxins present on barley and transformations that occur during processing. This information will be of valuable to maltsters and brewers in the development of strategies to handle Fusarium infected barley

PCR-based methods to detect mycotoxigenic fungi have provided rapid and inexpensive alternatives to techniques based on morphology. Committee members from PA and ND will collaborate to expand the use of this PCR identification technique and related DNA technologies for surveillance. Others will continue to develop more multiplex assays from mycotoxin-specific genes. We will also work with university diagnostic labs to implement these techniques. Understanding the cause for high mycotoxin in asymptomatic grains PA will quantify mycotoxin levels in grains at different developmental stages and under different conditions of growth of the host plants.

Objective 3: Research methodologies and approaches will include: genetic and molecular genetic analyses of fungal genes involved in mycotoxin production and spore production in model Fusarium fungi. We use techniques of genetics and genomics to understand how gene expression is influenced by environmental conditions and the different hosts. Genes perceived to be important to life cycle and survival will be specifically disrupted and mutants will be used to determine the roles in survival and mycotoxin production. Histological techniques will be used to determine when and where the fungus establishes itself and how it survives environmental extremes.

Maize germplasm exhibiting resistance or susceptibility to Aspergillus flavus infection and aflatoxin accumulation are used for genome wide comparative analysis on gene/RNA/Protein expression levels. Samples are collected from field maize plants with appropriate experimental design for statistical analysis. Kernels from ears with or without inoculation of Aspergillus flavus are collected at different time points. Maize protein/DNA/RNA are then extracted from resistant and susceptible maize samples. Genomics/proteomics/transcriptomics/bioinformatics tools are incorporated to facilitate the identification of genetic factors involved in maize resistance to Aspergillus flavus infection and aflatoxin accumulation. Candidate gene sequences will be provided for use as potential molecular markers to assist resistant maize breeding (MS).

Maize hybrids with varying aflatoxin resistance have been inoculated with either toxigenic (NRRL 3357) and atoxigenic (NRRL 21882) strains of Aspergillus flavus or combinations of the two, in test plots in MS. Tissue samples were collected from plants inoculated with both fungal strains, those inoculated with water, and from plants that were uninoculated. Using qRT-PCR and strain specific molecular marker we have begun to determine the A. flavus biomass. Additionally, we are developing a handheld FT-IR method for fungal detection in the field. The volatile metabolome and other small non-protein molecules of maize in response to A. flavus infection and aflatoxin accumulation are currently being profiled with SPME and liquid extraction coupled to GC/MS and GC-QTOF technologies. Identification of A. flavus induced phytoalexins has also been initiated. A detailed time-course analysis of acidic terpenoid phytoalexins, namely zealexin sesquiterpenoids and kauralexin diterpenoids, present in individually inoculated maize kernels was performed. The two primary maize varieties focused on included Va35 and Mp719, representing established lines susceptible and resistant to aflatoxin accumulation, respectively. Challenged at 18 days after pollination, Mp719 kernels displayed significantly greater zealexin accumulation 3 days post inoculation (dpi) compared to Va35 using the same toxigenic Aspergillus flavus isolate 3357. Similarly, kauralexin levels were significantly greater in Mp719 at 7 dpi compared to Va35. In contrast to the more rapid accumulation of zealexins and kauralexins in Mp719, levels of these defenses present in Va35 were significantly greater than those of Mp719 at 21 dpi. Inoculated kernels, adjacent kernels, uninoculated kernels, and cob sections have been collected at various times after inoculation in field trials conducted in MS for several analyses: RNAseq technology is being used examine the gene response of different germplasm lines to both toxin producing and non-toxin producing A. flavus strains and to identify genes that are integral to the host/pathogen interaction. Tissues collected at Mississippi State have been provided to collaborators at other locations. Candidate genes are being evaluated by using qRT-PCR on kernels with or without inoculation of a toxin-producing A. flavus strain in different corn germplasm lines. Polymorphic DNA markers were designed and tested on the selected differentially expressed genes. Genes contributing to resistance with high statistical significance are being identified using Dr. Warburton's QTL and association study pipeline. Aflatoxin is being quantified by LC/MS-MS and cob sample are being analyzed via qRT-PCR for the expression of resistance related genes. Additionally, aflatoxin quantification (LC-MS/MS single kernel) procedures are being performed on control samples and those inoculated with both the toxigenic and atoxigenic strains of A. flavus.

