FOOD SAFETY, TOXICOLOGY AND UTILIZATION OF CONTAMINATED GRAIN

Chairperson: Stephen Neate

RAPD ANALYSIS OF *FUSARIUM GRAMINEARUM* ISOLATES FROM ELECTRON BEAM IRRADIATED BARLEY

Anuradha Boddeda[1] and C.E. Wolf-Hall[1*]

[1]Department of Veterinary and Microbiological Sciences, North Dakota State University, Fargo, ND, USA
[*]Corresponding Author: PH: (701) 231-6387; E-mail: Charlene.Hall@ndsu.nodak.edu

ABSTRACT

Barley is affected by Fusarium head blight. The mycotoxins produced by *F. graminearum* affect the grain safety, malting and beer quality. Barley can be irradiated with electron beam radiation to reduce the fungal contamination, but there may be some *Fusarium* strains which can survive, grow and produce mycotoxins as irradiated barley is malted. Random amplified polymorphic DNA (RAPD) analysis was done to screen for mutations in *Fusarium* isolates from irradiated barley. FHB-infected and control barley samples, which were irradiated using electron beam radiation at doses of 0, 2, 4, 6, 8 and 10 KGy were obtained. *Fusarium* infection (FI) analysis was done on 100 barley seeds which were aseptically placed on half strength potato dextrose agar (HPDA) plates incubated at 25ºC for 5 days. Single germinated spores of *Fusarium* isolates confirmed by FI analysis were transferred to carnation leaf agar and incubated at 25ºC for 7 days. The fungal growth on the plates was identified to the species level microscopically based on morphological characteristics. The morphology, pigment and growth of these fungal colonies were confirmed by growing conidia of *Fusarium* isolates on HPDA plates which were incubated at 25ºC for 7 days. Nine *F. graminearum* isolates were used for RAPD analysis to screen for mutations by comparing with a control *F. graminearum* isolate (FRC R-9821) and were grown on 25 g of autoclaved rice (40% moisture content) at 25ºC for 14 days and analyzed for their ability to produce deoxynivalenol and related mycotoxins by high pressure liquid chromatography. The cluster analysis of the various *F. graminearum* isolates' RAPD profiles have confirmed that they can be divided into two groups. Group A consists of the reference strain (FRC R-9821) and four isolates obtained from control and irradiated (2KGy) barley. Group B consists of three isolates which were obtained from barley irradiated to 4, 6 and 10 KGy and two control strains. Deoxynivalenol (DON) produced by both control and irradiated isolates ranged from none to 31.1 µg and 15-Acetyl deoxynivalenol (15-ADON) produced ranged from none to 105.34 µg. Some of the isolates obtained from irradiated barley retained their DON producing ability to some extent (ranged from 0.65-4.76 µg) but produced no ADON. The results from mycotoxin analysis indicate that irradiation may decrease mycotoxigenesis in *F. graminearum.* Further research has to be done to know whether irradiated barley can be used in malting industry, as irradiation is found to reduce the mycotoxigenecity of *F. graminearum* isolates obtained from irradiated barley.

BEAUVERICIN AND ENNIATINS IN FUSARIUM HEAD BLIGHT GRAINS

Bottalico A., A. Logrieco* and A. Visconti

Institute of Sciences of Food Production (ISPA), National Research Council (CNR),
Via G. Amendola 122/O, 70125 Bari, Italy
*Corresponding Author: PH: 39 080 5929357; E-mail: Antonio.logrieco@ispa.cnr.it

ABSTRACT

Epidemics of Fusarium head blight (FHB) on cereal grains caused by several species of *Fusarium*, mainly reported as a complex, are becoming more frequent around the world. The disease reduces grain yield and quality, often causing grain to be unsuitable for human consumption, because some toxigenic *Fusarium* strains are capable to accumulate mycotoxins in cereal grains and derived foods and feeds. The pathogens mainly reported as causal FHB agents are strains of *F. graminearum* deoxynivalenol (DON) producer, but especially in cooler regions of Europe, other species of *Fusarium*, such as *F. culmorum*, (also a DON producer) and *F. avenaceum* (a moniliformin producer), can predominate. At this regard, recent investigations reported an increasing occurrence of strains of *F. avenaceum*, and its strictly related species *F. arthrosporioides*, as well as of *F. poae*, *F. sporotrichioides* and *F. tricinctum*, all producers of the esadepsipeptides beauvericin (BEA) and enniatins (ENs). The natural occurrence of high amounts of beauvericin (up to 3.5 mg/kg) and enniatins (up to 18.3 mg/kg of enniatin B) in FHB small grains, together with the relevant phytotoxic and zootoxic properties, suggest an examination of the potential role of these *Fusarium* metabolites in contributing to the severity of FHB and the toxicity of cereal grains.

Keywords: Fusarium Head Blight (FHB), Esadepsipeptides, Enniatins, Beauvericin, Mycotoxins, *Fusarium avenaceum*.

Fusarium Head Blight (FHB) of cereal grains - Fusarium head blight (FHB) of wheat and other small cereals is a severe disease world wide, causing reduction in crop yield often estimated up to 30 percent. In addition certain *Fusarium* strains are also capable of producing mycotoxins which can be formed already in infected plants standing in the field and then accumulate in stored grains under favourable fungal growth conditions. The occurrence of mycotoxins in cereal grains is of great concern, because their presence in foods and feeds is often associated with chronic or acute mycotoxicoses in livestock and, to a lesser extent, also in human. The *Fusarium* species predominantly reported around the world as causal FHB agent is *F. graminearum*, and the toxicological connected greatest problem is related essentially with the occurrence of deoxynivalenol or vomitoxin (DON). However, especially in cold northern European regions, other species of *Fusarium*, such as *F. culmorum* (also a DON producer), and *F. avenaceum* are important FHB agents (Bottalico, 1998).

Many strains of *F. avenaceum* from FHB small grains were found to produce several cyclic esadepsipeptides, including enniatins (ENs) and beauvericin (BEA) (Logrieco *et al*., 2002b; Morrison *et al*., 2002). In addition, some other *Fusarium* species from FHB grains, such as *F. poae*, *F. arthrosporioides* (*F. avenaceum*), *F. tricinctum* and *F. sporotrichioides* were proven to have the same relevance as esadepsipeptide producers (Nicholson *et al*., 2004). On the other hand, there are increasing evidences of the occurrence of high amounts of ENs and BEA in Finnish scab small grains (wheat, rye, oats and barley) (Logrieco *et al*., 2002b; Jestoi *et al*., 2004).

Enniatins were known for a long time as phytotoxins and associated with plant diseases characterized by wilt and necrosis (Gäumann *et al*., 1960), and some phytotoxic properties were recently reported also for BEA (Sagakuchi *et al*., 2000). Moreover, due to their ionophoric structure, BEA and ENs are able to exhibit many toxic effects on animal systems, starting from

the alteration of the ion transport across membranes, which may lead to the disruption of the cationic selectivity of cell wall, and ultimately to induce DNA fragmentation and cell death by apoptosis (Logrieco *et al*., 2002a; Macchia *et al*., 2002). These basic mechanisms, represent the behaviour leading to a large array of toxic ability, such as antimicrobial, insecticidal; and a strong cytotoxic activity on several cell lines of invertebrate, rodents, farm animals, and human (Ganassi *et al*., 2002; Calò *et al*., 2003, 2004; Fornelli *et al*., 2004). These findings on toxic potential of BEA and ENs in plant and animal systems, obviously stimulate us to a deeper examination of the significance of such esadepsipeptides and their producing *Fusarium* species in contributing to the FHB severity and grains toxicity.

Natural occurrence of Beauvericin and Enniatins - Beauvericin (BEA) is the main natural component of the beauvericin group, belonging to ENs family (Hamill *et al*., 1969). Besides BEA, five other compounds were purified from fumgal cultures, and designated as beauvericin A, C, D, E and F (Gupta *et al*., 1995; Fukuda *et al*., 2004), but they are not yet found as natural contaminants. The most important ENs, reported as natural contaminants, include: Enniatin A (ENA), Enniatin A1 (ENA1); Enniatin B (ENB); and, Enniatin B1 (ENB1) (Savard and Blackwell, 1994). Three new enniatins of the B series, designated as B_2, B_3 and B_4 were characterized from liquid culture of *F. acuminatum* and *F. compactum* by Visconti *et al*. (1992), but as far as we are aware they were not yet found as natural contaminants.

BEA was found in Finnish and Norwegian wheat and other small cereal grains mainly referred to the colonization of *F. avenaceum* (Logrieco *et al*., 2002b), but also to those of *F. poae* and *F. sporotrichiodes* (Yli-Mattila *et al*., 2004a; Jestoi *et al*., 2004; Uhlig and Ivanova, 2004). In particular, BEA was found in all samples of Finnish rye (up to 3.5 mg/kg) (Logrieco *et al*., 2002b); in Norwegian samples of wheat, barley and oats (up to 0.12 mg/kg) (Uhlig *et al*., 2004); in Finnish samples of wheat, barley and oats (up to 0.019 mg/kg), and at trace levels in Italian and Finnish samples of grain-based products (Jestoi *et al*., 2004). The natural occurrence of ENs in grains has been investigated less extensively than BEA. In fact, ENs have been found only in a few investigations in samples of small cereal grains and grain-based products from Finland and Norway (Logrieco *et al*., 2002b; Jestoi *et al*., 2004; Uhlig and Ivanova, 2004). Interestingly, ENs were reported at ppm levels in wheat (ENB, ENB1), rye (ENA1, ENB, ENB1), and barley (ENA1, ENB, ENB1), with concentrations of ENB as high as 18.3 and 9.76 mg/kg (ppm) in Finnish wheat and barley, respectively (Logrieco *et al*., 2002b; Uhlig and Ivanova, 2004; Jestoi *et al* , 2004). In particular, very high amounts of ENA (up to 6.9 mg/kg), ENB (up to 4.8 mg/kg); and ENB1 (up to 1.9 mg/kg) were found in Finnish rye samples by Logrieco *et al*. (2002b); whereas Uhlig and Ivanova (2004) found relevant amounts of ENs type B in Norwegian wheat, oats, and barley, but only trace levels of ENs type A.

Potential toxic role of BEA and ENs in FHB severity and toxicity of small cereals - The *Fusarium* species-complex causing FHB in northern Europe, depending on year crop season and cereal host, seems to be predominantly composed by *F. avenaceum*, *F. poae*, *F. arthrosporioides*, *F. sporotrichiodes* and *F. tricinctum* (Jestoi *et al*., 2004; Nicholson *et al*., 2004; Yli-Mattila *et al*., 2004b). As a consequence, the greatest mycotoxicological problem derived from FHB appears to be associated mainly with the occurrence of BEA and ENs and, at a lesser extent, with zearalenones and trichothecenes which are produced by *F. gaminearum*, *F. culmorum*, *F. poae* and *F. sporotrichiodes*. Both BEA and ENs exhibited phytotoxic and zootoxic activities in many bioassays, therefore, like for other mycotoxins formed in plant hosts, it could be suggested a possible role of BEA and ENs in plant pathogenesis and in grain toxicity.

