FOOD SAFETY, TOXICOLOGY AND UTILIZATION OF MYCOTOXIN-CONTAMINATED GRAIN

Chairperson: Stephen Neate

MULTIPLEX REAL-TIME PCR METHOD TO SIMULTANEOUSLY DETECT AND QUANTIFY DEOXYNIVALENOL AND OCHRATOXIN A PRODUCING FUNGI

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ABSTRACT

Cereal crop plants are colonized by many fungal species such as *Aspergillus ochraceus* and *Penicillium verrucosum,* which produce ochratoxins, and *Fusarium graminearum,* which produces trichothecene mycotoxins. A multiplex real-time PCR method using TaqMan probes was developed to simultaneously detect and quantify trichothecene producing *Fusarium* species and ochratoxin A producing *Penicillium* and *Aspergillus* species in cereal grains. Primers and probes were designed targeting the *Tri5* gene in trichothecene producing *Fusarium, rRNA* gene in *Penicillium verrucosum* and polyketide synthase gene in *Aspergillus ochraceus.* The method was highly specific to the species containing these genes and sensitive, detecting 3 pg of genomic DNA. These products were detectable over five orders of magnitude (3 pg to 30 ng of genomic DNA). Thirty barley samples were evaluated for the presence of deoxynivalenol (DON) and ochratoxin A (OTA) producing fungi using the above method. Among these samples, 9 tested positive for *Fusarium* spp, 5 tested positive for *Penicillium* spp and 2 tested positive for *Aspergillus* spp. Results were confirmed by traditional microbiological methods. These results indicate that DON and OTA producing fungi can be detected and quantified in a single reaction tube using this multiplex real-time PCR method.

TOWARD BICHROMATIC OPTICAL SORTING OF SCAB-DAMAGED WHEAT

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ABSTRACT

Our previous optical work exhaustively examined the best single wavelength and best two-wavelength combination that maximized class separation between normal and scab-damaged wheat kernels, using linear discriminant analysis. This was accomplished by single kernel visible (410-865 nm) and near-infrared (1031-1674 nm) scanning of more than 4500 kernels from 100 commercial varieties, equally divided between normal and scab-damaged categories. Generally, the best two-wavelength models were approximately 95% accurate in classification. From there, we have studied the application of a good-performing wavelength pair (675 nm and 1480 nm, as selected from a handful of manufacturers stock combinations) in a high-speed commercial sorter. Unknown until this study was the effectiveness of such classification models when applied under real-time, high-speed sorting conditions. More than 40 samples of soft red winter and soft white wheat, each approximately 5 kg, obtained from commercial mills in the eastern United States, primarily from the 2003 harvest. Of these, 35 samples were from regular process streams (selected because of a priori knowledge of elevated *Fusarium* damage), with the remaining 7 samples taken from discard piles of cleaners. The sorter, which used detectors and interference filters, one in the visible region and the other in the near-infrared region (see above), consisted of a series of parallel inclined channels. The level of DON ranged from 0.6 to 20 mg/kg. Samples were processed by the sorter operating at a throughput of 0.33 kg/(channel-min) (four of ten available channels used) and a kernel rejection rate of 10%. Visual measurements of the proportion of *Fusarium*-damaged kernels were collected on incoming and sorted specimens. Sort effectiveness was assessed by two means: percentage reduction of *Fusarium*-damaged kernels and percentage reduction in DON concentration. Results indicated that the fraction of DON contaminant level in the sorted wheat to that in the unsorted wheat ranged from 18 to 112 percent, with an average of 51 percent. Nine of the 35 regular samples and all 7 of the discard pile samples underwent a second sort, with 5 from this second set undergoing a third sort. Multiple sorting was effective in yielding product whose DON concentration was between 16 and 69 percent of its original, unsorted value. In summary, sorting resulted in a reduction of DON concentration by approximately one half on average, with further reduction arising from the resorting of accepted material. This study is fully described in our recent publication [Delwiche, et al., Plant Disease, vol. 89, p. 1214-1219 (2005)].

