الكشف والتعريف والتقدير الكمي لفطور *Fusarium graminearum* و *F. culmorum* في حبوب القمح باستخدام تقانات PCR

Detection, Identification and Quantification of *F. graminearum* and *F.culmorum* in Wheat Kernels by PCR Techniques

Received 27 May 2011 / Accepted 10 March 2011

Rabab Sanoubar [(1)], Astrid Bauer [(2)], and Luitgardis Seigner[(3)]

(1). MSc student, Department of Horticulture, Agriculture Faculty, Damascus University, Syria.

(2). PhD student, Iinstitute of Plant protection (BayerischeLandesanstaltfürLandwirtschaftInstitutfürPflanzenschutz, LfL), Freising, Germany.

(3). Head of Phytopathology Diagnosis Department, Iinstitute of Plant protection (BayerischeLandesanstaltfürLandwirtschaftInstitutfürPflanzenschutz, LfL) Freising, Germany.

المُلَخَّص

نُفذ هذا البحث في عام 2006 على 172 عينةً من القمح الشتوي المحصود في عام 2005. تتألف هذه العينات من أصناف مختلفة جُمعت عشوائياً من حقول المزارعين من منطقة بافاريا / جنوب ألمانيا. يهدف هذا البحث للكشف عن وجود المورثة Tri-5 المسؤولة عن انتاج سموم Trichothecene لاسيما Deoxynivalenol (DON)، عن طريق استخدام تفاعل بوليميراز التسلسلي التقليدي (PCR)، وتحديد العلاقة بين وجود المورثة Tri-5 وتركيز سموم DON وتقدير الإصابة بفطور *Fusarium graminearum* و *Fusarium culmorum* باستخدام تفاعل بوليميراز التسلسلي بالوقت الحقيقي Real-Time PCR وتقييم الارتباط بين محتوى DON ودرجة الإصابة بأنواع الفيوزاريوم *F. graminearum* و *F.culmorum* أظهرت هذه الدراسة أن 86 % من مجموع العينات المصابة تملك المورثة Tri-5 وتقوم بمضاعفة قطعة من DNA بطول 544bp وقد ترافق وجود هذه القطعة بوجود كميات من سموم DON تراوحت بين 10 - 2990 ميكروغرام/كغ. برهنت هذه الدراسة أن النوع *F. graminearum* هو النوع السائد والمرافق لمرض لفحة الفيوزاريوم (FHB)، كما أنه يُعد المنتج الأساس لسموم DON المترافقة مع مرض لفحة الفيوزاريوم حيث وجدت علاقة ارتباط قوية ($R^2 = 0.7$) بين محتوى سموم DON وبين كمية DNA لهذا الفطر وذلك بالمقارنة مع الارتباط الضعيف ($R^2 = 0.03$) بين مستوى سموم DON وكمية DNA لفطر *F. culmorum* في حبوب القمح المصابة.

الكلمات المفتاحية: القمح، لفحة الفيوزاريوم ، مورثة Tri-5، سموم DON، تفاعل بوليميراز التقليدي بالوقت الحقيقي (Real-Time (PCR.

Abstract

This study was carried out in 2006 on 172 samples of winter wheat harvested in 2005. The samples

consisted of various cultivars that had been randomly collected from farmer's fields in differentareas of Bavaria, South Germany. The objectives of this study were detecting the presence of Tri-5 gene producing fungus that generate stricho the cenemycotoxins, especially DON, by using conventional qualitative PCR; determining the correlation between the presence of Tri-5 gene and DON content; evaluating the *Fusarium graminearum* and *Fusarium culmorum* infectionby Real-Time PCR andestimatingthe correlation between DON content and the severity of *F.graminearum* and *F.culmorum* contamination. This study showed that 86 % of all infected samples had a Tri-5 gene and amplified a single 544bp fragment associated with a detectable amount of DON (ranged from 10 to 2990 μgkg^{-1}). This study demonstrated that *F. graminearum* is the predominant species associated with *Fusarium* head blight (FHB) and was considered as the predominant trichotheceneproducer that associated with FHB since there was a highly significant correlation ($R^2 = 0.7$) between DON and *F.graminearum* DNA content, compared to a weak correlation ($R^2 = 0.03$) between DON and DNA content of *F.culmorum* infected wheat kernels.

