SESSION 1:

FOOD SAFETY, TOXICOLOGY AND UTILIZATION OF MYCOTOXIN-CONTAMINATED GRAIN

Co-Chairpersons: Jim Pestka and

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RISK ASSESSMENT AND BIOMARKERS FOR DEOXYNIVALENOL

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ABSTRACT

Deoxynivalenol (DON) is the most commonly detected trichothecene fungal metabolite in cereal grains and processed food globally. Upon exposure, DON is rapidly distributed in animal tissues and induces proinflammatory cytokines (< 2 h). Longer term (> 2 wk) DON exposure reduces weight gain in many species through a poorly understood mechanism, thus creating uncertainties in human safety assessment and DON regulatory limits. Understanding of mechanism(s) and identification of biomarker(s) will increase the precision of DON regulatory limits. We hypothesized that DON-induced weight reduction is preceded by a dysregulation of proteins in the growth hormone pathway. Models of acute and chronic DON exposure were used to test this hypothesis. The results indicate that DON acutely induces hepatic suppressors of cytokine signaling (SOCS). The effect of SOCS on growth pathway was evaluated by measuring forms of insulin-like growth factor acid-labile subunit (IGFALS), a growth-related protein. Acute DON exposure (0.1-12.5 mg/kg) impaired growth hormone-induced IGFALS mRNA by 60-80%. Furthermore, dietary DON (20 ppm for 8 wk) suppressed IGFALS mRNA (65%), circulating IGFALS (66%), weight gain and elevated plasma DON (≤ 63 ng/ml). Circulating insulin-like growth factor 1 (a binding partner of IGFALS) was equally suppressed by dietary DON. Together, these data indicate that dietary DON consistently suppresses IGFALS in mice, while elevating plasma DON. Therefore, circulating IGFALS is a potential biomarker for DON's effect. Validation of this biomarker in human population will enhance epidemiological surveillance and might increase the precision of human risk assessment.

MULTI-YEAR SURVEYS ON FUSARIUM HEAD BLIGHT AND MYCOTOXINS IN COMMERCIAL WHEAT GRAINS FROM RIO GRANDE DO SUL STATE, BRAZIL

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ABSTRACT

Spring wheat is grown mainly in the states of Paraná and Rio Grande do Sul (RS), the major producers in Southern Brazil. In spite of the current significance of this disease in Brazil, systematic epidemiological surveys on FHB epidemics and mycotoxins on commercial grain are scarce. Since 2006 we collected extensive field and laboratory data to monitor and assess FHB disease-related parameters and mycotoxins in wheat growing regions of RS state. Assessment of kernel damage on 139 samples originating from 54 different municipalities surveyed in the 2006-2008 period revealed that over 90% of the samples presented some physical damage by FHB. Sixty-five samples taken from the same period were analyzed for the occurrence and levels of deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA) quantified using LC-MS/MS. DON was found in all but one sample. NIV and ZEA were present in 83% and 40% of the samples, respectively. DON levels averaged 0.55 mg/kg (ppm) with most samples showing DON<0.5 mg/kg (44/65) and 10 samples exceeding 1 mg/kg. NIV levels averaged 0.3 mg/kg with 10 samples in the range of 0.5 to 1 mg/kg. ZEA concentration was in trace levels. While mean DON varied among years and regions mean NIV was more consistent among the years. In our field surveys on FHB, 62 fields arbitrarily selected in the main growing regions were assessed for incidence in 2008 growing season. Mean incidence was lower than 10% with several fields showing trace levels. In the current 2009 season, both FHB incidence and severity were assessed and our preliminary results for 36 fields showed disease incidence averaging 41% (10-90%). However, severity levels averaged approximately 2%. The widespread occurrence of DON, but specially NIV, in commercial grain from the surveyed regions supports our previous molecular evidence of a toxin potential for a less predominant *F. graminearum* population spread across the state. Moreover, the monitoring of NIV should be regularly performed given its relatively high levels found and toxicological implications to animal and human health. Our further epidemiological analysis of survey data on several disease/toxin-related parameters will help to better determine local and regional factors associated to FHB and mycotoxin contamination in southern Brazil.