CORRELATION OF SEED SIZE AND DON ACCUMULATION IN SPRING WHEAT

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ABSTRACT

Fusarium head blight (FHB) reduces grain yield, test weight, grade, and may also contaminate kernels with mycotoxins. The most common mycotoxin associated with FHB infected grain, deoxynivalenol (DON or vomitoxin) reduces the marketability of grain since milling, baking, and feed quality may be adversely impacted. In this study we explored the relationship between DON accumulation within, and seed size of, thirty six spring wheat varieties and advanced breeding lines, where seed size represents an indirect estimate of the glume: endosperm ratio. Test entries were selected from South Dakota State University (SDSU) and North Dakota State University (NDSU) spring wheat breeding programs and represented a sample of germplasm which resulted from FHB resistance breeding efforts conducted from 1998 to 2003. Field tests were carried out as a Randomized Complete Block Design with four replications at Brookings, SD and Prosper, ND during the 2004 and 2005 growing seasons. Seed weight was obtained by weighing a 1000 seed sample of each entry, while DON concentration were collected on ground wheat samples by NDSU Veterinary Diagnostic Services. Analysis of seed size and DON concentration data over years from Brookings revealed the significant effect of years for both seed size and DON concentrations. Entry mean values for seed size and DON concentrations were calculated over years at Brookings for correlation analysis. Pearson's product moment correlation coefficients between seed size and DON concentration were computed individually for each year, the results showed negative association between seed size and DON concentration ($r=-0.35906$, $p=0.0315$; $r=-0.40772$, $p=0.0136$) in 2004 and 2005 respectively. Results from Brookings over years revealed a negative association between seed size and DON concentration but not statistically significant ($r=-0.22957$; $p=0.1780$). Both seed size and DON concentration observations were highly correlated over years ($r=0.79216$, $p=<.0001$; $r=0.56983$, $p=0.0003$) respectively. Breeders may eventually favor the selection of large-seeded lines as a means of lowering DON concentration in wheat varieties, although additional studies will be required to fully understand these relationships.

THE TRICHOTHECENE TRIANGLE: TOXINS, GENES AND POPULATIONS

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ABSTRACT

Fusarium trichothecenes have been classified based of structural properties. Type A and Type B trichothecenes differ in their A ring oxygenation pattern. Type A trichothecenes have a C-8 hydroxyl (neosolaniol), C-8 ester (T-2 toxin) or no C-8 substitution (diacetoxyscirpenol) and Type B trichothecenes having a C-8 keto group (deoxynivalenol, nivalenol). *F. graminearum* Type B trichothecenes can be divided into two chemotaxonomic groups. The DON chemotype produces deoxynivalenol and its acetylated derivatives (3-ADON, 15-ADON) and the NIV chemotype produces nivalenol and its acetylated derivative (fusarenone X). Nivalenol production is the ancestral trait based on sequence analysis. DON producers are more aggressive and virulent than those producing nivalenol (Desjardins et al. 2005). DON producers occur worldwide and predominate in North American, South America and Europe.

Trichothecenes are produced by a complex series of oxygenations, isomerizations, cyclization, and esterifications. Biochemical and genetic investigations of *Fusarium sporotrichioides*, *F. graminearum*, *F. culmorum* and other species have elucidated both the steps required (Zamir et al. 1999; Hesketh et al. 1991; McCormick et al. 1990) as well as the genes controlling trichothecene biosynthesis. Trichothecene genes have been mapped to four loci: the main 26 kb trichothecene cluster of twelve genes (*Tri8, Tri7, Tri3, Tri4, Tri6, Tri5, Tri10, Tri9, Tri11, Tri12, Tri13, Tri14*), a smaller cluster containing *Tri1* and *Tri16*, and two other loci with *Tri101* and *Tri15*, respectively (Jurgenson et al. 2002; Meek et al. 2003; Brown et al. 2001; Kimura et al. 1998).