Keywords: Wheat; *Fusarium* head blight, Tri-5gene, Trichothecenedon, Conventional and Real-Time PCR.

Introduction

Fusarium head blight (FHB) of small grains was first described over a century ago and was considered as a major threat to wheat and barley during the early years of last century (Dickson and Mains, 1929). Head blight or scab of wheat caused epidemics in many wheat area worldwide (Dubin *et al.*, 1997; McMullen et *al.*, 1997b). The International Maize and Wheat Improvement Centre (CIMMYT) have considered FHB as a major factor limiting wheat production in many parts of the world (Dubin et *al.*, 1997). FHB is also known as "tombstone" kernels of wheat because of the chalky and lifeless appearance of the infected kernels (Tuite *et al.,* 1990). It has the capacity to destroy a potentially high-yielding crop within few weeks (McMullen et *al.*, 1997a). FHB is a significant disease of small-grain cereals throughout Europe (McMullen *et al.*, 1997b), United States (Liu and Wang, 1990), Canada (Hart et *al.*, 1998), South America (Hanson et *al.*, 1950), Asia (Mathre, 1997) and Australia (Bechtel et *al.*, 1985). FHB was identified more than 120 years ago, in 1884, in England. The United States Department of Agriculture ranked FHB as the worst plant disease to appear since the 1950's (Wood et *al.*, 1999). It has increased worldwide (Parry et *al.*, 1995) and itwas considered as a major threat to wheat and barley during the early years of the twentieth century (Muriuki, 2001; Stack, 2003).

FHB is caused by a number of different fungal species of the genus *Fusarium* (*Fusarium* spp.) However, F. *avenaceum, F. culmorum, F. graminearum* (teleomorph, Gibberellazeae), *F. poae*, and *Microdochium nivale* (Teleomorph, Monographella nivalis) are the species which are most commonly associated with the FHB disease (Edwards et *al.*, 2001).

The threat possessed by *Fusarium* spp. is multifaceted. It causes yield and quality losses due to sterility of the florets and formation of discolouration, which reduces kernel size and losses light weight kernels (Mathre, 1997). In addition, grain quality factors such as protein content and germination can be severely affected by the pathogen (Schwarz et *al.,* 1995). Several *Fusarium* species which cause FHB are able to produce trichothecene mycotoxin.

F. culmorum, F. graminearum, and *F. poae* produce type B trichothecenes such as nivalenol (NIV), deoxnivalenol (DON), and fusaenon-X (McMullen et *al.,* 1997c), while other species are not (Marasas et *al.,* 1984). DON is the predominant Trichothecenes found in Europe and North America (Bottalico and Perrone, 2002). Trichothecene produced by this fungus possessd a serious hazard to human and animal health (Bechtel et *al.,* 1985) because these toxic materials are potent inhibitors of eukaryotic protein biosynthesis (Boyacioglu et *al.,* 1992; van Eeuwijk et *al.,* 1995). Acute adverse effects of the toxin in animals causes weight loss and feeding refusal in non-ruminant livestock, high rates of abortion, diarrhoea, emesis, alimentary haemorrhagy and contact dermatitis (Bennett and Klich, 2003). Human ingestion of grain contaminated with *F. graminearum* has been associated with alimentary toxicity as well as illness characterized by nausea, vomiting, anorexia, and convulsions (Murphy and Armstrong, 1995). Trichothecenes are also powerful modulators of human immune function and may promote neoplasms, cause autoimmune disease, or have long-term effects on resistance to infectious disease by altering immune response (Berek et *al.*, 2001; Lindsay, 1997).

Several genes of *Fusarium* are involved in the biosynthesis of trichothecene and most of them are localized in a Trigene cluster. The Tri-5 gene encodes the enzyme trichodiene synthase (Bai et *al.*, 1999), which catalyzes the first step in the trichothecene biosynthetic pathway in trichothecene-producing strains of *Fusarium* species. The development of Tri-5 gene specific primers has allowed trichothecene-producing *Fusarium* spp. to be distinguished from nonproducing species using PCR-based assays (Niessen and Vogel, 1998). The nucleotides sequence of the Tri-5 gene has been characterized in several *Fusarium* species (Fekete et *al.*, 1997; Hohn and Desjardins, 1992).