COMPARISON OF WEIGHT GAIN AND PLASMA INSULIN-LIKE GROWTH FACTOR ACID LABILE UNIT (IGFALS) SUPPRESSION FOR DETERMINING THE NO-OBSERVED EFFECT LEVEL (NOAEL) IN MICE FED DEOXYNIVALENOL

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ABSTRACT

Deoxynivalenol (DON) is a trichothecene mycotoxin that contaminates wheat and barley during Fusarium head blight in temperate regions throughout the world. The potential for adverse health effects in persons consuming DON is a public health concern, particularly with respect to growing children. The current no observed adverse effect level (NOAEL) for DON exposure in mice is 100 µg/ kg body weight / day which is based on impaired growth observed in a 2-year Canadian feeding study. This level has been used as a basis for current European Union (EU) regulations.

Recently, our laboratory discovered that acute DON exposure decreases the mRNA expression of insulin-like growth factor 1's (IGF1) binding partner, acid labile subunit (IGFALS). Furthermore, upon subchronic exposure to DON, mice exhibit decreased IGF-1 and IGFALS suggesting these to be sensitive biomarkers for DON adverse health effects. These hormones are critical for growth and likely will impact weight gain.

In this study, we compared the NOAELs for weight gain and IGFALS suppression in weanling (4-week old) B6C3F1 mice fed DON subchronically (9 wk) in a pelleted diet at concentrations of 0, 0.4, 0.7, 1.9, 3.6, and 5.8 ppm. To determine the NOAEL, average daily feed consumption was measured (2.7 g). Then the dose with no adverse effect was multiplied by the amount eaten per day, and divided by the average weight of mice (0.02 kg) to yield the NOAEL reported as "DON consumed per kg bw/day".

Mice fed DON did not exhibit weight gain inhibition at 3.6 ppm which is equivalent to a NOAEL of 490 µg/kg bw/ day. Using IGFALS as a biomarker of expression, the NOAEL was 260 µg/ kg bw/ day based on no significant decrease in plasma IGFALS after at 9 weeks at 1.9 ppm. Like IGFALS, there was also a trend toward suppression of IGF-1 by DON at 5.8 and 3.6 ppm but not at 1.9 ppm. The results suggest that IGFALS suppression is a predictive biomarker of weight gain inhibition. In both cases, the observed NOAELS in this subchronic study were higher than that of the aforementioned 2 year Canadian chronic study (ie. 100 µg/ kg bw/ day) currently employed to establish current EU regulations. Future studies will determine how food matrix (pellet vs. powder), exposure duration, gender, strain and age affect NOAEL determination using IGFALS as a biomarker of DON toxicity.

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FUSARIUM MYCOTOXIN CONCENTRATIONS IN THE STRAW, CHAFF, AND GRAIN OF SOFT RED WINTER WHEATS EXPRESSING A RANGE OF RESISTANCE TO FUSARIUM HEAD BLIGHT