Differentially expressed proteins across multiple samples can be identified and quantitatively evaluated by computational analysis of sets of 2D protein gel images. We have explored the use image processing methods (Matlab Image Processing Toolbox) for quantification of protein expression levels, and identified differentially expressed maize proteins associated with resistance to Aspergillus flavus infection and aflatoxin accumulation through the quantitative data analysis of 2D gel images. Resistant (Mp715, Mp719) and susceptible (Va35, Mp04:87) maize inbred lines were selected and developing kernels were collected from the primary ears of corn plants at 14 days after inoculation with A. flavus. Proteins were extracted using TCA/acetone precipitation in combination of a phenol extraction step. The 2-D protein gel electrophoresis was

performed using PROTEAN IEF Cell (Bio-Rad) and PROTEAN II XL cell (Bio-Rad). Proteins were visualized with florescent dye Oriole (Bio-Rad). Gel images were obtained with an Alpha imager. We have established a working protocol for quantitative proteomic studies of maize proteins by computational and statistical analysis of 2D protein gel images. It is a novel analysis procedure for profiling and comparative investigations of differentially expressed proteins to facilitate the development of DNA markers for maize resistance breeding.

The rachis samples from aflatoxin-resistant (Mp313E) and aflatoxin-susceptible (SC212m) line have been collected, sliced, frozen in liquid nitrogen and stored at -80ºC. The extraction of metabolites will be performed in quintuplets using the robotic sample preparation instrument Barocycler Nep2320 (Pressure Biosciences). Briefly, two grams of tissue from each inbred will be ground to a fine powder and lyophilized to remove water. To maximize recovery, the powder will be divided to two parts and metabolites will be extracted by methanol and acetonitrile using ultra-sonication. The use of a robotic sample preparation system will translate to an improvement in reproducibility and higher confidence in principal component analysis (PCA) of mass spectral data. The latter will be collected on a high resolution ORBITRAP (Thermo) mass spectrometer connected on-line to Ultimate 2000 (Dionex) HPLC system. A ninety minutes gradient and data collection will be performed in both positive and negative MS mode, with mass window of 100 - 1000 m/z, at the highest resolution of 100,000. The raw mass data files will be analyzed by SIEVE (Thermo) software. For positive and negative mode, ten technical samples will be grouped into two biological samples: A. flavus susceptible (S) and resistant (R), with five replicates each. First, the PCA of m/z values for detected peaks will be performed. Then, the S- and R-specific peaks will be tentatively identified based on accurate m/z measurement of monoisotopic mass, with tolerance of 1ppm. The SIEVE includes a "small molecule identification" module linked to metabolite databases (e.g. KEGG, ChemSpider and PubChem).The ultimate measure of the success of the project will be measured by the degree to which this preliminary data informs development of larger scale studies focused on A.flavus colonization of corn.

Genes are functionally defined in the model plant Physcomitrella patens, which (uniquely among plants) undergoes efficient homologous recombination and can be used to create site-specific gene knockouts. Genes are selected for functional studies based on knowledge of the existing literature, important genes flagged in other pathosystems, on transcriptome databases obtained in infected wheat, barley and other plants and from functional screens conducted in simple eukaryotes, such as yeast. Knockout plants that exhibit enhanced susceptibility to Fusarium Head Blight (FHB) and to mycotoxins reveal genes that normally contribute to resistance and mycotoxin sensitivity and whose over-expression may be used to enhance resistance in crop plants. Knockout plants that exhibit enhanced resistance to FHB and to mycotoxins reveal genes that are required for plant susceptibility to resistance and mycotoxin sensitivity and whose down-regulation may be used to enhance resistance in crop plants. The corresponding genes in barley or wheat are targeted for over-expression or down-regulation through the introduction of transgenes or RNAi-based constructs into

barley or wheat. These plants are then assayed for altered susceptibility to FHB, for altered sensitivity to mycotoxins and for the absence of any abnormalities associated with introduction of the transgene constructs. Research on the mode of action of Fusarium-derived mycotoxins is carried out using the model nematode, C. elegans. Comparison of the responses of wild type and mutant strains has allowed us to discern which animal pathways are required for sensitivity to mycotoxins. To-date, this approach has allowed use to delineate (and in some cases, exclude) a number of independent cellular pathways, including cell death and the endocytosis pathways. Ongoing research in this are combines the use of existing, defined, mutants, with the availability of transgenic reporter lines, and (more recently) the use of RNAi libraries to reveal novel pathways involved in toxin perception and sensitivity.