The main objectives of this study were detecting the presence of Tri-5 gene producing fungus which encodes the key enzyme in trichothecene production, especially DON, by using conventional PCR; determining the correlation between the presence of Tri-5 gene and the DON content, which was analysed by the chemist Dr. Puttner. J. Lepschy ;evaluating the amount of *F. graminearum* and *F. culmorumin* fection through Real-Time PCR assay; investigating the relationship between DON content and the degree of *F. graminearum* and *F. culmorum* contamination and determining the aggressiveness of FHB towards plant host.

Materials and methods

1. Fungal reference material

50 ngof extracted DNA from *F. graminearum* isolates were applied in tenfold serial dilutions (10^{-1} to10^{-4}) as a quantitative standard in Real-Time PCR (RT-PCR) using a *F. graminearum* specific Taqman® hybridization probe for beta-tubulin gene. In parallel, 50ngof DNA of *F. culmorum* strains were used in tenfold serial dilutionalso as a quantitative standard for RT-PCR using SYBR Green®1.

2. Plant material

At harvest time, 172 winter wheat spikes samples of various cultivars have been randomly collected from farmers' fields in different areas of Bavaria, South Germany. Directly after harvest, samples were sent to the Institute of Plant Protection, LfL and preserved at - 20 °C.

3. DNA extraction

DNA of infected wheat kernels was extracted

by homogenising 10 mg of dried kernels in a mixer with the presence of 1 ml DNA extraction buffer (2% CTAB, 1.4 M NaCl, 100 mMTris, 20 mM Na-EDTA, and 1% PVP -40). The mixture was vortexed and the flow was transferred to microcentrifuge tubes. 1 ml chloroform/isoamylalkohol (24:1) was added, well mixed and spined at 5000×g for 10 minute at 20° C. The aqueous phase containing DNA molecules was transferred into 2 ml fresh tubes where 100 µl Na-acetat (3M, pH 5.2) and 1 ml isopropanol (-20° C) were added and mixed by inverting the tubes many times. Tubes wereplaced in a freezer (-20° C) for at least 1 hour. For each sample, the lysate mixture was transferred to SV Minicolumn placed in 1.5 ml tubes and spined at 16.000×g for 1 minute at 4oC. The supernatant was discarded, and the SV Minicolumns were washed with ethanol many times as described by Bauer et *al.,* (2004). Finally, 50 µl of distilled sterile water was added directly into SV Minicolumn which was placed in 1.5 ml microcentrifuge tube and incubated at room temperature for 5 minutes then spined at 16.000×g for 2 minutes at 4° C to collect the eluted DNA (Bauer et *al.*, 2004).

4. PCR assay

Two Tri-5 specific primers have been used to detect the presence of Tri-5gene in *Fusarium* spp. infected wheat kernels. 172 wheat samples were tested with a sample of *F. graminearum* used as a positive control. The sequences of these primers are: forward primer Tri-5F: (5'- A G C G A C T A C A G G C T T C C C T C -3') and reverse primer Tri-5R: (5'- A A A C C A T C C A G T T C T C C A T C T G -3'). These primers were derived from the conserved region of Tri-5genein *Fusarium* spp.Tri-5primers (Tr5F and Tr5R) amplified a single 544bp fragment in both DNA extracted of *F. graminearum* and *F. culmorum* and DNA of infected wheat grains. The total volume of reaction master mix was 22.7 µl. The PCR amplification was performed using (2.7 µl of 25 µg ml^{-1}) of both fungal *F. graminearum* DNA and DNA from 10 mg dry weight of wheat material, 0.5 µM of each of the Tri-5-specific primers, 0.8 mM concentration of nucleotides dNTPs, 0.5 unit of Taq polymerase and 2.27 µl of PCR buffer with1.5 mM $MgCl_2$. The PCR negative control was a reaction master mix with 2.7 µl of distilled water instead of DNA template. Cycler programme was set as the following: one cycle at 95°C for 75 s then 32 cycles of 94° C for 20 s, 62° C for 17 s, 72° C for 45 s, and a final cycle at 72° C for 4 min and 15s. DNA banding were revealed by electrophoresis at 90 v on 2% agarose gels in 1xTris-acetate EDTA buffer (TAE) (where 50X TAE contained: 2M Tris, 1M Acetic Acid, and 0.1M Na-EDTA x $2H_2O$ at PH 8.0) using ethidium bromide staining (30 µg of a ethidiumbromide for 100 ml of 1X TAE buffer) and photographed under UV light using a camera and a photo print image visualizer.

