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Participants in the previous annual meetings of the project are available at the link noted below after the summary of meeting minutes.

Brief summary of minutes of annual meeting:

Institutional updates included a discussion of potential opportunities for collaboration on mycotoxin research with Feed the Future Laboratories, such as at Kansas State. Dr. Cardwell discussed NIFA funding for mycotoxin research and initiatives that may align governmental research funding to support such research. Dr. Jackson reiterated the need for the project to be more than the sum of its parts, so that subgroups within each objective should continually coordinate efforts.

Annual research progress reports were given by IA, IL, KS, KY, MI, MS, MO, NE, NJ, PA and VA.

The group went over each of the 3 objectives of the project and discussed how the different groups would meet their specific project goals. The need to update or change the project's website was discussed and Heather Hallen-Adams volunteered to investigate this further, and potentially move the hosting of the site to UNL. There was a discussion about when and where to hold the meeting that would be associated with a national or regional meeting in either food safety or plant pathology. The consensus was to aim for a meeting that coincided with an ASPP meeting in 2017. The next meeting will be held in late September, 2016 at Rutgers, NJ. Suzanne Hendrich will step down as chair and will be succeeded by Lisa Vaillancourt, who, in turn will be succeeded as vice-chair by Michael Lawton. David Schmale will serve as secretary. The Chair reminded all participants of the need to submit an Annual Report (termination report) within 2 months of this meeting. The meeting was adjourned.

See http://www.nimss.org/lgu_v2/homepages/saes.cfm?trackID=11976 for participants and

minutes from prior annual meetings (2011-2014).

Accomplishments:

Objective 1. Develop data for use in risk assessment of mycotoxins in human and animal health.

- A mycotoxin binder study was completed in rats showing efficacy of a commercially available binder against fumonisin B1 and deoxynivalenol, according to effects on serum sphingolipids and body weight.
- The Fusarium/ Poultry Research Laboratory evaluated a large number of mineral and organic adsorbents for binding mycotoxins in in vitro and in vivo studies in poultry, swine and cattle. Naturally occurring antioxidants (eg. curcumin) were evaluated for reducing mycotoxin toxicity in poultry. The laboratory continues to produce mycotoxins in culture (kg quantities aflatoxin, zearalenone, ochratoxin A, fumonisin B1) for in house as well as for other researchers doing animal feeding trials with mycotoxins. Diagnostic testing for mycotoxins in contaminated feedstuffs was improved utilizing affinity column methodology in combination with HPLC analysis. These data were published in refereed journals and/or provided to commercial companies which used the data to produce efficacious products for agriculture to prevent mycotoxicosis.
- To validate RNAseq data and further study the toxicity mechanisms of DON, we employed the recently developed CRISPR/Cas9- (clustered regularly interspaced short palindromic repeats/associated endonuclease 9) gene editing technology to completely knock-out the key up- and down-regulated genes in C. elegans. The C. elegans CRISPR vector pDD162 (Peft-3::Cas9 + empty sgRNA) was purchased from Addgene (www.addgene.org). The C. elegans F11D11.3 gene (unknown protein coding) as the highest expresser after DON exposure and the Y39G10AR (ugt-31) gene were chosen to be edited/mutated by CRISPR technology. The 20-bp target sequences conforming to the G(N19)NGG requirement for Cas9 were identified in the F11D11.3 and ugt-31 genes. The CRISPR/F11D11.3-sgRNA and CRISPR/ugt-31-sgRNA vectors have been constructed (pRD217 and pRD219).The effect of gene mutations will be evaluated following DON treatment compared to WT worms.

Objective 2. Establish integrated strategies to monitor and reduce mycotoxin contamination in cereal grains and distillers grains

- 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 50/57 batches. The 7 batches showing lesser increase of FB are planned to be investigated further.
- There is increased concern amongst growers, buyers, millers and other agriculture professionals about apparent increased levels of the mycotoxin deoxynivalenol in wheat and corn produced in Pennsylvania. We conducted a survey of the wheat head scab organism, F. graminearum that produces deoxynivalenol, to determine the incidence and prevalence of the more toxigenic 3-acetyl-deoxynivalenol strains in Pennsylvania. Corn and wheat debris were collected from fields at 75 locations in 18 PA counties in January through March 2013. Five pieces per site were plated on medium selective for the fungal