Regarding the phytotoxic activities of ENs, it was reported that these metabolites are toxic in several plant systems, including: loss of turgor, leaf yellowing and marginal necrosis in tomato cuttings (Gäumann *et al*., 1960); inhibition of growth of wheat seedlings (Burmeister and Plattner, 1987); necrotic lesions on leaves (Hershenhorn *et al*., 1992) and on potato tu-

ber slices (Herrmann *et al*., 1996b); and inhibition of germination of a parasitic weed (Zonno and Vurro, 1999). Information on the toxicity of BEA against plant systems is limited to the induction of premature death of melon and tomato protoplasts (Sagakuchi *et al*., 2000; Paciolla *et al*., 2004).

In analogy with the role of DON in FHB severity, also for ENs was proposed a role during the plant infection process by *Fusarium* species synthesizing enniatins. To this regard, there are evidences that the virulence of modified strains of *F. avenaceum* was significantly reduced after disruption of the *esyn1* gene, which encodes for the multifunctional enzyme enniatin synthetase involved in enniatin biosynthesis (Herrmann *et al*., 1996a)

It appears that ENs exhibit more phytotoxic capability than BEA, which instead appears endowed with stronger zootoxic properties.

It was well established that the toxicity of BEA and ENs derives from their peculiar ionophoric property, and their capability to cause DNA fragmentation and cell death by apoptosis (Logrieco *et al*., 2002a). Consequently, the high cytotoxicity, especially for BEA, was confirmed in several cell line bioassays, including invertebrate, insects, rodents, livestock and human cell lines, and the ability of these metabolites to induce apoptosis is currently used as positive control in many physiopathological investigations. But, the data obtained on the biological activity of BEA and ENs in the few *in vivo* studies indicated a general low activity at the concentrations tested. In fact, besides a relatively low acute toxicity observed by intraperitoneal administration of ENs on mouse (McKee *et al*., 1997), no adverse effects were observed on growth and health parameters in several feeding trials on broiler and turkey (Leitgeb *et al*., 2000; Zollitsch *et al*., 2003).

The high contamination levels of the Finnish cereal grains, both for BEA (up to 3.5 mg/kg) (Logrieco *et al*., 2002b) and ENs (up to 18.3 and 9 mg/kg of ENA in wheat and barley, respectively) (Uhlig *et al*., 2004; Jestoi *et al*., 2004), besides the high toxigenic potential of FHB causing strains, suggest a deeper investigation on the chronic toxicity of these esadepsipeptides, particularly for ENB. Strains causing FHB were capable of producing in culture amounts of BEA (especially *F. poae*) and total ENs (especially *F. arthrosporioides*) up to 130 and 3000 mg/kg, respectively (Jestoi M., personal communication). In addition, the possible toxic interactions or synergistic effects of ENs and BEA with mycotoxins co-occurring in infected grains (especially MON, DON and NIV) (Golinski *et al*., 1997; Yli-Mattila *et al*., 2004a) should be deeper explored.

Finally, the phylogenetic and toxigenic relationships among the new entities within the monophyletic group *F. avenaceum*/*F. arthrosporioides*/*F. tricinctum*, which appear to play an important pathogenic and toxigenic role in FHB of small cereal grain of northern European countries (Yli-Matila *et al*., 2004a) should be better assessed. To this purpose it seems now possible the use of several molecular markers (Nicholson *et al*., 2004; Yli-Mattila *et al*., 2004b) to separate such strictly related molecular and morphological species even directly from the infected spikes, and then to evaluate the possibility to reconsider the taxonomic importance of *F. arthrosporioides* Sherb.

In conclusion, the widespread occurrence of *F. avenaceum* as the predominant agent of FHB of small cereal grains, together with its ability to produce high amounts of BEA and ENs which can occur at high levels in naturally infected grains, warrant more accurate investigations to establish the role of these *Fusarium* metabolites in the severity of FHB and the toxicity of scabby grains.

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HIGH-SPEED OPTICAL SORTING OF SOFT RED WINTER WHEAT FOR REMOVAL OF FUSARIUM-DAMAGED KERNELS

S.R. Delwiche[1*], T.C. Pearson[2] and C.S. Gaines[3]

[1]USDA/ARS, Beltsville Agricultural Research Center, Instrumentation and Sensing Laboratory, Beltsville, MD 20705, USA; [2]USDA/ARS, Grain Marketing and Production Research Center, Manhattan, KS 66502, USA; and [3]USDA/ARS, Soft Wheat Quality Laboratory, Wooster, OH 44691, USA
[*]Corresponding Author: PH: (301) 504-8450; E-mail: delwiche@ba.ars.usda.gov

ABSTRACT

Our previous work has examined the accuracy of a semi-automated wheat scab inspection system that is based on near-infrared (NIR) reflectance (1000 to 1700 nm) of individual kernels. Classification analysis has involved the application of various statistical classification techniques, including linear discriminant analysis (LDA), soft independent modeling of class analogy (SIMCA), partial least squares (PLS) regression, and non-parametric (*k*-nearest-neighbor) classification. Recent research has focused on the determination of the most suitable visible or near-infrared wavelengths that could be used in high-speed sorting for removal of FHB-infected soft red winter wheat kernels. Current technology in high-speed sorters limits the number of spectral wavelengths (regions) of the detectors to no more than two. Hence, the critical aspect of this study has been the search for the single wavelengths and best two-wavelength combinations that maximize class separation, using LDA. Four thousand eight hundred kernels from 100 commercial varieties, equally divided between normal and scab-damaged categories, were individually scanned in the extended visible (410-865 nm) and near-infrared (1031-1674 nm) regions. Single- and all combinations of two-wavelength LDA models were developed and characterized through cross-validation by the average correctness of classification percentages. Short visible (~420 nm) and moderate near-infrared (1450-1500 nm) wavelengths produced the highest single-term classification accuracies (at approximately 77% and 83%, respectively). The best two-term models occurred near the wavelengths of 500 and 550 nm for the visible region alone (94% accuracy), 1152 and 1248 nm for the near-infrared region alone (97%), and 750 and 1476 nm for the hybrid region (86%). These wavelengths are, therefore, considered of importance in the design of monochromatic and bichromatic high-speed sorters for scab-damage reduction. Ongoing research is presently examining the efficiency of high-speed sorting for Fusarium-damaged kernels, as measured by reduction in DON concentration. Approximately 40 5-kg commercial samples of soft red winter wheat have undergone as many as three successive sorts, using a commercial sorter outfitted with filters at 675 and 1470 nm. Results indicate a significant reduction in DON is achieved through sorting; however, this comes at the expense of false positives (good kernels diverted to reject stream) and the overall reduction in material available for processing.

INVESTIGATION OF FUSARIUM MYCOTOXINS IN UK WHEAT PRODUCTION

S.G. Edwards

Harper Adams University College, Newport, Shropshire, TF10 8NB UK
Corresponding Author: PH: 44 1952 815226; E-mail: sedwards@harper-adams.ac.uk

OBJECTIVES

Determine the effects of agronomic factors on *Fusarium* mycotoxin levels in UK wheat grain over a five year period (2001 – 2005).

INTRODUCTION

Fusarium mycotoxins are produced on cereal grains by a wide range of *Fusarium* species. Many mycotoxins are produced in culture but the main ones found in cereals are the trichothecenes (which include deoxynivalenol (DON, also known as vomitoxin), nivalenol (NIV), HT2 and T2), zearalenone (ZEAR) and fumonisins. The *Fusarium* mycotoxins are produced predominantly in the field but levels can increase if stored under adverse conditions. On cereals the predominant mycotoxins worldwide are DON and zearalenone. Fumonisins are only usually found on maize.

The European Commission is currently considering maximum levels of *Fusarium* mycotoxins for unprocessed cereals and cereal foodstuffs intended for human consumption. Legislation for DON, zearalenone, HT2+T2 and fumonisins should be introduced within the next two to three years. Previous one year surveys of *Fusarium* mycotoxins have shown that levels are generally low within the UK (Turner et al., 1999; Prickett et al., 2000). However, it is not known how the levels of mycotoxins in wheat vary over different seasons. It is also not known how agronomic factors may affect mycotoxin levels in UK wheat.

MATERIALS AND METHODS

Three hundred grain samples are collected at each harvest. An equal number of samples were requested from each region: South, East, Central, West, North of England, Scotland and Northern Ireland.

An even number of samples were requested from each of the following categories:

1. Organic production
2. Conventional production with no head spray
3. Conventional production with straight strobilurin head spray
4. Conventional production with strobilurin /triazole mixture head spray
5. Conventional production with straight triazole head spray

Samples were analysed by RHM Technology, High Wycombe, UK by GC-MS analysis for ten trichothecenes and by the Central Science Laboratory, York, UK by HPLC analysis for zearalenone. The trichothecenes analysed were deoxynivalenol (DON), nivalenol (NIV), 3-acetylDON, 15-acetylDON, fusarenone X, T2 toxin, HT2 toxin, diacetoxyscirpenol (DAS), neosolaniol and T2 triol.

RESULTS AND DISCUSSION

Incidence of *Fusarium* mycotoxins in UK wheat in the first three years of the project, 2001-2003, was generally low with only four mycotoxins detected in more than 5% of samples tested. The five most dominant mycotoxins found are detailed in Tables 1, 2 and 3. Data for HT2 and T2 were combined as T2 is rapidly metabolised into HT2. HT2 was the major component of this combined data. It should be noted these results are from selected samples and not a strati-

fied survey so the average values obtained may not accurately represent the true UK averages.

The incidence and concentration of *Fusarium* mycotoxins in UK wheat were similar in each year tested. The most noticeable difference in the three years was the higher incidence of HT2+T2 in 2003 (Table 1, 2 and 3). The vast majority of samples were well below the current EU proposed maximum limit for DON in unprocessed wheat (1250 ppb) The concentration of DON found in UK wheat from 2001 to 2003 was generally low (average was 140 ppb) compared to levels found in other European countries and elsewhere in the world (Anon, 2001).

Preliminary statistical analysis of the combined data from 2001 to 2003 has shown a number of agronomic factors can affect DON levels in wheat grain. Other factors may be determined to have an effect once data from all five years have been analysed. The results to date indicate:

·Region where wheat is grown was a major factor and this can change with year. The South and East had higher levels of DON than the rest of the country.

·Maize as the previous crop increased the risk of higher DON levels.

·Minimum cultivation increased the risk of higher DON levels if following a cereal, in particular maize.

·The Fusarium head blight resistance in winter wheat varieties reduced DON levels in harvested grain.

·There was no measurable effect of fungicides used at current rates on DON levels and there was no difference between wheat samples from conventional and organic farms.

Visual assessments, using *Fusarium* damaged grain counts, were poor and inconsistent indicators of trichothecene levels in UK wheat.

Analysis of all 1500 samples over five years will provide a clear picture of *Fusarium* mycotoxin levels in UK wheat over a range of different seasons and will allow powerful statistical analysis of all agronomic factors. Results will aid the cereal industry to prepare for EU legislation on the maximum permissible levels of *Fusarium* mycotoxins in cereal grains and products. Results will also be used to advise growers of "Good Agricultural Practice" to minimise *Fusarium* mycotoxin levels in UK wheat production.