5. Quantification of *F.* species by Real-Time PCR

5.1. Quantification of *F. graminearum* by RT-PCR using a TaqMan probe assay

Two primers, derived from the consensus beta-tubulin sequence which is associated with head blight in wheat, were used for *F. graminearum* quantification. The forward primer FGtubf: (5′- G T C T C G A C A G CA A T G G T G T T -3′) and reverse primer FGtubr: (5′- G C T T G T G T T T T T C G T G G C A G T-3′) specifically amplified a 111bp fragment of the beta-tubulin gene of *F. graminearum* which was quantified by the TaqMan probe FG tub TM (FAM-the TaqMan probe FG tubTM (FAM5′A C A A C G G A A C G G C A C C T C T G A G

C T C C A G C3′-TAMRA). PCR was monitored on a Real-Time 7000 Sequence Detection System. PCR Master Mix contained: HotStartTaq DNA polymerase and PCR buffer specifically adopted for quantitative PCR analysis using species-specific probes. The total volume of master mix reaction (23 µl) contained: optimal primer concentrations 0.3 µM of FGtubf and FGtubr primers, 1 x PCR buffer, 50 ng of template wheat DNA samples, and 0.2 µM of TaqMan probe, and 50 ng of *F. graminearum* dilution template DNA as a standard curve. There were four series of diluted standard curves, with1:10 fold of dilution factor of *F. graminearum* DNA. The number of cycles in the PCR was set at 40, as the 40th cycle represented the extrapolated threshold cycle for a reaction with a theoretical single copy of the template DNA. PCR program was asthe following: 95° C for 15 min, 40 cycles of 95° C for 15 s and 67° C for 1 min. All reactions were performed in triplicates. PCR efficiency was calculated from threshold cycles of the standard dilution curve.

5.2. Quantitation of *F. culmorum* by Real-Time PCR using DNA binding dye assay

Two specific primers were used for thedetection of *F. culmorum* by amplifying 140bp fragment of *F. culmorum*. Forward primer sequence Fc03: (5'- T T C T T G C T A G G G T T G A G G A T G-3') and reverse primer sequence Fc02: (5'- G A C C T T G A C T T T G A G C T T C T T G -3') were specifically amplified a 140bp fragment of *F. culmorum* genome that was quantified by the DNA binding dye, SYBR® Green 1. The SYBR Green 1 assay is similar to that of TaqMan assay except the presence of an intercalating agent such as fluorescent dye SYBR Green 1 instead of fluorescent probes TaqMan.

Results and Discussion

Results:

1. Detection of *Tri-5* gene

The Tri-5 specific PCR assay could provide a screening tool for detection of trichothecene-producing *Fusarium* species in plant tissues. In this study, 172 DNA samples of infected wheat kernelswere analyzed using Tri-5gene primersin a PCR reaction to detect trichothecene producing *Fusarium* species. The separation of PCR products on agarose gels showed that 86% of DNA samples possessed a unique fragment of 544bp representing part of the Tri-5gene while 14% of samples didn't show any amplification product (Fig. 1).

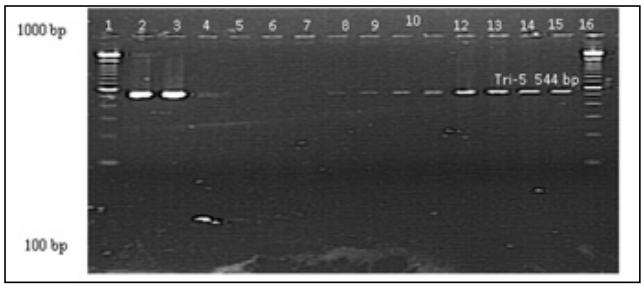


Fig. 1. Analysis of DNA samples with specific primers (Tri-5 gene) on 2% agarose gel.