genus Fusarium. Individual pure cultures created from these plates resulted in nearly 400 cultures. All but a few were determined visually to be Fusarium. The species of the cultures was determined by amplification of the five prime end of the EF1-alpha gene followed by DNA sequencing and comparison the sequence to the Fusarium ID database. About ¼ of the cultures (95) were identified as F. graminearum the causal agent of head scab of wheat and ear and stalk rot of corn. Of these 95 all were of the 15-acetyl-deoxynivalenol chemotype except 3 isolates that were confirmed to be the 3-acetyl-deoxynivalenol type. This data indicates that an incursion of the more toxigenic 3-acetyl-deoxynivalenol strains into PA is not a likely explanation for the apparent increase in overall deoxynivalenol levels in wheat and corn.

- Wheat infected with Fusarium head blight (FHB) has been collected from across the state of Nebraska since 2010 (and earlier), and the strains involved in FHB have been characterized using molecular biology (confirmation of all isolates to date as Fusarium graminearum, identification of putative toxin chemotype), ELISA testing for the mycotoxin deoxynivalenol, and aggressiveness infecting wheat in the greenhouse.
- Aflatoxin B1 did not affect ethanol yields in the dry-grind ethanol process. Yeast performance, as inferred by fermentation rate, was not affected by aflatoxin B1 up to a concentration of 775 ppb. In the downstream dry-grind ethanol process, 45–55% of the aflatoxin B1 was found in wet grains. The lower starch content of naturally contaminated corn should be considered while analyzing the results.
- We have adopted Brachypodium (Bd21 variety) and Arabidopsis as model systems for assessing infection by F. graminearum and the responses to application of DON. We have used the CRISPR/Cas-gene editing technology to engineer FHB resistance in Brachypodium, Arabidopsis and barley. We have produced CRISPR constructs to target the ugt (UDP- Glucuronosyl Transferase) genes in Brachypodium, Arabidopsis and barley. Gene editing-out of ugt will facilitate our understanding of the DON detoxification function of this gene in these plants. We have produced ugt-KO Arabidopsis plants, and are in the process of characterizing these plants. We have also made plant expression vectors containing the following constructs to engineer barley: FTLi to knock-down the Fusarium Transducin Beta-Like gene that is essential for Fusarium pathogenesis, GFPi to silence the green fluorence protein gene in WT Fg-GFP and tri5 Fg-GFP to monitor F. graminearum infection, HPGP to over-express the hydroperoxide glutathione peroxidase gene to elevate the anti-oxidative level, and snakin-1 to over-expression this plant anti-microbial peptide. We are currently transforming these constructs into barley (cv. Conlon).

Objective 3: Define the regulation of mycotoxin biosynthesis and the molecular relationships among mycotoxigenic fungi.

- 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 are studying the maize pathogen and endophyte Fusarium verticillioides and are interested in identifying genes involved virulence. Homologs of three genes identified in other fungi were identified in the genome sequence of F. verticillioides. These genes are involved in hyphal fusion and generation and regulation of reactive oxygen species

production. Strains of F. verticillioides with disruptions in each of the genes were made previously using DNA transformation procedures. Strains with a disruption of the gene involved in hyphal fusion were non-pathogenic on maize ears, stalks and seedlings and showed several development growth defects. Overall conidial production was decreased and the average conidial size was decreased compared to wild type. Additionally, these isolates grew more slowly than wild type. Strains with disruptions in either a gene encoding a NADPH oxidase or a gene regulating NADPH oxidases had significantly reduced pathogenicity on maize ears, stalks and seedling but showed normal in vitro growth rates. Production of the mycotoxin fumonisin was significantly lower than wild type in all three of the disruption strains.