The DON and NIV chemotypes have a clear genetic basis. DON-producing strains lack functional *Tri7* and *Tri13* genes (Kim et al. 2003). *Tri13* encodes the C-4 oxygenase and *Tri7* encodes a C-4 transacetylase. Similarly, differences for Type A and Type B trichothecene producing strains can be found in the small *Tri1-Tri16* gene cluster. T-2 toxin producing strains of *F. sporotrichioides* have *Tri1* that controls C-8 hydroxylation adjacent to *Tri16* that controls C8 esterification (Meek et al., 2003; Peplow et al., 2003). In DON-producing strains, *Tri16* is non-functional and a *Tri1* homolog controls hydroxylation at both C-7 and C-8 (McCormick et al. 2004). Finding the genetic basis for additional chemotypes (3-ADON/DON or 15ADON/DON) (Ward et al., 2002) may require the analysis of the genes involved in C-3 and C-15 acetylation and deacetylation. *Tri101* and *Tri3* encode the C-3 acetyltransferase and C-15 acetyltransferase, respectively (Kimura et al., 1998; McCormick et al., 1996). *Tri101* acts in concert with *Tri8*, the C-3 esterase gene (McCormick et al. 2002) to add and remove the C-3 acetyl group during biosynthesis. *F. graminearum Tri8* mutant strains lack the C-3 esterase and accumulate 3,15-diADON. Since biosynthesis of both 3-ADON and 15-ADON is likely via a 3,15-diADON intermediate, the esterase genes may be more important in distinguishing the 3ADON and 15ADON chemotypes. A C-15 esterase gene has not been identified. Infection with either 3-ADON or 15-ADON producing strains can result in the accumulation of DON in infected plant tissue. Defining chemotypes based on which toxins are found in infected plant tissue is problematic since plant esterases can modify the toxins normally produced in culture.

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PERSPECTIVES ON THE RISK ANALYSIS OF THE TRICHOTHECENE MYCOTOXINS

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ABSTRACT

Risk analyses for trichothecene mycotoxins and other food contaminants, which are to a significant extent unavoidable, present considerable challenges. The international risk assessments completed for deoxynivalenol, T-2 and HT-2 toxins, and nivalenol have noted a number of issues regarding the lack of adequate intake data for exposure assessment and significant gaps in toxicological studies for hazard characterizations. This is in spite of the fact that animal health problems have been associated with these mycotoxins for at least twenty-five years and occasional outbreaks of human illness have been reported. Risk assessment is constrained by uncertainties associated with the lack of adequate data, and risk management must consider the fact that mycotoxin contamination can have serious impacts on trade and food sufficiency. Risk assessment must be an iterative process, since the problem formulation and the risk assessment may need to be revised to reflect new data and theories. In addition to providing advice to risk managers, risk assessment should provide a blueprint for future research by illustrating what observations will influence a prediction. The objective, unbiased, and adequate evaluation of the association of exposures to specific agents or mixtures of agents with particular health outcomes can be extremely difficult. This is particularly true of complex, interactive phenomena, such as risk-risk and risk-benefit analyses, where there is often a lack of a common currency for comparisons. Two analytic approaches that can provide a more structured framework for such considerations are weight-of-evidence (WOE) and value-of-information (VOI). WOE approaches to human health risk analyses provide a framework for the interpretation of information about the harmful effect, including quality of testing methods, size and power of study designs, consistency of results across studies, biological plausibility of the exposure-response, and statistical associations. While most risk assessments involve WOE considerations, no general criteria or guidance have been established. VOI approaches can provide the basis for deciding whether it is better to make a decision now based on an inherently uncertain risk assessment or to collect additional information first and then decide. VOI analyses, based on decision analysis principals, can focus on the potential cost, including health impacts, from errors due to the uncertainties in decision making (based either on false-negatives or false positives) and identify the most valuable information for reducing the uncertainties. Addressing these uncertainties would provide risk managers with better guidance for control measures. The World Health Organization/International Programme on Chemical Safety has been developing criteria for WOE approaches and has initiated work on general considerations and guidance for VOI approaches.