Line 1, 16 standards 100 bp. Lines 2, 3 *F. graminearum* DNA used as positive control. Lines (4-15) some tested wheat samples: lines (5-7) free of DNA band, Lines (4,8-11) faint bands, and lines (12-15) bright bands at 544bp.

The same wheat samples were used to estimate the content of DON in their tissues determined by HPLC (results provided by the chemist Dr.puttner J. Lepschy, LfL). The quantity of DON varied between the samples leading to regroup the samples into 3 categories according to their DON content.

The comparison between the amplification products produced with Tri-5gene primers and the DON contents demonstrated that the samples free of Tri-5gene products (14% of all samples) showed

either absence of DON in their tissues (in 7% of all samples) or detected an amount of DON ranged from 11 - 226µg kg^{-1} (in 7% of all samples).

For all samples possessing 544bp DNA fragments (86% of all samples), an amount of DON was detected in their tissues. Approximately 76% of them (59% of all samples) were considered DON high-producing strains (101 - 2990µg kg^{-1}) and possessing an intensive DNA bandon agarose gels, while 14% of them (27% of all samples) were considered DON low-producing strains (10 - 99µg kg^{-1}) and showing a faint DNA band at 544bp.

2. Quantification of *F. graminearum* and *F. culmorumusing* Real-Time PCR

The specific primers and TaqMan hybridization probe targeting the beta-tubulin gene amplifies DNAs from *F. graminearum* infected wheat. Based on determination of threshold cycle (Ct-values) in individual samples and known DNA standards during Real-Time PCR, amounts of target DNA present in the samples were calculated. The amplification of standard dilution curves of *F. graminearum* in Real-Time PCR gave linear and reliable results (R^2 values were between 0.997 and 0.989). The concentration of *F. graminearum* DNA ranged from 0.04 to 4945 µg kg^{-1} (mean of triplicate samples ranged from 9.19E-07 to 1.29E-01 µg ml^{-1}) of dry weight wheat kernels (Fig. 2), while, *F. culmorum* DNA content was limited and ranged between 0.04 and 39.22 µg kg^{-1} (mean of triplicate samples ranged from 8.00E-07 to 7.13E-04 µg ml^{-1}) of dry weight wheat kernels over all Bavaria (Fig.3).

By comparing the results of Tri-5 specific PCR assay, DON quantification by HPLC technique, and RT-PCR DNA quantification of *F. graminearum* and *F. culmorum* for the same wheat samples, we found that the same Tri-5 DNA intensive bright samples that associated with the largest amounts of DON were contained also the highest amount of *F. graminearum* DNA (49.45 -100.82 µg kg^{-1}) and trace amounts of *F. culmorum* DNA (39.22 to 10.67 µg kg^{-1}).Alternatively, faint Tri-5 DNA samples which had a trace amount of DON (10 - 99 µg kg^{-1}) showed trace detectable amounts of both *F. graminearum* DNA ranged from 0.04 to 47.15 µg kg^{-1} and *F. culmorum* DNA ranged from 0.04 to 1.99 µg kg^{-1}. In case of absence of Tri-5 gene products which associated