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Table 1. Mycotoxin content of UK wheat from the 2001 harvest (283 samples).

		Mycotoxin concentration (ppb)				
	%>10ppb	Mean	Median	90th%	95th%	Max
DON	80	80	32	133	223	5175
NIV	80	34	23	71	97	428
HT2+T2	30	<20	<20	22	32	214
ZEAR	4.9	<5	<5	6	9	188

Table 2. Mycotoxin content of UK wheat from the 2002 harvest (343 samples).

		Mycotoxin concentration (ppb)				
	%>10ppb	Mean	Median	90th%	95th%	Max
DON	78	116	30	211	470	3065
NIV	55	21	11	46	68	430
HT2+T2	16	<20	<20	<20	22	75
ZEAR	17	10.6	<5	19	38	707

Table 3. Mycotoxin content of UK wheat from the 2003 harvest (328 samples).

		Mycotoxin concentration (ppb)				
	%>10ppb	Mean	Median	90th%	95th%	Max
DON	89	218	38	346	594	10626
NIV	82	34	22	77	106	237
HT2+T2	69	22	18	44	55	199
ZEAR	13	7	<5	14	28	209

Means are based on an imputation of 1.67 (0.83 for zearalenone) for all samples below the limit of quantification (10 ppb; 5 ppb for zearalenone).

DOES BIOCHEMICAL COMPOSITION OF DURUM WHEAT KERNELS INFLUENCE THE TRICHOTHECENES B CONTAMINATION LEVELS?

L. Favre[1], M.N. Verdal-Bonnin[1], L. Pinson-Gadais[1], P. Roumet[2], C. Barreau[1], F.Richard-Forget[1*]

[1]INRA, Unité MYCSA, 71 av Ed. Bourleaux, BP81, 33883 Villenave d'Ornon, France ; and [2]INRA, UMR DGPC, Domaine de Melgueil, 34130 Maugio, France
[*]Corresponding Author: PH: 33 5 57 12 24 83; E-mail: fforget@bordeaux.inra.fr

ABSTRACT

Commercial durum wheat varieties do not significantly differ for their level of resistance to Fusarium Head Blight (FBH). However, this does not exclude different sensitivities to trichothecenes B (TCTB) accumulation. In addition to FBH resistance, several mechanisms may influence the TCTB content of kernel such as degradation/conjugation of TCTB or occurrence of compounds inhibiting the toxinogenesis.

The aim of the present work was to investigate the eventual occurrence of different sensitivities to TCTB contamination among a collection of durum wheat lines derived from crosses involving *dicoccoïdes*, *dicoccum* or *polonicum* accessions with high yielding durum varieties and to identify the factors involved in this variability. Fifteen cultivars were inoculated at anthesis by a Nivalenol (NIV) producing *Fusarium* strain. Ergosterol and TCTB amounts were quantified on the harvested kernels. The TCTB contamination rates (measured as TCTB/ergosterol ratios) showed great variations depending on the considered genotype. Some lines appeared therefore as able to limit TCTB biosynthesis.

Two *Fusarium culmorum* strains, a Deoxynivalenol (DON) producing strain and a NIV one, were inoculated *in vitro* on kernels, bran and semolina from the Nefer durum wheat variety. If semolina resulted to be an excellent substrate for TCTB biosynthesis, bran induced a strong decrease in both DON and NIV yields compared to whole kernels. Moreover, in 30 days liquid cultures of both DON and NIV producing strains supplemented with 0.05g.l^{-1} of bran, the TCTB amounts were 10 fold lower than in the control flasks, meanwhile the fungal growth was similar in the different conditions. These results allowed us to conclude for the occurrence of some biochemical compounds inhibiting the TCTB biosynthesis in brans of Nefer durum wheat.

In accordance with the literature data and the biochemical composition of bran fractions, these inhibitors could be phenolic compounds, and more precisely some acid phenols. Thus, ten benzoic and cinnamic acids including ferulic and *p*-coumaric acids which are predominant in wheat bran, were added to liquid cultures of a DON producing strain at concentrations which do not affect fungal growth. Whatever the considered phenolic acid, higher TCTB amounts were obtained in the supplemented media. Therefore, in our experimental conditions, phenolic acids were shown to activate TCTB production. Moreover, this efficiency of activation appeared to strongly depend upon the antioxydant potential of the phenolic acid, the most antioxydant leading to the highest TCTB amount.

Our studies demonstrated that the choice of durum wheat genotype may influence the TCTB accumulation level of the yielded kernels. The different sensitivities to TCTB contamination may be ascribed to the biochemical composition of kernels. Brans were shown to contain TCTB biosynthesis inhibitors. Phenolic acids appeared

as not involved in this inhibition. Further studies are carried out in order to purify and identify the compounds of durum wheat bran inhibiting toxinogenesis.

|Abbreviations used: TCTB: trichothecene B; DON: deoxynivalenol; NIV: nivalenol

SIMPLE AND RAPID IMMUNOQUANTIFICATION OF FUSARIUM IN BARLEY AND ITS RELATIONSHIP WITH DON AND FHB SCORES

N.S. Hill[1*], P. Schwarz[2], R. Horsley[2], S. Neate[3], B. Steffenson[4], L. Dahleen[5], B. Cooper[6], B. Kittle[1] and A. Jones[1]

[1]Dept. Crop and Soil Sciences, University of Georgia, Athens, GA 30602, USA; [2]Dept. Plant Sciences, North Dakota State University Fargo, ND 58105, USA; [3]Dept. Plant Pathology, North Dakota State Univ., Fargo, ND 58105, USA; [4]Dept. Plant Pathology, University of Minnesota, St. Paul, MN 55108, USA; [5]USDA-ARS, Fargo, ND 58105; and [6]Busch Agricultural Research Institute, Ft. Collins, CO., USA

[*]Corresponding Author: PH: 706-769-2397; E-mail: nhill@uga.edu

ABSTRACT

A simple and accurate method of quantifying the presence of *Fusarium* graminearum limits our ability to understand disease epidemiology, develop control measures, or improve resistance via breeding or biotechnology. Breeding based upon visual scores of head blight incidence have greater heritability than selecting low deoxynivalenol (DON), but visual scoring is not easily standardized. Head blight scores (mean r = 0.21, std. dev. = 0.24) and DON (mean r = 0.46, std. dev. = 0.22) were poorly correlated among locations in the 2003 NABSEN field trials. Both visual scoring and DON analysis are time consuming, making neither amenable to multiple analysis for inter- or intra-experiment quality control assessments. Thus a better method of *Fusarium* quantification is needed to measure head blight that can be used as quality assurance methods for end users.

An ELISA test for *Fusarium* in small grains and corn was developed. Experimental error associated with visual scoring, DON analysis, and ELISA evaluation of *Fusarium* presence was evaluated in three replicated field experiments grown in Osnabrock, Langdon, and Casselton, ND. Each were visually scored for FHB, analyzed for DON by GC-EC, and analyzed for *F. graminearum* by species-specific indirect ELISA. Coefficients of variation and a correlation matrix were calculated for each response variable. We obtained 89 samples from grain elevators in North Dakota and correlated FHB, DON, and ELISA data with one another. In another study, we developed a method to sequentially extract DON and *Fusarium* antigens to quantify both within individual seeds, thus eliminating variability to sampling.

Mean coefficients of variability for the ELISA values were lower than FHB or DON in the field studies. Mean correlation coefficients among the field experiments were greater for ELISA vs. DON than for ELISA vs. FHB or DON vs. FHB. Similarly, correlations coefficients between ELISA and DON were greater than ELISA vs. FHB or DON vs. FHB for the grain elevator samples. Analysis of individual seeds for DON and ELISA did not improve goodness of fit among samples. Thus, we grew 3 isolates of *Fusarium graminearum* in 4 different media with 10 replications. Mycelial growth was less on Shenk Hildebrandt medium than other media, but other media did not differ from one another. *Fusarium* antigen per g of mycelium was not different among media or isolates. DON was highly variable within each medium, ranging from 0 to 26 ppm. Subsequent to these studies we have analyzed as many as 1000 barley samples (in duplicate) in one day using ELISA with an R^2 = 0.97 for the repeated measures. Consistency, speed, and ease of analyses make ELISA a superior method for quantifying *F. graminearum*.

DETOXIFICATION OF ZEARALENONE BY GENETICALLY MODIFIED ORGANISMS

Tomoko Igawa, Naoko Takahashi-Ando, Arisa Higa-Nishiyama, Tetsuko Ochiai-Fukuda, Kaori Kadokura, Isamu Yamaguchi and Makoto Kimura*

Laboratory for Remediation Research, Plant Science Center, RIKEN, 1-7-22 Suehiro, Tsurumi, Yokohama, Kanagawa 230-0045, Japan
*Corresponding Author: PH: 81 48 467 9796, E-mail: mkimura@postman.riken.go.jp

ABSTRACT

Zearalenone (ZEN) is a nonsteroidal estrogenic mycotoxin produced by numerous Fusarium species in pre- or post-harvest cereal grains, and causes severe reproductive problems on livestock. Previously, we isolated a ZEN-detoxifying gene, zhd101, from a fungus Clonostachys rosea (Takahashi-Ando et al., 2002). To reduce the mycotoxin-contamination level in food and feed, this gene is expected to be useful in establishing a reliable detoxification system with genetically modified organisms.

We constructed a codon-optimized zhd101 gene for expression in yeast (Takahashi-Ando et al., in press), which was recently reported to be applicable as a live vehicle for transgenic detoxification of toxic substances (Blanquet et al., 2003). When the transgenic yeast cells were added to the medium containing 2 µg/ml of ZEN, this mycotoxin was completely eliminated within 48h of incubation at 28°C. We also generated several transgenic rice and maize using an egfp::zhd101 fusion gene. Preliminary experiments with transgenic rice demonstrated in vivo and in vitro detoxification of ZEN by calluses (Takahashi-Ando et al., 2004) and leaf extracts, respectively. Transgenic maize also showed ZEN detoxification activity. The ZEN-detoxification activity of these transgenic cereal plants will be evaluated by an artificial inoculation assay using mycotoxigenic Fusarium graminearum.

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MYCOTOXINS AND CYTOTOXICITY OF FINNISH *FUSARIUM*-STRAINS GROWN ON RICE CULTURES

M. Jestoi[1*], S. Paavanen-Huhtala[2], S. Uhlig[3], A. Rizzo[1] and T. Yli-Mattila[2]

[1]National Veterinary and Food Research Institute (EELA), Department of Chemistry, P.O.BOX 45 (Hämeentie 57), FIN-00581, Helsinki, Finland; [2]Laboratory of Plant Physiology and Molecular Biology, Department of Biology, FIN-20014, University of Turku, Finland; and [3]National Veterinary Institute, Department of Food and Feed Hygiene, Section for Chemistry, N-0033, Oslo, Norway
*Corresponding Author: PH: 358-9-393 1728; E-mail:marika.jestoi@eela.fi

OBJECTIVES

To screen the mycotoxins produced by Finnish *Fusarium*-strains in pure cultures grown on rice at three different environmental conditions, and to test the cultures for cytotoxicity in a bioassay using porcine kidney –cells.