Global regulators of mycotoxin synthesis will be identified through mutagenesis, biochemical and/or genetic means in the model fungus Aspergillus nidulans. Orthologs will be found in common mycotoxigenic species (e.g. A. flavus and Fusarium spp) and assessed for conservation of action. Strategies to inhibit function of global regulators will be assessed.

We hypothesize that genes regulated by LaeA (a protein required for mycotoxin production) are involved in mycotoxin production. We will delete and overexpress candidate LaeA regulated genes to examine this hypothesis. Assessment of such mutant strains will be measured for mycotoxin production to refute or support the hypothesis.

PROGRESS:

Target Audience:

Corn growers in Arkansas and other states in the southern U.S., as well as the international mycotoxin research community, faculty and staff studying mycotoxins, food and feed industry professional, industry and start-up companies in the biofuel and bioproducts arena agricultural producers, malting and brewing industries, regulatory agencies (FDA, FSIS), USDA-NIFA panel for A1121 Program Priority Area Agriculture and Food Research Initiative Program: Plant Health and Production and Plant Products: Understanding Plant-Associated Microorganisms, Participants of 62nd ASMS Conference on Mass Spectrometry and Allied Topics (June 2014) and readers of Conference Proceedings, general public.

Changes/Problems:

One major change in the project is that rapid progress in the use of CRISPR/Cas9 technology makes this a more attractive choice for gene editing in barley and Brachypodium than the use of TALENS, which was our original intention. The former is quicker, cheaper and more specific and allows mulitplegenes to be targeted. Consequently, we haves witched our efforts to constructs that use this new technology

which we will test in Arabidopsis, Brachypodium before exploiting it for knocking out genes in Barley.

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

Investigators enhanced knowledge and practical ability regarding bioinformatic analyses of fungal genome sequencing and assembly and advanced in transition from proteomics to metabolomics via hands-on experience and interacting with respective colleagues during the American Society for Mass Spectrometry meeting. A research associate received training in operation of liquid chromatography - mass spectrometry instrumentation for analysis of small molecules and the software-based analysis of raw mass spectrometry data. We developed new bioenergy curricula. We have been providing trainings in molecular lab techniques and data acquisition and analysis to MSU graduate students, undergraduate students, and also high school students from the community to provide opportunities that students can be exposed to up to date molecular biology techniques in the research area. We also hosted international visiting scholars. We maintained outreach services to the community by hosting agricultural and food/feed safety experiences within our laboratory. Individuals from the community (74) participated in the following activities: “What killed my bees and contaminated my honey?” activity as a forensic experience for Bug Camp; “An Agricultural Crime Case-Focusing on Mycotoxin Analysis” activity as a forensic/feed safety experience for Winston Academy; Facility tours were provided to 4-H Participants during Summer 2014 highlighting food and feed safety. We also offered a demonstration of the practical application of analytical testing for petroleum products for environmental and mechanical engineers (50 individuals). 2 PhD students graduated in Toxicology with work related to this project, 2 other PhD students graduated; 6 other graduate students were mentored. Six undergraduate students were engaged in these studies.

How have the results been disseminated to communities of interest?

Professional Master's students have benefited from the new curricula. Results have been presented at professional meetings in chemistry, plant pathology and veterinary medicine. Presentations were made at the NCC annual meetings at Purdue University, and at Iowa State University, The Genetics Society of America, Gordon Research Conference, Food Research Institute (FRI) annual meetings, the ASMS Conference on Mass Spectrometry and Allied Topics, June 2014 and at the American Society of Brewing Chemists, and informally at the annual US Wheat and Barley Scab Forum. Scientists received a report at the MSA meetings hosted by MSU; results were shared with 2 companies. Two graduate students working on these projects attended and presented material at national research conferences. Both of these students provided extended abstracts and they were awarded ACS National Travel Grants. A number of papers were published.

What do you plan to do during the next reporting period to accomplish the goals?