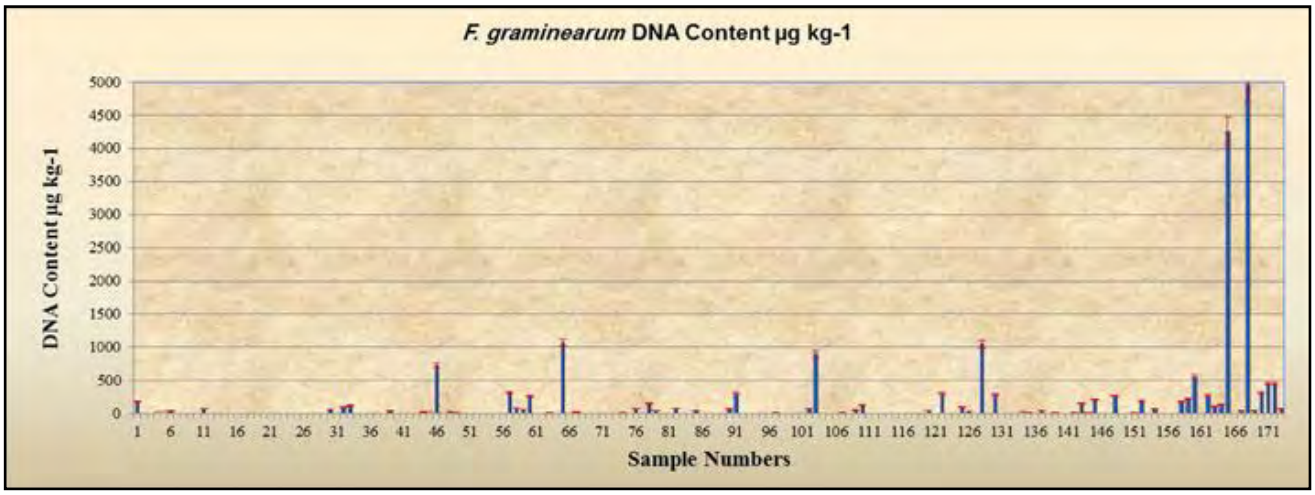


Fig. 2. Infestation by *F. graminearum* in wheat samples from 2005 harvest by Real-Time TaqMan probe. Samples were tested in triplicates.

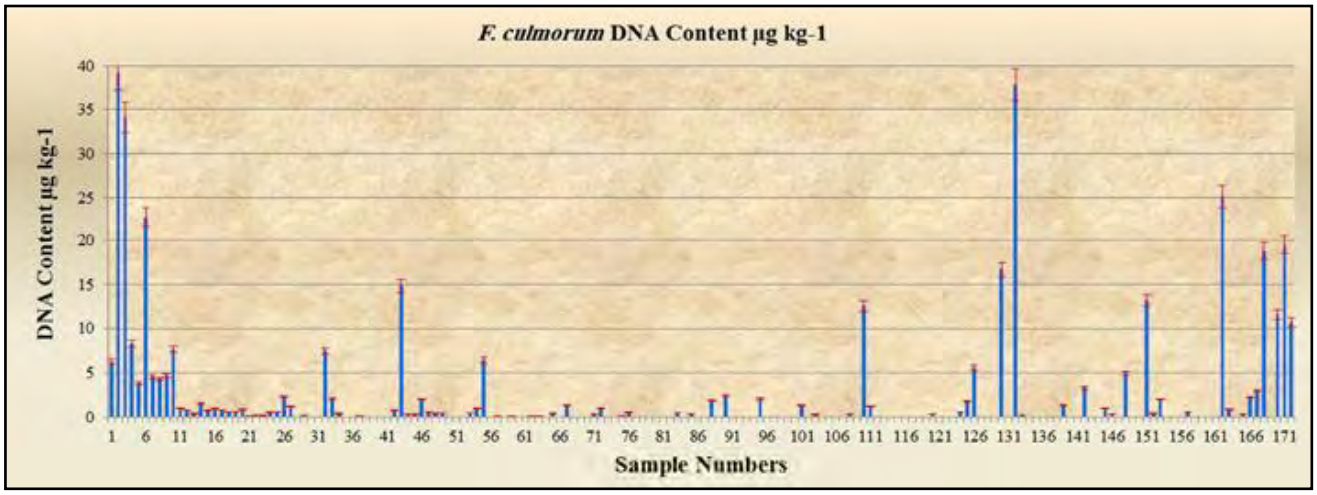


Fig. 3. infestation by *F. culmorum* in wheat samples from 2005 harvest by Real-Time SYBR Green 1 dye. Samples were tested in triplicates.

with free DON contents (7% of all samples), there was trace amounts of both *F. graminearum* and *F. culmorum* DNA (0.72 and 0.78 µg kg^{-1}, respectively). While in case ofabsence of Tri-5 gene products on agarose gelsbut with presence of DON in their tissues (the other 7%), there was trace detectable amounts of both *F. graminearum* DNA (0.66-1.83 µg kg^{-1}) and *F. culmorum* DNA (0.08-39.22 µg kg^{-1}).