INTRODUCTION

Several species of *Fusarium* are capable of producing a range of mycotoxins. Generally, the production of mycotoxins is a very complex and diverse process, the details of which have not been totally understood, although several affecting factors have been recognized (Hesseltine, 1976).

The type and amount of mycotoxin produced is determined mainly by the fungus, substrate and environmental conditions (Lacey, 1986). However, the different strains of the same species of fungi may differ remarkably in their toxin production capability due to the genetic differences. This fact may partly, in addition to the environmental conditions applied, explain the differences in the reported cases of *Fusarium*-species to produce specific mycotoxins.

Attention must be paid to the true identification of the species as well as to the metabolites produced. In fact, the current view is that the metabolite profile of a strain is regarded as an important parameter in the systemic taxonomics of a fungus supporting the genetic and morphological studies, thereby preventing possible misidentifications. The modern chemical techniques, e.g. mass spectrometry, enable the reliable determination of fungal metabolites providing more detailed data on the capabilities of the species to produce specified mycotoxins. In addition to the chemical analyses, *in vitro* –studies with cell cultures may provide complementary data on the biological properties of mycotoxins.

An identification and understanding of the factors that affect the mycotoxin production of fungi is crucial to the success of preventive actions to minimize the exposure of humans and animals to these toxic compounds.

MATERIALS AND METHODS

Fifteen different *Fusarium*-strains (Table 1.) were isolated from Finnish raw cereal samples harvested in 2001-2002 and identified by morphology and species-specific primers (Jestoi et al., 2004a). Polished rice (100 g) was autoclaved with 25 ml or 150 ml of deionized water (corresponding to a_w-values 0.973 and 0.997, respectively) and inoculated with 10 ml of spore suspension obtained from pure cultures on potato dextrose agar (PDA) –plates. The rice cultures were incubated (a_w 0.997 at 15°C and 25°C; a_w 0.973 at 25°C) for four weeks. After incubation, the cultures were air-dried and ground with a laboratory-mill.

The air-dried cultures were analysed for trichothecenes (deoxynivalenol – DON, fusarenon X – FX, 3-acetyldeoxynivalenol – 3-AcDON, diacetoxyscirpenol – DAS, nivalenol – NIV, HT-2 –toxin and T-2 –toxin), fusaproliferin (FUS), beauvericin (BEA), enniatins (ENN A, ENN A1, ENN B, ENN B1), moniliformin (MON) and zearalenone (ZEN) (Jestoi et al., 2004a,b; Eskola et al., 2001) using gas chromatography–mass

spectrometry (GC-MS), liquid chromatography–tandem mass spectrometry (LC–MS/MS) or high-performance liquid chromatography (HPLC) combined with fluorescence detection.

Porcine kidney (PK15) –cells were exposed to rice culture extracts and their cytotoxicity was assessed using the alamarBlue™ -assay (Nakayama, et al., 1997). In the assay, the metabolic activity of the exposed cells results in a chemical reduction of the colour reagent (resazurin à resorufin) and the absorbance of the affected cells is inversely proportional to their viability. Thus the absorbance can be used as an index of cell viability (O'Brien et al., 2000). PK15 -cells were exposed for 24 hours to filtered rice culture extracts (84 % acetonitrile in water), corresponding to 1 g of culture, and cytotoxicity was calculated relativ to the solvent control.

RESULTS AND DISCUSSION

All tested Finnish *Fusarium*-strains produced mycotoxins on rice media at the investigated culture conditions (Table 1.). BEA was produced by both strains of *F. poae* and *F. sporotrichioides*. Interestingly, *F. avenaceum/F. arthrosporioides*-, *F. tricinctum*- and *F. langsethiae* -strains did not produce BEA. Especially type-B ENNs were produced by one strain of *F. poae* and all strains of *F. avenaceum*/*F. arthrosporioides* and *F. tricinctum*. MON was produced by all *F. avenaceum/F. arthrosporioides*- and *F. tricinctum*-strains.

DON and 3AcDON were produced by *F. culmorum* and *F. graminearum*. FX was produced by only one *F. poae* –strain (p53). High concentrations of DAS were produced by *F. langsethiae*, but also in one culture of *F. poae* (p53) and in two cultures of *F. sporotrichioides* remarkable amounts of DAS could be detected. NIV was produced by all *F. poae*-, *F. sporotrichioides*- and *F. langsethiae* -strains. *F. culmorum* and *F. graminearum* produced only small amounts of NIV, suggesting that Finnish strains belong to chemotype IA, producing particularly DON and 3AcDON. HT-2 and T-2 were produced mainly by *F. sporotrichioides* and *F. langsethiae*. Significant production of ZEN was observed in the cultures of *F. culmorum* and *F. graminearum*. Smaller amounts of ZEN were also detected in one *F. tricinctum*- and *F. langsethiae* -cultures. FUS was not produced by any of the Finnish *Fusarium*-strains tested.

The capabilities of the Finnish *Fusarium*-strains to produce mycotoxins were generally in accordance with the available literature (e.g. Bottalico, 1997). Traces of specified mycotoxins were, however, detected in several cultures (Table 1.). These findings may be due to the contamination of the rice matrix used, rather than the toxins being produced by these strains, as the levels detected were clearly lower than the levels measured in cultures of recognised producers. Nevertheless, it is possible that very small amounts of mycotoxins can be produced also by other species than those earlier reported.

Environmental conditions had a tremendous effect on the production of some mycotoxins on the Finnish strains examined, as for some mycotoxins only minor changes in the production rates could be observed (Table 1.). For instance, the mycotoxin production of *F. poae* p57 was mainly favoured by high temperature and high water activity (a_w). *F. culmorum* p241, instead, produced higher amounts of mycotoxins at lower a_w or temperature (Figure 1.). Based on the data collected, it can be concluded that environmental (stress) factors may affect the nature and the amount of mycotoxins produced. This conclusion is in line with other published studies (e.g. Fanelli et al., 2003). To better understand the influence of different temperature/water activity-combinations on Finnish *Fusarium*-strains more studies are needed.

The metabolite profiles of *F. poae* p57 and *F. culmorum* p241 at three different environmental conditions, y-axis: mycotoxin produced (log µg/kg), x-axis: determined mycotoxins.

The cytotoxicity of Finnish *Fusarium*-strains grown on rice cultures was significantly ($p<0.05$) correlated (Spearman Rank Correlation) to the total mycotoxin concentrations determined (Figure 2.). In terms of single compounds, only DAS, HT-2 (main producers *F. sporotrichioides* and *F. langsethiae*) and NIV (main producer *F. poae*) correlated with

the cytotoxicity observed, this being in line with the findings of earlier studies (Visconti et al., 1992; Morrison et al., 2002a). Although *F. culmorum* was reported to be more toxic than *F. graminerum in vitro* (Morrison et al., 2002b), we could not confirm that observation. However, more data on toxic metabolites produced by *Fusarium* spp. is needed before the cytotoxicity of the complex mycotoxin mixtures can be resolved (Abbas et al., 1984).

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Table 1. The capabilities of Finnish *Fusarium*- strains to produce mycotoxins on rice cultures under three different environmental conditions (aw 0.997, 25 ºC; aw 0.973, 25 ºC; aw 0.997, 15 ºC).

Strain	BEA	ENN A	ENN A1	ENN B	ENN B1	DON	FX	3AcDON	DAS	NIV	HT-2	T-2	ZEN	MON
***F.poae* p57** range (µg/kg)	+++ 90-3930	+++ 1470-11200	+++ 8200-77700	+++ 34100-235000	+++ 41400-254900	±± n.d.-<100	± n.d.-<100	n.d.	± n.d.-<100	+++ 340-2150	n.d.	± n.d.-<100	± n.d.-<100	n.d.
***F.poae* p53** range (µg/kg)	+++ 2540-131400	n.d.	n.d.	± n.d.-<100	n.d.	n.d.	++ n.d.-11700	n.d.	++ n.d.-10800	+++ 2370-35000	±± n.d.-<100	±± n.d.-<100	n.d.	n.d.
***F.culmorum* p241** range (µg/kg)	n.d.	n.d.	n.d.	n.d.	n.d.	+++ 200-118700	± n.d.-<100	+++ <100-3500	n.d.	+ n.d.-320	n.d.	+ n.d.-150	+++ 320-2618000	n.d.
***F.culmorum* p251** range (µg/kg)	± n.d.-<50	n.d.	n.d.	± n.d.-<100	n.d.	+++ 550-36500	n.d.	+++ 260-12800	± n.d.-<100	± n.d.-<100	n.d.	n.d.	+++ 262000-1726000	n.d.
***F.sporotrichioides* p139** range (µg/kg)	+++ (150-4310)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+ n.d.-240	+ n.d.-6570	+ n.d.-7900	+++ 5790-24650	+++ 124000-182000	± n.d.-<100	n.d.
***F.sporotrichioides* p46** range (µg/kg)	+++ (100-11500)	± n.d.-<50	n.d.	n.d.	n.d.	n.d.	n.d.	+ n.d.-110	++ n.d.-7590	++ n.d.-1950	+++ 4660-30000	+++ 5130-219000	± n.d.-<100	n.d.
***F.graminearum* p6** range (µg/kg)	±± n.d.-150	n.d.	n.d.	n.d.	n.d.	+++ 240-26300	n.d.	+++ <100-57300	n.d.	++ n.d.-270	n.d.	± n.d.-<100	+++ 38200-174000	n.d.
***F.graminearum* p11** range (µg/kg)	± n.d.-50	n.d.	n.d.	+ n.d.-500	± n.d.-<100	+++ 220-57700	n.d.	+++ <100-12100	± n.d.-<100	+ n.d.-250	n.d.	±± n.d.-250	+++ 343000-1944000	n.d.
***F.arthrosporioides* p84** range (µg/kg)	n.d.	++ n.d.-310	+++ <50-3990	+++ 2890-226400	+++ 1340-46700	±± n.d.-<100	n.d.	n.d.	n.d.	n.d.	n.d.	± n.d.-<100	+ n.d.-340	+++ 39200-59650
***F.arthrosporioides* p75** range (µg/kg)	n.d.	+++ 2280-25100	+++ 13500-144400	+++ 183700-1900000	+++ 115400-1155000	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+ n.d.-270	+++ 120-22800
***F.avenaceum* p191** range (µg/kg)	n.d.	+ n.d.-700	+ n.d.-4830	+++ 270-213400	++ n.d.-50400	± n.d.-<100	n.d.	n.d.	n.d.	n.d.	n.d.	± n.d.-<100	n.d.	+++ 250-45550
***F.avenaceum* p228** range (µg/kg)	n.d.	++ n.d.-280	++ n.d.-4970	+++ 110-130800	++ 9600-13800	n.d.	n.d.	n.d.	± n.d.-<100	n.d.	± n.d.-<100	± n.d.-<100	+ n.d.-100	+++ 3800-91650
***F.tricinctum* p105** range (µg/kg)	n.d.	+++ 60-11100	+++ 410-70600	+++ 600-724900	+++ 800-339500	+ n.d.-230	n.d.	n.d.	± n.d.-<100	n.d.	± n.d.-<100	+ n.d.-260	n.d.	++ n.d.-14700
***F.tricinctum* p113** range (µg/kg)	n.d.	+++ <50-11900	+++ 70-52200	+++ 170-227200	+++ 240-164400	± n.d.-<100	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	++ n.d.-34000	+++ 310-2140
***F.langsethiae* 113** range (µg/kg)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+ n.d.-120	++ n.d.-60700	+++ 2830-4440	+++ 3770-10630	+++ <100-49000	++ n.d.-1990	n.d.