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ABSTRACT

Fusarium graminearum Schwabe (teleomorph *Gibberella zeae* (Schwein.), which causes Fusarium head blight (FHB) or scab, is an increasingly important problem in the north-central region of the United States. During years of heavy FHB infection, veterinary diagnostic laboratories have, on occasion, unexpectedly found unusually high concentrations of both deoxynivalenol (DON) and zearalenone (ZEA) in wheat straw, as well as FHB-infected grain. Because swine are sensitive to concentrations of DON and ZEA as low as 1 $\mu g\ g^{-1}$, these mycotoxins are particularly problematic when wheat straw is used for bedding in less than optimal production settings. Similarly, where straw is used as a source of roughage for cattle in total mixed rations (TMRs), concentrations of mycotoxins, to which ruminants are fairly resistant, might be found at clinically relevant, high concentrations in straw. Although there is a large body of literature on mycotoxin content in FHB infected grain, little is known of both the range and concentrations of mycotoxins in wheat straw. As such, a preliminary study was undertaken to investigate mycotoxin concentrations in the straw, chaff, and grain of the 60 soft red winter genotypes comprising the 2008 Uniform Northern Fusarium Head Blight Nursery. The nursery was grown at the Bradford Research and Extension Center near Columbia MO and spray-inoculated at 75% heading with a macroconidial suspension of *F. graminearum* concentrated to 50,000 macroconidia/mL. It was maintained under overhead mist irrigation through heading and evaluated for incidence and severity 18 - 21 d after inoculation. The field FHB index for each genotype was determined as incidence x severity expressed as a percentage. At harvest, a 3-meter long sample of each genotype was cut at ground level, dried and separated in three components including grain, chaff, and straw. Samples were sent to North Dakota State University where they were analyzed by GC/MS in the SIM mode for DON, 15-ADON, zearalenol, and ZEA. FHBI for the 60 genotypes evaluated ranged from a low of 9.9% to a high of 61.9% and averaged 35.7%. Significant concentrations of DON and ZEA were detected in the grain, chaff and straw while 15-ADON and zearalenol concentrations were negligible ($<0.5\ \mu g\ g^{-1}$) in the grain but higher levels were present in the chaff and straw. 3-ADON and nivalenol were not detected. In the grain, DON and ZEA concentrations were relatively low averaging 4.7 and 4.4 $\mu g\ g^{-1}$, respectively, across the 60 genotypes and were significantly correlated with resistance level (r=0.56 and r=0.51 for DON and ZEA, respectively). In chaff samples, both mycotoxins were present at higher concentrations averaging 16.9 $\mu g\ g^{-1}$ (DON) and 42.9 $\mu g\ g^{-1}$ (ZEA) and were poorly correlated with resistance (r=0.32 and r=0.37; DON and ZEA, respectively). In the straw, DON concentrations were again low, averaging 3.5 $\mu g\ g^{-1}$ over entries but, surprisingly, the ZEA concentrations were extraordinarily high (55.5 $\mu g\ g^{-1}$) and the correlation with resistance was much lower for both mycotoxins (r=0.21), indicating that *Fusarium* mycotoxin concentrations in the straw could not be predicted by the resistance level of the cultivar. These findings are, potentially, very clinically relevant to livestock producers. The need for a more rigorous, replicated study over different environments is warranted.

FORMATION OF THE BIOMARKER ZEARALENONE-4-O-GLUCURONIDE BY HUMAN UDP-GLUCURONOSYLTRANSFERASES AND ENGINEERED YEAST

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ABSTRACT

Various xenobiotic compounds (medicinal drugs, plant and fungal secondary metabolites, including *Fusarium* mycotoxins) are efficiently detoxified in animals and humans by formation and excretion of toxin-conjugates. The UDP-glucuronosyltransferases (UGTs) are the most relevant enzymes of phase II detoxification (conjugate formation). The main goal of the project was to test whether it is possible to engineer this detoxification pathway in *Saccharomyces cerevisiae* (baker's yeast), where it does not naturally occur. The toxicologically relevant *Fusarium* mycotoxin zearalenone served as model substances in this study.

A ZON-4-O-glucuronide standard was synthesized via the Koenigs Knorr procedure using a bromosugar activated by silver carbonate. LC-MS techniques to detect the conjugate were developed.

Since the catalytic domain of mammalian UDP-glucuronosyltransferases is located in the lumen of the endoplasmatic reticulum, and yeast does not contain the UGT co-substrat UDP-glucuronic acid (UDP-GlcUA), a gene encoding a UDP-glucose dehydrogenase (UGDH) for synthesis of UDP-GlcUA and in addition a gene encoding a membrane transporter allowing UDP-GlcUA to enter the ER were introduced. Such yeast strains additionally expressing different cDNAs of human UDP-glucuronosyltransferases formed predominantly zearalenone and zearalenol glucosides instead of the desired glucuronides, despite the strong overexpression of UGDH, which led to depletion of UDP-Glc in favour of UDP-GlcUA. Therefore, relocation of the enzyme into the cytosol as N-terminal GST-fusion protein was also attempted.

Only small amounts of glucuronides of the tested mycotoxins were found in the supernatant of toxin treated yeast cultures. It was therefore not possible to utilize the engineered yeasts as cell factory for production of mycotoxin conjugates, as previously demonstrated for ZON and ZOL-glucosides. A problem of the yeast heterologous expression system is that the product ethyl-glucuronide is formed by the expressed UDP-glucuronosyltransferases in the presence of the competitive inhibitor ethanol. The enzymes expressed in yeast or in insect cells are useful to test *in vitro* which of the multiple UGT isoforms are most relevant for inactivation of ZON.