- We developed novel markers and used them to analyze a population of Fusarium graminearum from Kentucky. We learned that most of these isolates belonged to the dominant chemotype, but that they showed signs of being an isolated divergent population. Two species were recovered from wheat heads that had not previously been described from symptomatic wheat heads, but these did not cause symptoms when inoculated onto healthy wheat. These strains may colonize tissues secondarily that are killed by the scab fungus. This is significant because the other species produce different types of mycotoxins. One other study suggested that the mating type genes of Fusarium graminearum are important for aggressiveness to wheat: knockouts of the MAT1-1-1 and MAT1-2-1 genes were less aggressive than controls on winter wheat, but were not different from controls on corn stalks. Other experiments suggested that selfing or crossing among strains produced transgressive progeny that were more or less aggressive, or more or less toxigenic than their progenitor strains.
- Genes and enzymes for key steps in loline alkaloid biosynthesis by epichloid fungi (endophytes) symbiotic with grasses were identified. Three genes were characterized and roles confirmed for steps leading to a variety of loline alkaloids. The lolO gene encodes an oxygenase that converts 1-acetamidopyrrolizidine (AcAP) into the first loline alkaloid, N-acetylnorloline (NANL). In the process of elucidating the role of lolO, AcAP was discovered and found to be the pathway end product in some endophytes of native grasses such as Canadian wild rye (Elymus canadensis) and long-awned wood grass (Brachyelytrum erectum). Similarly, NANL is the end product in some other native grasses such as foul managrass (Glyceria striata) and some lines of Canadian wild rye. The enzyme encoded by lolN catalyzes conversion of NANL to norloline, which is then N- methylated by the enzyme encoded by lolM to give loline and N-methylloline (NML). A plant enzyme converts loline to N- acetylloline, and the fungal LolP monooxygenase converts NML to N-formylloline (NFL). NAL and NFL are the most abundant alkaloids in the tall fescue with common strains of E. coenophiala and are well characterized protectants against invertebrates.
- Characterized the fungi-specific velvet regulators that play a key role in regulating sporulation and production of mycotoxins.
- Revealed that fungal sporulation and aflatoxin production are intimately associated via bridging activities of the velvet family proteins VeA, VelB and VelD in Aspergillus flavus.
- Revealed that velvet proteins interact with each other, alone (“homodimers”), in various combinations (“heterodimers”), and also with other proteins including the master regulator of mycotoxins LaeA.

- Further revealed that velvet proteins are a family of fungus-specific transcription factors having a NF-kB-like domain that directly binds to target DNA.
- The fungi-specific velvet regulators are conserved in many agriculturally important fungi, affecting growth, development, pathogenicity and toxigenesis.
- Characterized functions of 15 G-protein coupled receptors (GPCRs) in aflatoxigenic A. flavus.
- Revealed that the G-protein coupled receptors GprC and GprD play a crucial role in governing oxylipin signaling and quorum sensing in A. flavus.
- The velvet genes in A. flavus are ideal targets for control strategies, as disruption of these genes can reduce the fungus ability to spread and produce toxin. We 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-null mutant no longer produced AFB1. The deletion of vosA causes earlier conidiation and higher conidia number. Besides, the vosA-null mutant produces significantly less AFB1 comparing to WT. velB- and vosA-null mutant conidia contain less trehalose compared to wild type, suggesting that both velB and vosA are required for the spore viability in A. flavus. velC- and velD-null mutants don't show disrupted spore viability, stress tolerance, growth rate, ortrehalose amount. However, velC- and velD-null mutans form more sclerotia comparing to wild type under dark conditions, while velD-null mutant shows no significant difference in sclerotia formation under light conditions. Some Velvets are involved in aflatoxin biosynthesis. In submerged culture and liquid culture, veA-, velB-, and velD-null mutants fail to produce AFB1. In comparison, velC-null mutant produces AFB1 in submerged culture, but fail to produce AFB1 in liquid culture.
- Other than the Velvets, we also characterize the function of two key development regulators, OsaA and WetA, 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. WetA is an evolutionary conserved central developmental regulator in certain Ascomycetes. The wetA-null mutant forms wet and white conidia, which have reduced viability and autolyzes in few days. The wetA-null conidium has a smaller size, lacks of the crenulated structure, and eventually loses the spore content. Loss of wetA leads to decreased trehalose level in conidia, which is a major conidia content and a protectant against various environmental stresses. Loss of WetA reduces the aflatoxin accumulation.
- The Velvet proteins, OsaA, and WetA are involved in either sporogenesis and/or mycotoxin production, which make them excellent potential broad-spectrum anti-fungal target. By dissecting the regulatory mechanisms of these regulators in A. flavus, we have more confidence to control both fungal dissemination and mycotoxin production in fields and diminish fungal hazards in food industry.
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Impacts:

Obj. 1

- The effectiveness of a mycotoxin binder against fumonisin and deoxynivalenol provides additional assurance for prevention of mycotoxin problems in pet foods and animal feeds.
- Safe utilization of low level mycotoxin contaminated grains in animal feedstuffs can be

increased with the development of new proprietary adsorbents and use of naturally occurring antioxidants to reduce or eliminate the toxicity of mycotoxins. In vitro analysis of mycotoxin sequestration can be very useful in identifying potential dietary sequestering agents and in helping to determine mechanisms and conditions favorable for sequestration to occur. Providing mycotoxins (fumonisin, ochratoxin A, moniliformin, zearalenone, and aflatoxin B1) in culture material to mycotoxin research groups makes it economically feasible to undertake animal feeding studies that would be nearly impossible if mycotoxins were purchased commercially. A number of proprietary adsorbents were evaluated for efficacy to bind or detoxify mycotoxins in animal feedstuffs. The information was used to help select products with the highest binding ability for further in vivo testing or to help in making decisions on how to improve the product. Information was used to determine inclusion rates, particle size, and limitations of adsorbents and yeast cell wall products.

- Information from in vitro and in vivo studies was reported to the feed additive industry. This information was utilized to develop products for commercialization. The information was disseminated to the livestock industry.

Obj. 2.

- This work shows that the continued development of genetically modified insect resistant maize prevents FB contamination of this crop, which will help to prevent FB mycotoxicosis in swine and poultry.
- The knowledge of the lack of 3-acetyldeoxynivalenol producing strains of F. graminearum suggests other explanations for the apparent increase in DON should be sought. These include changes in cultural practices, cultivar use and weather patterns. Similarly, surveys to document increases in DON in corn and wheat grain are warranted.

Obj. 3

- The presence of black Aspergillus spp. might alter FB1/FB2 ratios in maize, which could somewhat reduce mycotoxicosis risk, but the presence of these species also correlated with the presence of A. flavus, leading to the expectation of greater co-occurrence of FB and aflatoxins. This work shows the need for continued vigilance by grain producers and the food industry on surveillance and development of anti-mycotoxin strategies that target multiple mycotoxins, especially when grains are under drought stress.
- The data collected during these experiments increases the overall knowledge of virulence mechanisms of F. verticillioides. While fumonisin mycotoxins have been previously shown to have a role in virulence this is only for seedling blight pathogenicity. These results show that some genes, and their products are involved with all of the three types of disease this fungus causes on maize namely seedling blight, ear rot and stalk rot. The new knowledge generated indicating a role for reactive oxygen species in F. verticillioides pathogenicity on maize opens new opportunities for deeper understanding of virulence mechanisms and potential targets for control.
- Work on knocking out mating genes associated with aggressiveness of F. graminearum may lead to new ways of protecting winter wheat from this fungus.
- The identification of loline metabolic pathways lays a foundation for fungal gene modification to lessen the toxicity of these compounds in forage grasses.
- Outcomes of this project have provided the mechanistic insights into the novel regulatory roles of the velvet proteins in bridging fungal spore formation and mycotoxin biosynthesis.

- Our studies further illuminated the global functions of velvet regulators in controlling complex expression, cellular and metabolic responses in fungi.
- As the velvet regulators' functions in governing development and toxigenesis are conserved in many fungi, the results of our research would aid to the development of novel ways for the intervention of fungal infestation and mycotoxin production in fields, thereby protecting human and plant health.
- Studies of GPCRs can provide new ways to antagonize signaling for A. flavus aflatoxin production in fields.
- We have generated vosA-, velB-, velC-, velD-, osaA-, and wetA-null mutants and and elucidate their effects on sporogenesis and mycotoxin biogenesis in A. flavus. To better understand the molecular regulatory mechanism of these regulators, we conducted RNA-seq toward wild type, vosA-, velB-, osaA-, and wetA-null mutants in A. nidulans, the model organism of Aspergillus species. Partial data has been deposited to NCBI GEO database. Several papers has been published on highly reputed journals. Our findings provides several new potential anti-fungal targets to eliminate the mycotoxin problem and promote biosafety in food industry.

Overall impact summary:

As tested by members of this project, proprietary adsorbents added to animal feeds may help to assure feed quality with respect to preventing mycotoxicoses,. Genetically modified insect-resistance corn prevents fumonisin contamination in this crop and mycotoxicoses in swine and poultry. Both of these findings should help the production livestock economy.

The research of this project has generated knowledge of a number of new gene targets that are involved in the spread and toxicity of key mycotoxin-producing fungal species. These constitute new potential anti-fungal targets to eliminate mycotoxin problems and promote biosafety in food industry.

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