+++ = mycotoxin produced in all three environmental conditions; ++ = mycotoxin produced in two environmental conditions,
+ = abundant mycotoxin production in one environmental condition; = traces of mycotoxin detected, n.d. = mycotoxin not detected in any of the environmental conditions tested.

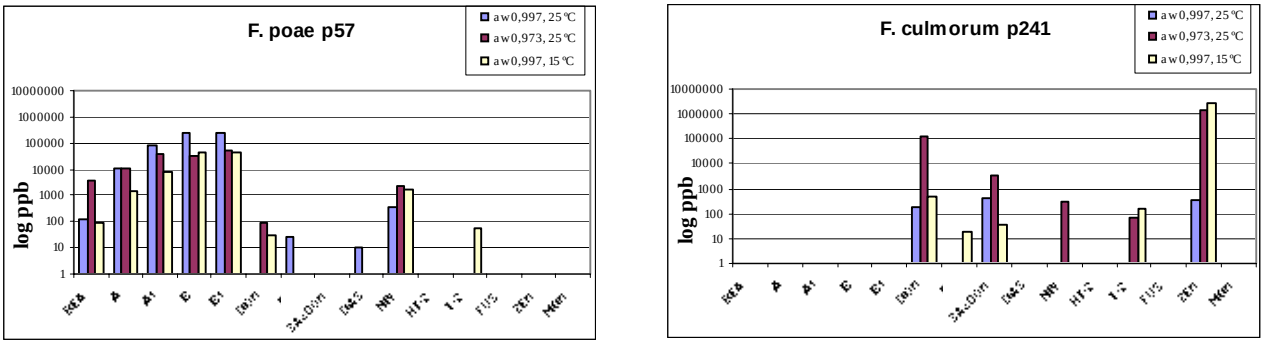

Figure 1. The metabolite profiles of *F. poae* p57 and *F. culmorum* p241 at three different environmental conditions, y-axis: mycotoxin produced (log µg/kg), x-axis: determined mycotoxins.

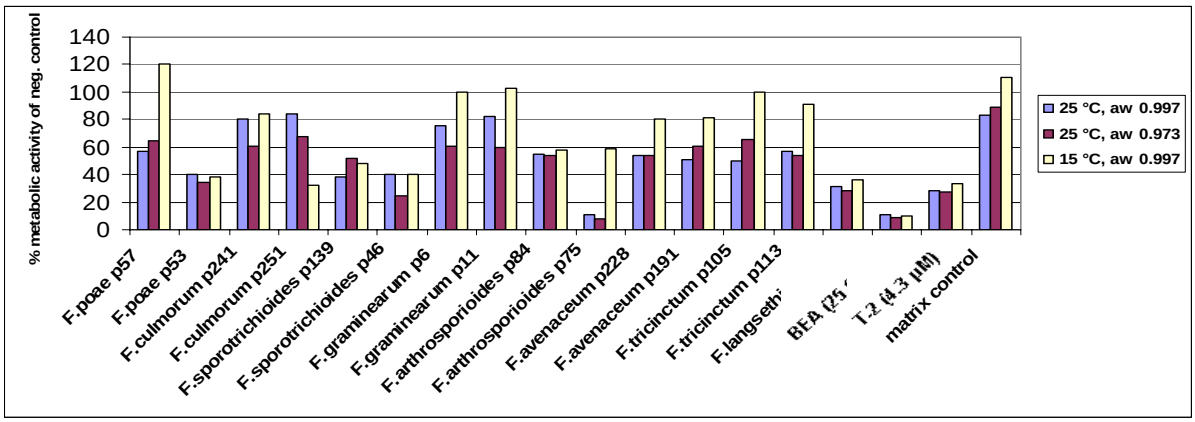

Figure 2. The cytotoxicity of Finnish *Fusarium* –strains (1 µg culture/2 × 10^4 cells) grown on rice medium at different environmental conditions. x-axis: the strains studied and BEA and T-2 standards (positive controls); y-axis the percentage of the metabolic activity of the exposed porcine kidney -cells compared with the extraction solvent (84 % acetonitrile in water).

DETECTION AND QUANTIFICATION OF *FUSARIUM* SPP. IN CEREAL SAMPLES

S.S. Klemsdal* and O. Elen

Norwegian Crop Research Institute, Plant Protection Centre, Norway
*Corresponding Author: PH: (47) 64949400, Email: sonja.klemsdal@planteforsk.no

ABSTRACT

To screen cereal samples for the presence of mycotoxin producing *Fusarium* spp. and to study the interaction between these fungi and their host plants, species-specific and highly sensitive detection methods are needed. In this study, species-specific PCR-primers designed for the detection of *F. poae, F. sporotrichioides* and the new species *F. langsethiae* are described. In cereal samples infections lower than 1% could be detected by this PCR assay. Four additional primers were developed to detect subgroups of *F. poae* representing the four different ITS genotypes present in *F. poae*. The primer pair developed for the detection of *F. langsethiae* could be used to distinguish between *F. langsethiae* and the morphologically similar *F. poae*. The described PCR assays are highly sensitive and enable the detection of *Fusarium* genomic DNA in concentrations as low as 5-50 fg. High levels of *F. poae* and/or *F. langsethiae* were detected in random field cereal samples but only low levels of *F. sporotrichioides* were found. To allow a quantitative detection of the species of *Fusarium* producing the mycotoxins most frequently detected in samples of small grain cereals in Norway, several TaqMan real-time PCR assays were developed. One assay allowed the detection and quantification of *F. avenaceum*. Another TaqMan assay was used for the quantification of the total amounts of *F. langsethiae* and *F. sporotrichioides*. In Norway these *Fusarium* species are the two most important producers of the highly toxic type A trichothecenes, T-2 and HT-2 toxins. To determine the total amount of trichothecene-producing *Fusarium* (both type A and type B trichothecenes) a TaqMan assay based on the *Tri5* gene of the trichothecene synthesis pathway was developed. The possible correlation between the amount of *Fusarium* DNA detected by these assays and corresponding mycotoxin content found in cereal samples will be discussed.

EVALUATION OF OZONE AND HYDROGEN PEROXIDE TREATMENTS FOR PREVENTING THE POST-HARVEST *FUSARIUM* INFECTION IN MALTING BARLEY

B. Kottapalli[1], C.E. Wolf-Hall[1*] and P.B. Schwarz[2]

[1]Department of Veterinary and Microbiological Sciences, North Dakota State University, Fargo ND; and
[2]Department of Plant Sciences, North Dakota State University, Fargo ND, USA
[*]Corresponding Author: PH: (701) 231-6387, Email: charlene.hall@ndsu.nodak.edu

ABSTRACT

Utilization of *Fusarium* infected barley for malting may lead to mycotoxin production and decreased malt quality. Methods for treatment of *Fusarium* infected barley may prevent these safety and quality defects and allow use of otherwise good quality barley. Gaseous ozone and hydrogen peroxide were evaluated for effectiveness in reducing *Fusarium* infection (FI) while maintaining germinative energy (GE) in two barley samples (sound and infected). Gaseous ozone treatments (GOT) included concentrations of 11 and 26 mg/g for 0, 15, 30, and 60 minutes. Hydrogen peroxide (HP) treatments included 0, 5, 10, and 15% concentrations with exposure times of 0, 5, 10, 15, 20, and 30 minutes. For GOT, in naturally *Fusarium*-infected barley, a statistically significant ($P<0.05$) decrease of 24-36% in FI occurred within 15 minutes of exposure at either concentration. GE was significantly ($P<0.05$) affected (11-20%) by 30 minutes at both concentrations in naturally *Fusarium* infected barley but not in sound barley. For HP, FI was significantly decreased (50-98%) within 5 minutes of exposure. With the exception of two treatments (10% and 15% HP agitated for 20 minutes) GE was not statistically significantly different from the control in naturally *Fusarium* infected barley. In sound barley, HP had no significant ($P>0.05$) effect on GE. The results suggest that GOT and HP may have potential for treatment of *Fusarium* infected malting barley.

CAFFEINE AS INTERNAL STANDARD FOR HIGH PRESSURE LIQUID CHROMATOGRAPHY ANALYSIS OF DEOXYNIVALENOL IN WHEAT SAMPLES

C.A. Landgren, P.A. Murphy and S. Hendrich*

Food Science and Human Nutrition, Iowa State University, Ames, IA 50011, USA
*Corresponding Author: PH: (515) 294-8059; E-mail: shendric@iastate.edu

ABSTRACT

A method was developed to utilize caffeine as an internal standard for analytical detection of deoxynivalenol (DON) in wheat samples using high pressure liquid chromatography (HPLC) with ultraviolet detection. Ground wheat samples (25 g) were extracted with 100 mL aqueous acetonitrile (84:16) and spiked with 30 µg caffeine (CAF) dissolved in extraction solvent. Samples were blended for 3 minutes at high speed then a 5 mL aliquot passed through an alumina-charcoal column for clean-up. The resulting extract was evaporated in a 50°C water bath under nitrogen. The residue was dissolved in aqueous methanol (20%) and microfiltered into autosampler vials. Analytes were separated with linear aqueous methanol solvent gradient (85:15 ramping to 80:20 over 20 min, flow rate 0.7 mL/min) with UV detection at 220 nm. Retention times for DON and CAF were approximately 9.0 min and 16.0 minutes, respectively. Fungal culture material (124 ppm) was added to commercial wheat flour (no detectable DON) to achieve mean DON concentrations of 0.26, 0.51, 1.99, and 5.96 ppm. Three replicates of each concentration were analyzed in one day and repeated four different days. An internal standard curve was developed plotting sample concentration against area response ratios (DON/CAF). The correlation coefficient (r) was 0.985 across days and there was no significant difference between or among days of analysis. Recovery analysis of spiked samples at 1.00 ppm was 100% in this system. This method will allow analysis of samples using an easily accessible, stable, and inexpensive compound that is not likely to be found in most food samples of interest.

POSSIBLE WAYS TO UTILIZE MYCOTOXIN CONTAMINATED GRAIN

Mesterházy, A.*

Cereal Research non-profit Co., 6726 Szeged, P.O.Box 391. Hungary
*Corresponding Author: PH: 36.30 415 9730, E-mail: akos.mesterhazy@gk-szeged.hu

ABSTRACT

There is a strong tendency that for securing food and feed safety requirements, more and more countries will set up limit values for toxin contamination. Industry applied until now toxin limits. When toxin limits will be officially introduced, millions of tons of grain can be qualified as dangerous waste material. In this case the farmers will bear all risks and they should pay for everything even they are not fully responsible for the damage. The social consequences are now clear in the Red River Valley and elsewhere (McMullen 2003). For this reason economic utilization(s) of the contaminated grain must be found. Without this a well working system cannot be developed.