FUSARIUM INFECTION, DON CONTENT AND MICROBIAL LOADS IN DURUM WHEAT FROM THE NORTHERN PLAINS: 2001-2004

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ABSTRACT

Durum wheat samples for years 2001 to 2004 were collected from five districts in North Dakota and one district in Montana and were analyzed for their microbiological quality, including deoxynivalenol (DON) content, aerobic plate count (APC), mold and yeast count (MYC), mesophillic aerobic spore formers (MASF), *Fusarium* infection (FI), and total mold infection (TMI). Composite samples from different regions were also analyzed for APC, MYC, FI, TMI, MASF. Processed samples such as bran, dust, flour, pasta, semolina, and shorts were also analyzed for APC, MYC and DON content. DON content for durum samples, district composite samples, and processed samples averaged 1.76 ± 2.05 (range: 0.47-4.79), 1.83 ± 1.81 (range: 0.5-3.89), and 1.76 ± 1.86 (range: 0.52-4.5) µg/g respectively. APC for durum samples, district composite samples, and processed samples averaged 8.18 ± 8.44 (range: 5.68-8.75), 7.91 ± 7.98 (range: 5.55-8.27), and 6.97 ± 7.13 (range: 5.12-7.46) $\log_{10}$ cfu/g respectively. MYC for durum samples, district composite samples, and processed samples averaged 4.49 ± 4.26 (range: 4.07-4.71), 4.25 ± 4.22 (range: 3.91-4.63), and 3.98 ± 3.88 (range: 2.41-4.27) $\log_{10}$ cfu/g respectively. MASF for durum samples and district composite samples averaged 2.5 ± 1.53 (range: 2.47-2.54) and 2.48 ± 0.76 (range: 2.47-2.48) $\log_{10}$ cfu/g respectively. FI for durum samples and district composite samples averaged 35.76 ± 19.42 (range: 11.13-57.36) and 40.76 ± 28.98 (range: 11.67-75.67) % respectively. TMI for durum samples and district composite samples averaged 83.32 ± 8.11 (range: 75.39-94.01) and 85.72 ± 8.44 (range: 77.17-97.17) % respectively. When the whole grains were processed, DON content was reduced significantly from an average of 1.76 ± 2.05 (whole grain) to 0.92 ± 0.67 (pasta) µg/g. APC were reduced from an average of 8.18 ± 8.44 (whole grain) to 5.48 ± 5.78 (pasta) $\log_{10}$ cfu/g. MYC were reduced from an average of 4.49 ± 4.26 (whole grain) to 2.51 ± 2.44 (pasta) $\log_{10}$ cfu/g. Overall, microbial loads and DON were reduced as a result of milling and processing. Data for all four years will be presented.

DEOXYNIVALENOL AND 15-ACETYLDEOXYNIVALENOL PRODUCTION BY *FUSARIUM GRAMINEARUM* STRAINS GROWN IN SEMI-DEFINED MEDIUM WITH DIFFERENT CARBOHYDRATE SOURCES

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ABSTRACT

Two *Fusarium graminearum* strains (FRC # R-9828 and FRC # R9832) were cultured in yeast extract peptone broth limited to single carbohydrate sources (at 1%), including xylan, cellulose, starch or glucose, to measure production of deoxynivalenol (DON), 15-acetyldeoxynivalenol (15Ac-DON) and mycelium. R-9828 was an isolate from South Africa belonging to Lineage 3 of *Fusarium graminearum*, while R9832 was a strain from the United States and belongs to Lineage 7. The two strains were chosen for the study because they demonstrated virulence to wheat in greenhouse trials and were good DON producers in solid substrates such as rice, corn, wheat and barley. A random complete block design with factorial arrangement was used. Each treatment was done in duplicate and repeated once on different days (n = 4). Analysis of variance with Duncan's multiple comparison was employed to test for treatment differences at a significance level of 5% ($p<0.05$). On average, xylan yielded the highest mycelial mass (22.22 ± 10.63 mg), followed by glucose (13.56 ± 8.73 mg), starch (11.36 ± 7.39 mg), and finally cellulose (4.21 ± 6.95 mg). There were significant differences between the four carbohydrate sources for DON production with glucose (12.94 ± 19.65 ppm/mg mycelia) and xylan (6.30 ± 9.17 ppm/mg mycelia) yielding significantly higher DON than starch (4.72 ± 8.84 ppm/mg mycelia) or cellulose (0.39 ± 0.70 ppm/mg mycelia). There were no significant differences between the four carbohydrates for 15Ac-DON production.

These preliminary results support the theory that *Fusarium graminearum* invades through xylan in the cell walls of wheat and barley and suggests that xylan may induce *Fusarium* growth and DON production to help with the invasion process.