Obj. 1) We will continue to develop data for use in the assessment of mycotoxins in human and animal health. We will seek funding to further study DON detoxification and mitigation by endogenous biotransformation in human cell/tissue models. We will continue to study mitigation of DON toxicity in swine production, especially in the context of diets including dried distillers grain solids. Obj. 2) We will outline strategies to mitigate mycotoxin contamination in food and feed products. We will continue to assess mycotoxin production by genetically modified maize variants. The capacity of barley and wheat genotypes to transform DON to DON3G will be evaluated during the next reporting period. DON will be applied directly to seeds during germination, and DON3G will be measured following 24, 48 and 72 hours. We will be analyzing two sets of samples for deoxynivalenol and deoxynivalenol-3-glucoside levels and for one sample set ergosterol levels as well as a measure of fungal growth. The first set of samples are from a previous field experiment. We will be looking at levels of deoxynivalenol and deoxynivalenol-3-glucoside at multiple time points from anthesis to grain maturity to learn about development of the masked mycotoxin deoxynivalenol-3-glucoside in different wheat varieties and over time. Currently we are working to re-establish a gas chromatography method for this purpose. The second of samples are from a growth chamber experiment that addresses the question of the impact of ambient temperature on in planta deoxynivalenol production relative to fungal growth measured by ergosterol. We have previous results indicating that lower temperatures are associated with higher levels of deoxynivalenol production relative to fungal biomass. This experiment will confirm or deny those previous observations. Obj. 3) We will continue to study novel mycotoxigenic fungal species and fungal species interactions, especially with Aspergillus spp. We will continue to evaluate Fusarium populations using other types of markers, and investigate the infection of corn by these strains of Fusarium wheat blight fungi. We will work with Diplodia zeae to understand the role of mycotoxins in pathogenicity to maize tissues. We plan to perform MSn experiments to confirm tentative identification of most differentially abundant metabolites present in cob tissue of fungi resistant maize genotypes vs. susceptible genotypes. We will characterize the first putative barley and Brachypodium transformants containing selected genes, verify their expression levels and determine function of the genes efficacy against DON and F. graminearum. We will engineer constructs designed to edit selected genes in Brachypodium, in order to test their role in FHB susceptibility. We will analyze the results of RNA-Seq from DON-treated C. elegans and select genes for further analysis. Gene expression levels will be verified by qRT-PCR. Genes whose expression is substantially induced or repressed by DON will be assessed through RNAi, gene editing or overexpression. The development of the C. elegans system paves the way for achieving one of our major goals, namely setting up screens for natural and synthetic compounds that can protect against DON toxicity in vivo. We are seeking funding to further explore the activities of anti-aflatoxin compounds and their practical application. To further understand molecular functions of the Velvet protein family, OsaA, and WetA in A. flavus, we will carry out more integrated genetic, genomic and physiological studies in the future. For example, we are planning to do RNA-seq analyses of the OsaA and WetA mutants. The RNA-seq data will be analyzed to find more mycotoxin- and conidiation-regulatory genes for further study. We will assess the role of BrlA regulated toxins on spore survival, determine the role of FleA on spore survival and if

FleA is required for adhesion to host surfaces or other microbes and ask if any GPCR receptor is involved in recognizing quorum sensing molecules. We will attempt to organize our project meeting in concert with Midwest AOAC in spring of 2015.

IMPACT: 2013/10 TO 2014/09
Obj. 1: A survey for mycotoxins in grain storage elevator (KS) was conducted in late September, 2014, early October, 2014, and early November, 2014. Seven different corn samples were collected and tested for the presence of aflatoxin, fumonisin, and deoxynivalenol (DON). Only three samples were contaminated with fumonisin. The fumonisin levels in seven samples were very low ranged (0.01 ppm to 7.71 ppm). All the samples showed a negative result for aflatoxin contamination. In all samples, DON level was also below the allowable limit and ranged from 0.00 to 0.01 ppm. This year's weather was not extreme therefore, the results were negative and it also showed proper harvesting and storage of the grains. As part of this work the formation of Deoxynivalenol-3-glucoside (DON3G) during malting was investigated. DON3G is a bound, or conjugated, form of deoxynivalenol (DON). There is concern over the presence of DON3G in commercial samples of barley and malt, as it is not measured by routine analytical methods for DON. It has been proposed that DON3G may be broken down or transformed, to free DON during digestion or in food processing, and if this is the case, DON3G and other conjugated forms could make a significant contribution to the tolerable daily intake (TDI), and should be measured. DON-3-G was observed to increase by an average of 48-fold during malting. Levels ranged from the limit of detection to 65.84 mg/kg on the malt. In all cases, the levels of DON3G, greatly exceeded those of DON. The formation of DON3G during malting is attributed to the presence of UDP-glucosyl-transferase enzymes in germinating barley. A preservative/antioxidant blend mitigated DON toxicity in swine in terms of improved average daily gain. DON at 5 ppm decreased liver selenium compared with controls in swine fed for 120 d. A system was developed to study the toxicity of DON that uses Caenorhabditis elegans. The lifespan and egg-laying capacity of DON-treated nematode worms is significantly reduced compared to controls. This assay system will allow us to study mechanisms of toxicity of DON and other mycotoxins, and to test potential mitigation strategies, in a simple animal model. We performed RNA-Seq using the C. elegans system in order to identify genes that are potential targets for DON and which may also be involved in detoxification mechanisms. Genes that are up-regulated by DON include a number involved in detoxification and innate immunity, while down-regulated genes are involved in metabolism and development. The significance of these genes can now be assessed through the use of RNAi suppression or overexpression in transgenic worms.