The food and feed industry is excluded, therefore other industrial uses remain. Now three possibilities have greater chances:

a. Direct energy production. Wheat grains have energy content comparable to middle quality coal (Ruckenbauer and Reichart 1994). When a coal price would be paid for the contaminated grain and some additional sum would be paid it would lessen the financial damage and could help farmers to survive. For this economists should count out, whether movable plants should be built and positioned into the given area for a while or existing electric plants can accept it. It should be decided whether heat or electricity is more economic. The use of straw may increase the profitability when not used otherwise.

b. Usage for gasoline production. The European Union plans that it will use the agricultural and forest waste materials for fuel production as renewable energy sources. With the present oil prices above 50 USD this is economic now. The EU will replace 30-40 % of the diesel fuel by this new resource within ten years. For this regional plants will be built with a capacity of 480 t fuel/day capacity. The first Sundiesel experimental plant is working now. It seems that oil need grows exponentially, but not the resources. The Middle East provides a number of risk factors for the world economy. The gasoline production of this art can lessen the tension.

c. Alcohol production for industrial use or as fuel. The problem is the large amount of waste material that is also toxin contaminated. Drying and burning could be a solution, the question: profitability.

d. Other industrial uses. From there is no special information, but their need is not in range of million tons, but may help.

We are convinced that the economic utilization of the toxin contaminated grain can be a good business for the farmers who cannot sell their contaminated grains now. For this reason national and international efforts are needed to find solutions that help producers and utilize products that are not marketable now. This would also stabilize commodity prices.

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THE RELATIONSHIPS BETWEEN FUSARIUM HEAD BLIGHT VISUAL SYMPTOMS, *FUSARIUM* BIOMASS AND DEOXYNIVALENOL LEVELS IN BARLEY

S.M. Neate[1*], P.B. Schwarz[2], N.S. Hill[3] and R.D. Horsley[2]

[1]North Dakota State University, Department of Plant Pathology. P.O. Box 5012, Fargo ND 58105, USA;
[2]North Dakota State University, Department of Plant Science. P.O. Box 5051, Fargo ND, 58105, USA; and
[3]Dept. Crop and Soil Sciences, University of Georgia, Athens, GA 30602, USA
[*]Corresponding Author: PH: (701) 231-7078; E-mail: stephen.neate@ndsu.nodak.edu

ABSTRACT

For more than a decade Fusarium Head Blight (FHB) has resulted in large portions of the upper mid-west six-rowed barley crop being rejected for malting due to levels of the toxin deoxynivalenol (DON) in the grain exceeding 0.5 ppm. Currently, screening for disease resistance involves a combination of visual scoring of disease symptoms and DON analysis. The Barley Pathology and 6-rowed Barley breeding program at NDSU undertake more than 5,500 DON analyses each year and to reduce costs and time also do more than 45,000 visual scores on barley heads, yet it is not clear that visual scores are a good predictor of DON. Recently a *F. graminearum* species-specific indirect ELISA technique has been developed that can quantify fungal biomass of *Fusarium* sp.. Our objectives were twofold, 1) to investigate the relationship between FHB visual score, DON and the Fusarium ELISA, and 2) to determine the impact on total spike DON by kernels with differing visual scores. Visual scoring was done at hard dough stage of development on seven cultivars grown in different environments in North Dakota in 2003. Individual kernels with characteristic dark brown lesions occupying > 25% of the kernel were scored and determined as a percentage of the total number of kernels on the head. Heads were dissected and kernels separated into those displaying FHB symptoms on >25% of the kernel, 1-25% of the kernels, or no symptoms. Single kernels were used for ELISA and DON analysis. For ELISA, antigens were extracted from whole seed, diluted in coating buffer and coated onto microtiter plates followed by indirect ELISA using monoclonal antibodies. DON in the single kernel barley samples was determined by gas chromatography with electron capture detection. When regressions were done between FHB and DON, R^2 values differed widely between cultivars and environments, but when pooled, gave a R^2=0.39. The R^2 values for ELISA and DON also differed widely between cultivars and environments, but when pooled, gave a R^2=0.73. When separated into different symptom classes, the R^2 values for both ELISA and DON or FHB and DON were greatest for the kernels showing >25% symptoms and very low for kernels showing 1-25% or zero symptoms. Kernels showing >25% symptoms had the highest average DON levels with individual kernels up to 700 ppm. However, 70/205 of these kernels tested zero for DON. Of kernels showing 1-25% lesion coverage, 140/205 tested zero for DON and the remainder between 1-65 ppm with one kernel 488 ppm. Of asymptomatic kernels, 173/215 tested zero for DON and the remainder tested 1-4 ppm. It is clear that the kernels showing zero or 1-25% lesion coverage are making a small contribution to the DON level and that some visual symptoms are not associated with DON accumulation.

ADVERSE HEALTH EFFECTS OF TRICHOTHECENE MYCOTOXINS

J.J. Pestka* and Z. Islam

Dept. of Food Science and Human Nutrition, Michigan State University, East Lansing MI, 48824-1224, USA*Corresponding Author: PH: (517) 353-1709; E-mail: pestka@msu.edu

ABSTRACT

During head blight of wheat and barley, deoxynivalenol (DON or "vomitoxin") and other trichothecene mycotoxins are elaborated that can potentially cause adverse health effects in individuals who consume the infected grain. Although DON is regulated in the U.S. at 1 ppm in finished food, the European Economic Union and Codex Alimenterius have proposed much lower limits for consumption based on rodent studies. A further concern is that although agricultural workers are exposed to airborne DON during harvest, threshing and milling of infected wheat and barley, virtually nothing is known about the adverse effects of inhaling this toxin. *In vitro* studies suggest that the keys steps for DON toxicity are induction of stress signaling and cytokine expression in white blood cells which ultimately can mediate acute and chronic illness. We have used multiparameter flow cytometric analysis to measure the sensitivity of human white blood cells to induction of cytokines by DON. In assessing blood from 8 individuals, some were found to be much more sensitive to DON's effects than others. The minimal threshold for induction of IL-1 beta, IL-6 and IL-8 was 100 ng/ml. Activation of the mitogen-activated protein kinase p38 was found to precede and be a requisite for inducing IL-1 beta, IL-6 and IL-8 with the minimum threshold for activation being 25 ng/ml. These are critical observations because they suggest: (1)p38 activation is a biomarker for DON toxicity and for which humans are 4-fold more sensitive than mice, (2) higher concentrations are needed for cytokine induction and (3) some people may be resistant to DON whereas others are sensitive perhaps due to genetic and non-genetic factors (eg.prior/ ongoing infections, diet, medication). To accurately measure the hazardous potential of trichothecene to humans, it is essential to relate these in vitro studies to threshold dose, duration of exposure, exposure route, and magnitude of toxic effects in the mouse model. The resultant data can be used by the regulatory agencies and the wheat and barley industries for improved accurate, safety assessments relative to consumption of grain products and inhalation of grain dust.

REAL-TIME PCR FOR QUANTIFICATION OF TOXIGENIC *FUSARIUM* SPECIES IN BARLEY AND MALT

T. Sarlin[1*], M. Jestoi[2], A. Rizzo[2], S. Paavanen-Huhtala[3], T. Yli-Mattila[3] and A. Haikara[1]

[1]VTT Biotechnology, Finland; [2]National Veterinary and Food Research Institute, Finland; and [3]University of Turku, Finland
[*]Corresponding Author: PH: 358 9 456 7141; Email: tuija.sarlin@vtt.fi

ABSTRACT

Fusarium species are potential mycotoxin producers in cereals. In near future EU is going to set the maximum limit values for some *Fusarium* toxins in unprocessed cereals and cereal products. Mycotoxin analyses are expensive and time-consuming. Hence, a rapid and reliable quantification method for toxigenic *Fusarium* species is needed for evaluation of the mycotoxin risk in cereal-based industry. We have applied real-time PCR technique for the quantification of trichothecene-producing *Fusarium* species present in barley and malt samples (the TMTRI assay, S. Klemsdal unpubl. sequences). PCR results were compared to the amount of trichothecenes in the samples. Furthermore, highly toxigenic *Fusarium graminearum* was quantified in cereals by real-time PCR (the TMFg12 assay, T. Yli-Mattila unpubl. sequences). DNA was extracted from ground kernels (0.1 g) using FastDNA Spin Kit for Soil and analysed in a LightCycler® system using fluorigenic TaqMan probes. Both naturally and artificially contaminated grains were analysed. The TMTRI assay and the TMFg12 assay enabled the quantification of trichothecene-producing *Fusarium* species and *F. graminearum* present in barley and malt samples, respectively. Both TaqMan assays were regarded as sensitive and reproducible. Linearity of the assays was at least 3-4 log units when determined using pure *Fusarium* DNA. The amount of *Fusarium* DNA analysed with the TMTRI-trichothecene assay correlated with the DON content in Finnish barley samples. The TMFg12 assay for *F. graminearum* gave a good estimation about the DON content in North American barley and malt samples. The amounts of DON and *F. graminearum* in Finnish barley were found to be naturally low.

ROLE OF GLUTATHIONE IN TRICHOTHECENE TOXICITY IN *SACCHAROMYCES CEREVISIAE*

H.F. Sigmund[1*], F. Berthiller[2], S. Goritschnig[1,3], H. Weindorfer[1], R. Schuhmacher[2], R. Krska[2] and G. Adam[1]

[1]BOKU - University of Natural Resources and Applied Life Sciences, Department of Applied Plant Sciences and Plant Biotechnology, Institute of Applied Genetics and Cell Biology, Vienna, Austria; [2]BOKU - University of Natural Resources and Applied Life Sciences, Department IFA-Tulln, Center for Analytical Chemistry, Tulln, Austria; and [3]Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4

[*]Corresponding Author: PH: 43 1 36006 6383; E-mail: harald.sigmund@boku.ac.at

ABSTRACT

In a yeast cDNA library screen intended to identify genes mediating increased DON resistance when overexpressed, we found the yeast *CYS4* gene. *CYS4* encodes cystathionine beta-synthase, which synthesizes cystathionine, the immediate precursor of L-cysteine. Cysteine is required for the two-step synthesis of glutathione (GSH) mediated by the *GSH1* and *GSH2* gene products. Since many toxins are known to be detoxified by GSH conjugation, we speculated about a possible role of GSH in DON detoxification by yeast. Yeast has a specific GSH uptake transporter, we therefore tested whether exogenous GSH protects yeast against DON toxicity. Exogenous GSH indeed increased resistance against DON and 3-Acetyl-DON (3-ADON), but not against the closely related trichothecenes nivalenol (NIV), trichothecin (TTC) and 15-Acetyl-DON (15-ADON). Using LC-MS/MS, clear evidence for formation of a previously unknown DON-glutathione conjugate was obtained. Efforts to prepare larger amounts of this conjugate in order to characterize its chemical structure (by NMR) and biological properties (using a wheat germ *in vitro* translation assay) are currently under way. In order to find out which enzyme is responsible for conjugate formation we have performed a systematic gene knock-out approach (gene disruption of all putative yeast glutathione-S-transferases (GSTs)). We identified one gene, where inactivation leads to increased sensitivity against DON. Deleting this candidate gene also increased sensitivity against 3-ADON and CDNB, the latter is a widely used test substrate for GSTs. We have generated overexpression constructs (with and without a *c-MYC-6xHis*-tag for affinity pruification). Isolation and biochemical characterisation of the protein encoded by this candidate gene is currently under way. In an attempt to identify *Arabidopsis* GSTs capable of DON-GSH conjugate formation, several DON-induced *Arabidopsis* GST genes where tested. The candidate *Arabidopsis* cDNAs where cloned into a yeast expression vector and tested for their ability to increase DON resistance in yeast. Unfortunately this approach was so far not successful.