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A TARGET FOR THE *FUSARIUM* MYCOTOXIN ZEARALENONE IN PLANTS: INHIBITION OF HSP90 ATPASE

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ABSTRACT

The *Fusarium* mycotoxin zearalenone (ZON) is well known for its strong estrogenic activity in animals. Plants do not have an estrogen receptor, and it is an open question whether the *Fusarium* metabolite ZON has a biological role in plant-pathogen interaction. We have identified a prominent target for zearalenone: Hsp90. Zearalenone and more strongly beta-zearalenol (bZOL) inhibit ATPase activity of purified yeast Hsp90 (ScHsp82p) *in vitro*. Hsp90 is necessary for the stability of many client proteins such as signal transduction components, and has been shown to be essential for plant defense [1]. Microarray experiments of ZON treated *Arabidopsis* plants showed marked changes in gene expression. ZON is able to suppress the root phenotype of a mutant with a defect in a cell wall biosynthetic gene, which leads to constitutive activation of ethylene responsive genes. Many genes encoding proteins with a role in cell wall remodeling and especially peroxidases were repressed by ZON. In contrast, small heat shock proteins and *AtHSP90-1* were upregulated by ZON treatment of *Arabidopsis*. Also many putative candidate detoxification genes (e.g. glucosyltransferases, sulfotransferase) were induced. ZON was found to be rapidly converted into ZON-4-O-glucoside and ZON-4-sulfate in plants. Both conjugates do not inhibit Hsp90 ATPase *in vitro*.

The finding that ZON and its biosynthetic precursor bZOL are Hsp90 inhibitors also raises the question about the mechanism of self resistance in the toxin producing fungus. We have engineered yeast strains with increased ZON sensitivity (deletion of several ABC transporters) and with deletions of the endogenous yeast Hsp90 genes (hsp82 hsc82), that express as sole source of Hsp90 either the yeast or *F. graminearum* gene. We further transformed *Fusarium graminearum* with a deletion construct removing both *PKS*4 and *PKS13*, which are required for ZON biosynthesis. These strains will be used to re-address the question whether zearalenone biosynthesis contributes to *Fusarium* virulence.

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ASSESSMENT OF THE ACCURACY OF SINGLE-KERNEL NEAR-INFRARED TECHNOLOGY TO SORT WINTER WHEAT KERNELS BASED ON SCAB AND DEOXYNIVALENOL LEVELS

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ABSTRACT

Fusarium head blight (FHB, scab), caused by *Fusarium graminearum*, causes significant losses in winter wheat by reducing yield and grain quality. Kernels damaged by FHB, commonly referred to as *Fusarium*-damaged kernels or FDK, appear shriveled and/or discolored. *F. graminearum* produces the toxin deoxynivalenol (DON) which accumulates in grain during the grain filling period. Grain contaminated by DON usually is discounted at the elevator or can be rejected altogether. For purposes of quality assurance, DON concentration usually is determined in grain or the products made from it. The most commonly used method for determining DON concentration is gas chromatography. This method is accurate and can determine DON concentrations as low as 0.5 ppm. However, the method is destructive because grain must be ground to flour before it can be tested for DON. For purposes such as breeding for resistance to FHB where grain (seed) destruction may be undesirable; thousands of samples may need to be screened for DON in a short period of time; and knowledge of whether the grain from a given line has a low or high DON concentration is all that is needed to make a decision on whether or not to advance the line in a breeding program, alternative methods of determining DON concentration in grain are needed.

Single-kernel near-infrared (SKNIR) technology provides a non-destructive and quick alternative to gas chromatography in the determination of DON concentration in wheat grain. The present study examined the accuracy of a SKNIR system to sort winter wheat grain based on FDK and DON calibrations. *Fusarium*-damaged kernels were visually sorted from grain (cultivar Jagalene) harvested in 2007 and 2008 from FHB-affected experimental plots at the University of Nebraska Agricultural Research and Development Center near Mead, Nebraska. The FDK from each year was mixed with healthy grain in increasing proportions by weight of FDK ranging from 0% FDK to 100% FDK in 5% increments. Six replicates of a total of 21 samples (treatments) each weighing 5 g were obtained for each year. The samples were first sorted using a scab calibration into four fractions (Bin 1 for sound kernels and Bins 2-4 for FDK with increasing severity of scab damage), and the number of kernels collected in each bin was recorded. Kernels were mixed together again and placed in the corresponding packets for later sorting on DON estimates.