Obj. 2: Research focused on the development of new protocols for production biofuels and chemicals. Genetically modified lines of maize containing newer Bacillus thuringensis (BT) genes were assessed for content of fumonisins (FB) and susceptibility to insect damage (IA). A new BT gene was very effective in reducing FB contents compared with the older BT versions. Ethanol was made from corn containing up to 8 ppm FB, which did not adversely affect ethanol yield. Spiking ethanol fermentation with even higher levels of FB also did not affect ethanol production. Dried distillers grains had about 3 fold enrichment of FB in 50 out of 57 batches. The 7 batches showing

lesser increase of FB are planned to be investigated further. The role of mycotoxins as virulence-enhancing factors in plant disease was studied in seedling maize, soybeans, and wheat. We have identified 4 compounds that affect the production of aflatoxin by Aspergillus and deoxynivalenol by Fusarium graminearum. We have examined their effect in culture using pure synthesized compounds. This project accomplished the first study describing a QuEChERS method for the quantitative determination of AFM1 in raw milk using HPLC-MS. The effectiveness of the binders to separate AFM1 from the milk using QuEChERS as an extraction method was possible as QuEChERS produced 3 layers separating the AFM1 in the organic layer, the middle layer contained the binder from the sample (and AFM1 if the binder was efficient), and the lower aqueous layer and the excess salts.

Obj. 3: The genome sequence data for Stenocarpella maydis was refined with a more complete assembly. This work was performed in close collaboration with other members of the multi-state working group (IN, KY). This work produced a genome assembly that is comparable to published de novo genome sequences in other filamentous fungal pathogens. Work on FB production by black Aspergillus spp. (IA) showed that a good portion of these isolates produce FB2 in the laboratory, but low levels compared with F. verticillioides or F. proliferatum. Drier regions had greater black Aspergillus in maize, which co-occurred with A. flavus. The interactions between fungal species and mycotoxigenesis are planned to be further studied. We developed some novel markers and used them to analyze a population of Fusarium graminearum from Kentucky (Bec et al., 2014). We learned that most of the isolates belonged to the dominant chemotype, but that genetically they showed signs of being an isolated divergent population, suggesting there is not a lot of mixing of isolates from outside of Kentucky. We found two species that had not been previously described on symptomatic wheat heads, but these did not cause symptoms. We postulate that these colonize the tissues killed by the scab fungus. This is significant because these other species produce different types of mycotoxins. We have developed Brachypodium distachyon as a model system for studying infection by F. graminearum and the effects of DON. Detached leaves of B. distachyon can be infected with GFP-labeled wild type F. graminearum and tri5-mutant F. graminearum strains, and are also sensitive to the DON application. We have now developed B. distachyon and barley tissue culture and transformation protocols using immature and mature seeds, and have started a program to exploit CRISPR/Cas gene editing technology to engineer FHB resistance in these plants. The model plant Arabidopsis thaliana is being used to test efficacy of CRISPR/Cas gene editing to improve FHB resistance. A number of genes required for FHB susceptibility and DON detoxification are currently being targeted using this technology. A number of transgenic barley plants expressing genes involved in resistance and susceptibility have now been introduced and are currently being verified for gene expression. Functional testing against FHB will proceed once gene expression levels are known.

This project's research can potentially be used to analyze large qRT-PCR datasets in combination with the corresponding maize DNA marker data and maize phenotypic data. This is essential to the identification of aflatoxin resistance DNA markers for incorporation of maize resistance into elite commercial maize lines. A number of

candidate genes differing between resistant and susceptible lines were identified. Newly found antifungal agents may have a wide array of applications – from crop protection, to wood decay prevention, to use in household cleaning products. In regards to major goals, the initial (year 1) research provided critical outcome in the form of a set of statistically significant and tentatively identified corn metabolites that are differentially abundant in fungus-resistant genotype versus fungus-susceptible genotype.