ACKNOWLEDGEMENT

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A NOVEL *FUSARIUM* METABOLITE WITH BIOLOGICAL ACTIVITY

S. Uhlig[1*], D. Petersen[2], A. Flåøyen[1] and A. Wilkins[3]

[1]National Veterinary Institute, P.O. Box 8156 Dep., N-0033 Oslo, Norway; [2]Department of Chemistry, University of Oslo, P.O. Box 1033 Blindern, N-0315 Oslo, Norway; [3]Chemistry Department, The University of Waikato, Private Bag 3105, Hamilton, New Zealand
[*]Corresponding Author: PH: 47 23216264; E-mail: silvio.uhlig@vetinst.no

ABSTRACT

A novel *Fusarium* metabolite with a hydrocarbon-like basic structure was purified from the methanolic extract from the rice culture of a *F. avenaceum* strain, which had been isolated from Norwegian grain. The metabolite was discovered by bio-assay guided fractionation of culture extracts and was cytotoxic against the porcine kidney epithelial cell line PK-15. Other *Fusarium* spp. that were found to produce the compound in rice culture include *F. tricinctum*, *F. langsethiae* and *F. poae*. The results from the structure elucidation using NMR- and mass spectroscopy will be presented.

WORLDWIDE REGULATIONS FOR *FUSARIUM* MYCOTOXINS

H.P. van Egmond* and M.A. Jonker

Laboratory for Food and Residue Analyses, National Institute for Public Health and the Environment (RIVM), P.O. Box 1, 3720 BA Bilthoven, The Netherlands
*Corresponding Author: PH: (31) 30 274-2440; E-mail: hp.van.egmond@rivm.nl

ABSTRACT

Regulations for mycotoxins have been established in food and animal feed in many countries since the late 1960s to protect the consumer from the harmful affects that mycotoxins may cause. Various factors influence the decision-making process of setting limits for mycotoxins. These include scientific factors such as data about effects on man and animals, the level of human exposure, as well as the availability of methods of sampling and analysis. Economical factors such as commercial interests and sufficiency of food supply have their impact as well. Over the last 2 decades various international inquiries on worldwide limits and regulations for mycotoxins were published. The latest completed inquiry resulted in the publication "Worldwide regulations for mycotoxins in food and feed in 2003 (FAO Food and Nutrition Paper 81, 2004). On a worldwide basis, approximately 100 countries had mycotoxin regulations or guideline limits for food and/or feed in 2003, an increase of approx. 30 % as compared to 1995. The total population in these countries represents approx. 90 % of the world's inhabitants. *Fusarium* mycotoxins for which (proposed) limits and regulations existed in 2003 include deoxynivalenol, diacetoxyscirpenol, the fumonisins B_1, B_2 and B_3, HT-2 toxin, T-2 toxin and zearalenone. Deoxynivalenol, zearalenone and fumonisins were among the most regulated. At least 41 countries have set regulatory or guideline limits for deoxynivalenol in food or feed. Whereas in 1995 this trichothecene was only sporadically regulated, it has become a toxin of high concern in monitoring programmes and amongst regulatory authorities since the late 1990's when mg/kg concentrations were reported to occur in cereals and cereal products, particularly in Europe. Tolerance levels for deoxynivalenol in wheat (flour) range from 300-2000 µg/kg, with a peak at 750 µg/kg. The latter is dominated by the countries of the EU, where this (unofficial) guideline tolerance level is applied since several years for deoxynivalenol in flour used as raw material. Zearalenone is now regulated in food and feed in 28 countries as compared to 6 in 1995. Limits for this toxin in maize and other cereals currently vary from 50 to 1000 µg/kg with more of the tolerance levels set at the higher end of this range than at the lower end. Whereas in 1995 fumonisins were only subject of regulations in one country, this number has now increased to 6, with limits for maize ranging from 1000-3000 µg/kg. Although proportionally a very significant increase, the number of fumonisins-regulating countries is too small to draw meaningful conclusions about generally agreed limits. Comparing the situations in 1995 and 2003, regulations for *Fusarium* mycotoxins have increased, and they have become more diverse and detailed with newer requirements regarding official procedures for sampling and analytical methodology. These developments reflect the general concerns that governments have regarding the potential effects of *Fusarium* mycotoxins on the health of humans and animals.

EFFECT OF HARVESTING TIME ON INCIDENCE OF *FUSARIUM* SPECIES AND ACCUMULATION OF DEOXYNIVALENOL IN KERNELS OF SILAGE CORN IN ONTARIO

A.G. Xue[1*], B.L. Ma[1], R.M. Clear[2], D.T. Chi[1], F. Sabo[1] and Y. Chen[1]

[1]Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada, 960 Carling Avenue, Ottawa, Ontario K1A 0C6, Canada; and [2]Grain Research Laboratory, Canadian Grain Commission, 1404-303 Main Street, Winnipeg, Manitoba R3C 3G8, Canada

[*]Corresponding author: PH: (613) 759-1513, Email: axue@agr.gc.ca

ABSTRACT

The effect of three harvesting times (75, 50, and 25% milkline) on the incidence of kernelborne *Fusarium* spp. was examined using four silage corn hybrids (Maizex Leafy 4, NK Enerfeast 1, Pioneer 37M81, and TMF94) in Ottawa, Ontario in 2001 and 2002. Eleven *Fusarium* spp. were isolated over the two years. *Fusarium subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas was the dominant species recovered from 28.8% of the kernels. Other frequently isolated species were *F. oxysporum* Schlecht. (2.6%), *F. graminearum* Schwabe (2.5%), *F. proliferatum* (Matsushima) Nirenb. (0.3%), and *F. sporotrichioides* Sherb. (0.2%). The remaining six species, *F. avenaceum* (Fr.) Sacc., *F. crookwellense* Burgess, Nelson, & Toussoun, *F. culmorum* (W.G. Sm.) Sacc., *F. equiseti* (Corda) Sacc., *F. poae* (Peck) Wollenw., and *F. solani* (Mart.) Sacc., were recovered from < 0.1% of the kernels. The incidences of *F. subglutinans* increased from 20.9 to 26.7, and to 38.7%; *F. graminearum* from 1.7 to 2.9, and to 3.1%; and, total *Fusarium* spp. from 28.7 to 32.2, and to 42.3%, when harvested at 75, 50, and 25% milkline, respectively. The incidences of the other species and deoxynivalenol (DON) levels in kernels were not affected by harvesting time. Of the four silage corn hybrids, TMF94 had significantly greater incidence of *Fusarium* spp. and DON concentration than the others, indicating a genotypic variation in resistance to kernelborne *Fusarium* spp.

REAL-TIME PCR DETECTION AND QUANTIFICATION OF *FUSARIUM POAE* AS COMPARED TO MYCOTOXIN PRODUCTION IN GRAINS IN FINLAND

T. Yli-Mattila[1*], S. Paavanen-Huhtala[1], P. Parikka[2], V. Hietaniemi[3], M. Jestoi[4] and A. Rizzo[4]

[1]Lab. of Plant Phys. and Mol. Biol., Dept of Biology, Univ. of Turku, FIN-20014 Turku, Finland; [2]Plant Production Research/Plant Protection, MTT Agrifood Research Finland, FIN-31600 Jokioinen, Finland; [3]MTT Chemistry Laboratory, MTT Agrifood; Research Finland, FIN.31600 Jokioinen, Finland; and [4]National Veterinary and Food Research Institute (EELA), Department of Chemistry, FIN-00581 Helsinki, Finland
[*]Corresponding Author: PH: 358-2-3336587; E-mail: tymat@utu.fi

OBJECTIVES

To develop a fluorogenic (TaqMan) real-time polymerase chain reaction (PCR) -assay for species-specific detection of *F. poae* in barley, wheat and oats based on species-specific differences in intergenic spacer region (IGS) sequences.

INTRODUCTION

Species-specific primers based on IGS sequences for end-point PCR have been developed (Konstantinova and Yli-Mattila, 2004), and in this study these primers were tested together with DyNAmo SYBR Green kit (Finnzymes) and rox dye. In addition, TaqMan primers and probe were designed for the same DNA region and the correlation between *F. poae* DNA and mycotoxins in grain samples was studied.

The mycotoxins produced by *F. poae* include e.g. diacetoxyscirpenol (DAS), monoacetoxycirpenols (MAS), scirpentriol (SCR), nivalenol (NIV) and fusarenon-X (FX) (Pettersson 1991, Liu et al., 1998, Torp and Langseth 1999, Thrane et al., 2004). The reports of *F. poae* isolates to produce type-A trichothecenes (HT-2 –toxin, T-2 –toxin and neosolaniol) are contradictory (Torp and Langseth, 1999, Abramson et al., 1994, Sugiura et al., 1993). These variable findings may, however, be due to the misidentification of *F. langsethiae* isolates, which are morphologically similar to *F. poae* (Yli-Mattila et al., 2004a). According to our results (Jestoi et al., 2004) Finnish *F. poae* isolates produce beauvericin (BEA) and enniatins (ENNs) in addition to DAS, NIV and FX.

MATERIALS AND METHODS

Grain samples - The specificity of the TaqMan primers and probe were studied using DNA from eight *Fusarium* species (Yli-Mattila et al., unpublished results). Finnish grain samples (15 barley, 16 one oats and 23 wheat) harvested in 2002 and in 2003 were used for the analysis (Yli-Mattila et al., 2004b, unpublished results). Some samples were from field plots, which were artificially inoculated with the spores of different *Fusarium* species (Jestoi et al., unpublished results, Yli-Mattila et al., unpublished results).

Molecular analyses - DNA was extracted from grain samples according to the modified method of Taylor et al. (2001). The final volume of DNA extract from 10 g of grains was ca. 50 µl and the total DN concentration was usually 1-10 ng/µl. Before PC the DNA concentration was diluted to 1 ng/µl or th results obtained were divided by the total DNA concentration.

For isolation of genomic DNA, fungi were grown for 4 to 6 days at 24°C on potato dextrose agar (PDA) plates. DNA was extracted with chloroform/octanol method as described in Yli-Mattila et al. (2004a,b). In barley, DNA was also extracted from ground kernels (c20-c25 and c27-c28) using FastDNA Spin Kit for Soil (Q-BIOgene, Sarlin et al., 2004, unpublished results).