Two SKNIR instruments were used for sorting. For each treatment/year combination, replicates 1-3 were sorted in SKNIR 1 (old machine) and replicates 4-6 were sorted using SKNIR 2 (new machine). Using a DON calibration, DON levels in single kernels were estimated and placed in respective bins as follows: BIN 1 - kernels with non-detectable DON levels; BIN 2 – kernels with DON values between 1 - 60 ppm; BIN 3 – kernels with DON values between 61-160 ppm; BIN 4 – kernels with DON val-

ues above 161 ppm. After sorting, the number and weight of kernels in each bin were recorded. The sorted kernels from each bin (4 bins x 21 samples x 3 replications x 2 SKNIR machines x 2 years) were bulked by replication for each bin, SKNIR machine, and year and sent to the North Dakota Veterinary Diagnostic Laboratory for DON determination using gas chromatography. The three replications from each sample were bulked because replication samples were too small for DON analysis. DON was also determined in three replications of each of 21 similarly prepared samples not sorted by the SKNIR system.

The average number of kernels from the 21 samples sorted into Bin 1 (sound kernels), Bin 2, Bin 3, and Bin 4 (increasing severity of scab damage) was 22 (range: 5-39), 111 (range: 98-130), 74 (range: 11-113), and 10 (range: 0-22), respectively, from 2007 samples and 27 (range:4-61), 76 (range: 55-97), 82 (range: 6-158), and 66 (range: 0-141), respectively, from 2008 samples. The majority of kernels sorted into Bin 1 were from samples with 0 to 50% FDK whereas the majority of kernels sorted into Bins 3 and 4 were from samples with 50 to 100% FDK. Kernels sorted into Bin 2 were evenly distributed among the 21 source samples (0 to 100% FDK).

DON results from the SKNIR 1 system are presented (bin number, range in ppm of actual DON as determined by gas chromatography, and average DON in ppm in the bin).

2007. Bin 1: range, 0-17.6 ppm; average, 4.2 ppm
Bin 2: range, 0.8-44.5 ppm; average, 16.1 ppm
Bin 3: range, 0.8-39.6 ppm, average, 25.3 ppm
Bin 4: range, 25.7-84.5 ppm, average, 63.0 ppm

Unsorted by the SKNIR system: 0.8 ppm in 0% FDK samples – 36.2 ppm in 100% FDK samples

2008. Bin 1: range, 0-23.1 ppm; average, 3.3 ppm
Bin 2: range, 0-10.5 ppm; average, 3.7 ppm
Bin 3: range, 3.9-35.0 ppm, average, 21.7 ppm
Bin 4: range, 80-173 ppm, average, 141.6 ppm

Unsorted by the SKNIR system: 0.0 ppm in 0% FDK samples – 50.5 ppm in 100% FDK samples

DON levels were higher in 2008 samples (up to 173 ppm) than in 2007 samples (up to 84.5 ppm). Based on the results obtained from this study, the SKNIR system's accuracy in estimating DON was highest for samples from both years sorted into Bin 2 (calibrated for 1-60 ppm) and for 2008 samples sorted into Bin 4 (calibrated for > 160 ppm). The SKNIR system was able to sort wheat kernels into four fractions with the Bin 1 fraction having the lowest DON concentration and the Bin 4 fraction having the highest DON concentration. Due to the high precision of the SKNIR system in sorting kernels based on scab and DON calibrations, kernels in Bin 4 had a much higher DON content compared to the visually sorted and bulked 100% FDK samples (84.5 ppm SKNIR versus 36.2 ppm visually sorted and bulked in 2007 samples and 173 ppm SKNIR versus 50.5 ppm visually sorted and bulked in 2008 samples. Compared to DON determination techniques that estimate DON in unsorted bulk samples, sorting wheat grain into several fractions based on DON levels would provide breeders with more detailed information and would be a way of enriching resistance in segregating populations.