3. Correlation between *F. graminearum* DNA and DON content

The plot of *F. graminearum* DNA content in 172 wheat samples, determined by Real-Time TaqMan probe PCR and the DON content in their tissues determined by HPLC, showed a strong positive linear correlation between both parameters. Correlation coefficient was 0.725 (Fig. 4). Moreover, the regression analysis of all data sets indicated a strong and highly significant correlation ($p < 0.05$) between DON contents in the plant tissues and *F. graminearum* DNA contents in wheat samples, and the regression equation was ($y = 0.4896x + 79.784$; $R^2 = 0.7252$).

4. Correlation between *F.culmorum* DNA and DON content

The plot of DON content against *F. culmorum* DNA standard curve (Fig.5) showed a slight correlation between DON and *F. culmorum* DNA, whereas the linear correlation coefficient was ≈ 0.2 which is very far from +1. The regression analysis of all data set showed a weak correlation ($p < 0.01$) between *F. culmorum* DNA and DON content, and the regression equation was ($y = 8.9319x + 117.51$; $R^2 = 0.0353$).

Moreover, comparison of the results of TaqMan Real-Time PCR for *F. graminearum* with analysis of DON content for the same samples showed that 73% of *F. graminearum* presence was associated with DON production. On the contrary, the results of SYBR Green 1 Real-Time PCR for *F.culmorum* with DON content showed that 56% of *F.culmorum* incidence was associated with DON production. However, in spite of the noticeable infections of wheat grains with *F. culmorum* 56%, its DNA content was low (0.04- 39.22 µg kg^{-1}) compared to that of *F. graminearum* (0.04 to 4945 µg kg^{-1}) (Fig. 6).