We established a mechanism of bZIP regulation of fungal secondary metabolites (SMs) through RsmA, a recently discovered YAP-like bZIP protein. RsmA greatly increases SM production by binding to two sites in the Aspergillus nidulans AflR promoter region, a C6 transcription factor known for activating production of the carcinogenic and anti-predation SM, sterigmatocystin. Deletion of aflR in an overexpression rsmA (OE:rsmA) background not only eliminates sterigmatocystin production but also significantly reduces asperthecin synthesis. Furthermore, the fungivore, Folsomia candida, exhibited a distinct preference for feeding on wild type rather than an OE:rsmA strain. RsmA may thus have a critical function in mediating direct chemical resistance against predation. Taken together, these results suggest RsmA represents a bZIP pathway hardwired for defensive SM production. We also used laeA mutants as tools to elucidate virulence attributes in Aspergillus flavus. Microarray expression profiles of ΔlaeA and over-expression laeA (OE::laeA) were compared to wild type A. flavus. Strikingly, several nitrogen metabolism genes were oppositely mis-regulated in the ΔlaeA and OE::laeA mutants. One of the nitrogen regulatory genes, the bZIP encoding meaB, was up-regulated in ΔlaeA. Significantly, over-expression of meaB (OE::meaB) phenocopied the decreased virulence attributes of a ΔlaeA phenotype including decreased colonization of host seed, reduced lipase activity and loss of aflatoxin B1 production in seed. However, a double knock-down of laeA and meaB (KD::laeA,meaB) demonstrated that KD::laeA,meaB closely resembled ΔlaeA rather than wild type or ΔmeaB in growth, aflatoxin biosynthesis and sclerotia production thus suggesting that meaB does not contribute to the ΔlaeA phenotype. MeaB and LaeA appear to be part of regulatory networks that allow them to have both shared and distinct roles in fungal biology.

The velvet genes in A. flavus are an ideal target for control strategies, as disruption of these genes can reduce the fungus ability to spread and produce toxin. We found a new member of velvet family, VelD, which only found in A. flavus and A. oryzae. We have generated vosA, velB, velC, and velD deletion mutants in A. flavus. The deletion of velB caused severely impaired (number, size and morphology) conidiation and the lack of sclerotia production. Moreover, the velB deletion mutant no longer produced AFB1. The deletion of vosA causes earlier conidiation and shows 2 fold more conidia number in 4 day culture. Besides, the vosA deletion mutant produces significantly less AFB1 comparing to WT. velB and vosA deletion mutant conidia contain only ~30% of trehalose compared to wild type spores, suggesting that both may be required for the spore viability in A. flavus. Deletion mutants of the osaA gene homologues in A. flavus show aberrations in development and aflatoxin biogenesis. For that reason, we conclude that OsaA is a key regulatory factor that participates in controlling the process of development and mycotoxin biosynthesis in Aspergillus species. We also did the first study to elucidate WetA function on mycotoxin production in A. flavus. The loss of wetA

leads to reduced conidia viability and conidia autolysis in 3 days after inoculation. The wetA null mutant showed a reduced growth rate. Deletion of wetA also decreased fungal stress tolerance. The Velvet proteins, OsaA, and WetA are involved in either sporogenesis and/or mycotoxin production. Understanding the regulatory mechanisms of these proteins, we have more confidence to control both fungal dissemination and mycotoxin production in fields.

We have identified several global regulators of mycotoxin production in A. nidulans, such as LaeB that regulates sterigmatocystin production. LaeA regulates all mycotoxins in all fungi. We have recently found that LaeA regulates production of spore toxins through the transcription factor known as BrlA. We found that LaeA regulates brlA expression via chromatin remodeling of the brlA promoter. A human immunotoxin in A. fumigatus, endocrocin, was found to be regulated by LaeA through BrlA. We proposed that G protein coupled receptors (GPCR) are the likely receptors involved in quorum sensing; we have published the deletion of 14 of 16 GPCRs (the 2 others were already known). We are now testing these mutants to see if they are impaired in density development, which will further advance understanding of mycotoxin regulation.

PUBLICATIONS (not previously reported): 2013/10 TO 2014/09

Type: Book Chapters Status: Published Year Published: 2014 Citation: Wang, Y., Janssen, H. and Blaschek, H.P. Fermentative biobutanol production: An old topic with remarkable recent advances. In: Bioprocessing of Renewable Resources to Commodity Bioproducts, Bisaria and Kondo (Editors), Wiley.

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