DyNAmo SYBR Green kit (Finnzymes) with rox dye was used for SYBR Green quantitative PCR in GeneAmp 5700 cycler (PE Biosystems). Amplification for quantitative PCR was performed in 96-well Optical Reaction Plates (ABgene) sealed with Optical Adhesive Covers (ABgene) in a GeneAmp5700 sequence detector. The PCR program for SYBR Green PCR consisted of 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 60°s at 60°C (Yli-Mattila et al., unpublished results). TaqMan amplification reactions were performed in the GeneAmp5700 sequence detector in 25 µl containing 12.5 µl Absolute qPCR Rox master mix, 100 ν M TMpoaef and TMpoaer primers, 100µM TMpoae probe and DNA sample containing 1-6 ng DNA with the standard PCR protocol. The probe was labelled at the 5´ends with TET (tetrachloro-6-carboxy-fluorescein) and at the 3´end with 3'Eclipse Dark Quencher (Yli-Mattila et al., unpublished results).

To determine the sensitivity and range of the assay, 1 ng of genomic DNA from *F. poae* strain 02-65 was serially diluted by a factor 10 and used as a template. The specificity of the primers was tested against genomic DNA of eight *Fusarium* species.

Mycological and toxin analyses - Fresh and dried grain samples (200 seeds per sample) from the years 2002-2003 were used for the analysis of *Fusarium* spp. The isolation and morphological identification of the fungi took place as described in Yli-Mattila et al. (2004b). With the samples of the year 2003 *Fusarium*-specific Peptone PCNB plates were used (Nash and Snyder Medium modified by Nelson et al 1983). Mycotoxins (deoxynivalenol, FX, DAS, 3-acetyldeoxynivalenol, NIV, HT-2, T-2, BEA, ENNs, moniliformin) were analysed as described in Jestoi et al. (2004).

Statistical analyses - R^2 (= square of regression coefficients), regression slope and P(a) (= significance of regression slope) were calculated by the program SigmaPlot 2001 version 7.1 (SPSS Inc). The original DNA and mycotoxin concentrations were transformed to logarithmic values.

RESULTS AND DISCUSSION

TaqMan reactions - The PoaeIGS/CNL12 primer pair displayed a linear range of at least two orders of magnitude from $10^{-3.5}$ to $10^{-1.5}$ µg/µ l with pure DN from *F. poae* isolates. In grain samples containing *F. poae* DNA the *F. poae* specific peak and occasionally also an unspecific peak was found in the melting curve, which made it difficult to estimate the exact amount of *F. poae* specific DNA. On gel, only one major specific PCR product (ca. 306 bp) was obtained by the primer pair in the DNA samples from *F. poae* and grain samples was found, but the unspecific products made it difficult to estimate the amount of *F. poae*-specific PCR product.

Primers and probe designed for *F. poae* successfully amplified DNA extracted from pure cultures and from grain samples. The primers and probe displayed a linear range of at least four orders of magnitude with pure DNA from *F. poae* isolates. The primers and probe amplified DNA extracted only from *F. poae* strains.

Correlation between molecular, morphological and mycotoxin results - NIV and ENNs contamination levels were generally higher in 2002 than in 2003. A significant correlation was found between *F. poae* DNA and NIV ($p<0.05$) and ENNs ($p<0.01$) levels in barley (Figure 1).

In wheat and oats the correlation was not as clear, which may be due to the lower levels of NIV and ENNs found. But also in wheat higher *F. poae* DNA levels were found in samples with high NIV levels, especially in 2002. No clear correlation was found between the levels of other mycotoxins and *F. poae* DNA (Yli-Mattila et al., 2004, unpublished results) in grain samples, although BEA levels were also somewhat higher in the few samples with high *F. poae* DNA levels in 2002.

This study is one of first reports of fluorogenic PCR detection assays for *F. poae* in grains (Waalwijk et al., 2004). Artificial infection increased *F. poae* contamination level only in barley in 2003. In 2002 there

was a strong natural infection in all artificially infected barley samples. In 2003 the highest *F. poae* contamination levels were found in oats.

SYBR Green real-time quantitative PCR was suitable for estimating the amount of *F. poae* DNA in pure cultures and it can also be used for estimating the amount of *F. poae* DNA from the melting curve obtained from grain samples. However, more manual calculations are required for the estimation and the results were not as reproducible as in Taqman real-time quantitative PCR. Also the correlation with toxin levels was not good. Due to variable amounts of unspecific PCR products in SYBR green realtime quantitative PCR, it was difficult to get comparable and reproducible results from the grain samples between separate PCR runs.

According to the results of the present study Taqman primers and probes designed are really specific for *F. poae*. With other *Fusarium* species at least one thousand times more DNA is required to get the threshold value with a cycle number below 35. Thus, the primers and probe can be used for estimating the DNA levels of *F. poae*.

For most of the samples, there was a clear correlation between *F. poae* DNA and NIV and ENNs contamination levels. For some samples, however, the determined mycotoxin contamination levels were high, even though the DNA levels were low. This may be due to a large number of small colonies or low number of bigger colonies on grains. The environmental conditions in the fields (Jestoi et al. 2004) may also have an influence on the type and amount of mycotoxin produced.

In 2002 one oats and seven barley samples having high levels of NIV also had high levels of *F. poae* DNA. In wheat both *F. poae* DNA and NIV levels were generally lower. In 2003 both NIV and *F. poae* specific DNA levels were lower in barley than in 2002.

It is possible to use the *F. poae* DNA concentration levels to eliminate the grain samples containing high amounts of NIV and ENNs in barley and oats. In the combined barley samples of 2002-2003 the average NIV level in the seven samples containing the highest levels (>2 x 10^{-3} ng/ng total DNA) of *F. poae* DNA was 4100 µg/kg, while in the rest of 29 samples the average level was 77µg/kg. In the combined oats samples of 2002-2003 the average NIV level in the five samples containing the highest levels (>10^{-4} ng/ng total DNA) of *F. poae* DNA was ca. 190 µg/kg, while in the rest of 13 samples the average level was 85 µg/kg.

Also in Sweden *F. poae* seems to be the most important NIV producer (Pettersson 1991), while strains of other *Fusarium* species, such as *F. culmorum, F. graminearum* (Bottalico et al. 2002), *F. sporotrichioides* and *F. langsethiae* (Jestoi et al., 2004) may also be able to produce NIV. The ENNs levels in the seven barley samples with the highest *F. poae* DNA (>2 x 10^{-3}ng/ng total DNA) was 10400 µg/kg, while in the rest of the 19 samples the average level was 320 µg/kg. A correlation was also found between *F. avenaceum/F. arthrosporioides* DNA and ENNs levels in barley (Paavanen-Huhtala et al., 2004, unpublished results). These results are in accordance with the results of Jestoi et al., (2004), according to which some *F. poae* strains are effective in producing NIV and ENNs.

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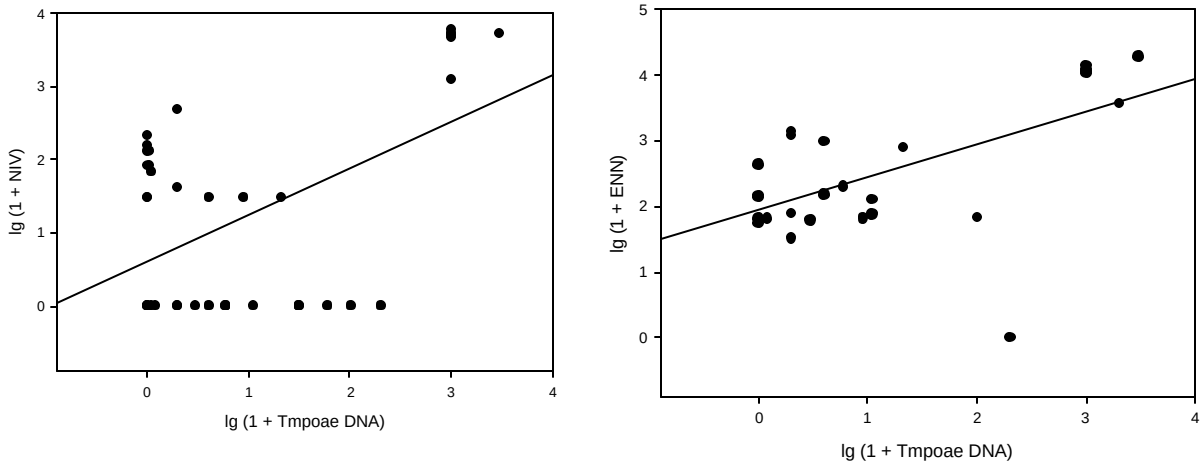

Figure 1. Correlation between *F. poae* DNA (ng/ng total DNA) and mycotoxins (µg/kg) nivalenol (left, R^2 = 0.21, P(a) = 0.043) and enniatins (right, R^2 = 0.35, P(a) = 0.0015) in Finnish barley samples harvested in 2002-2003.

PHENOTYPIC EXPRESSION BY DIFFERENT STRAINS OF *FUSARIUM GRAMINEARUM* AS AFFECTED BY SUBSTRATE AND TEMPERATURE

H. Zhang[1], C. Wolf-Hall[1*], C. Hall[2] and R. Stack[3]

[1]Department of Veterinary and Microbiology Science; [2]Department of Cereal and Food Sciences; and [3]Department of Plant Pathology, College of Agriculture, Food Systems and Natural Resources, NDSU, Fargo, 58105, USA

[*]Corresponding Author: PH: (701) 231-6387; E-mail: Charlene.hall@ndsu.nodak.edu

ABSTRACT

We investigated the phenotypic expression of biomass, pigments and mycotoxins by *Fusarium graminearum* as affected by strain type, substrate, and temperature. Twelve isolates known to have phenotypic and genetic diversity were used for this experiment. Each isolate of *F. graminearum* was inoculated in triplicate into 25 g of 40% moisture content autoclaved white rice, hard red spring wheat, corn, and barley in half pint canning jars. Approximately 10^4 spores in 1 ml were added to each jar. The cultures were incubated at 15°C, 25°C and 35°C and 80% humidity for two weeks with 12 hr light cycle in an environmental chamber. The fifth substrate was wheat that was grown at 22-25°C with supplemental lighting in a greenhouse. The heads of Grandin wheat, a moderately susceptible hard red spring variety, were sprayed with spore suspension of *Fusarium graminearum* isolates at a concentration of 10,000 macroconidia per ml until dripping at anthesis stage. The samples were analyzed for deoxynivalenol (DON),15-acetyldeoxynivalenol (15Ac-DON), 3-acetyldeoxynivalenol (3Ac-DON), nivalenol (NIV) and zearalenone (ZEN). Ergosterol was measured as an estimate of fungal biomass. We found that all the Fusarium strains used in this experiment belonged to 15Ac-DON chemotype, and ZEN normally was produced along with B trichothecene. The mycotoxin production and the ratio among DON, 15-AcDON and ZEN varied with *Fusarium graminearum* strain type, substrate and temperature. Even though interactions among media, temperature and substrate existed, the following trends were found. Rice was the best medium for mycotoxin production, followed by corn and wheat, while barley and greenhouse wheat were the worst media for mycotoxin production. The 15°C and 25°C promoted mycotoxin production much more than 35°C did. And also visible mycelium growth and pigment production were observed in the four grain cultures incubated at 15°C and 25°C, while at 35°C, sparse white mycelium was observed in some samples of rice culture only. Ergosterol contents in samples were in agreement with the observations mentioned above. Additional detailed results and further discussion will be presented.