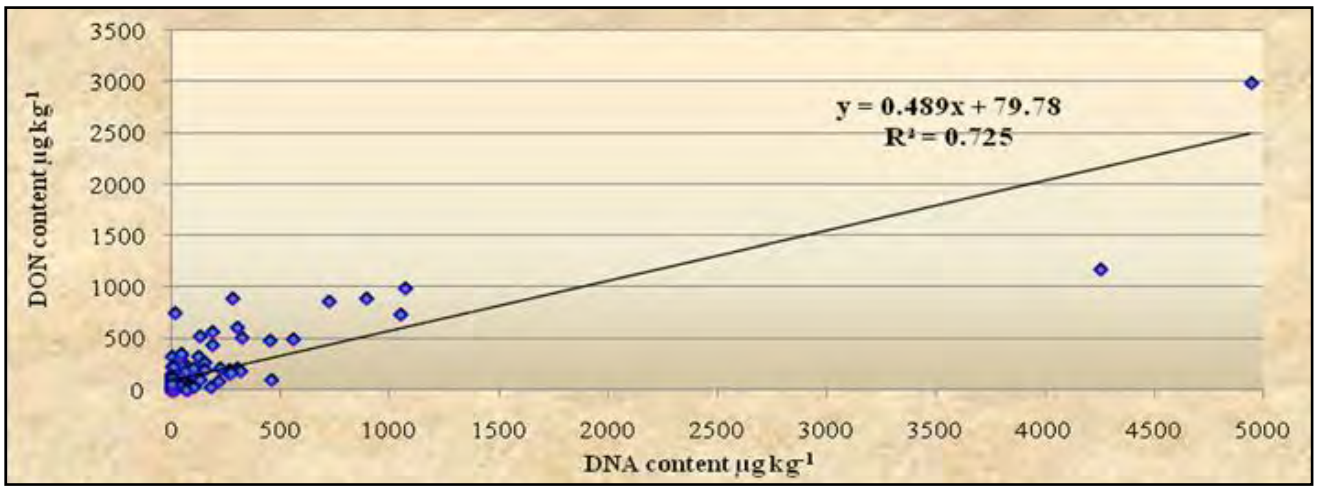


Fig. 4. Relationship between DON and *F.graminearum* DNA content in all samples

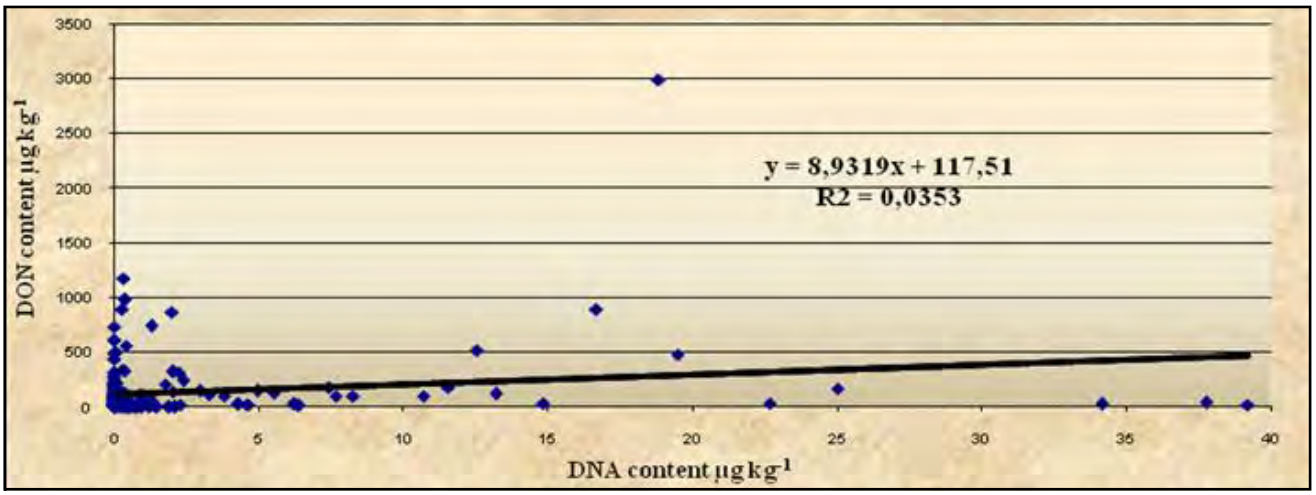


Fig.5. Relationship between DON and *F. culmorum* DNA content in all samples.

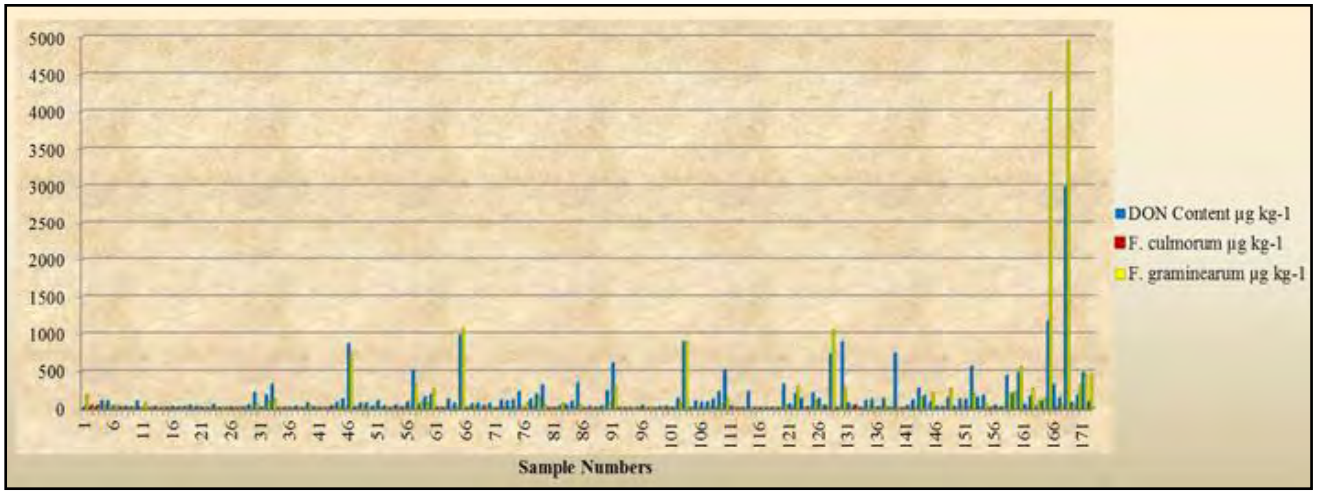


Fig. 6. Correlation between DON, *F. graminearum* and *F. culmorum* DNA content in all samples.

On the other hand, although the *F. graminearum* DNA was not detected in 20% of samples, but DON was found (10-320µg kg^{-1}) and *F.culmorum* DNAwas detected in some of these samples(2-39µg kg^{-1}) (Fig.7).

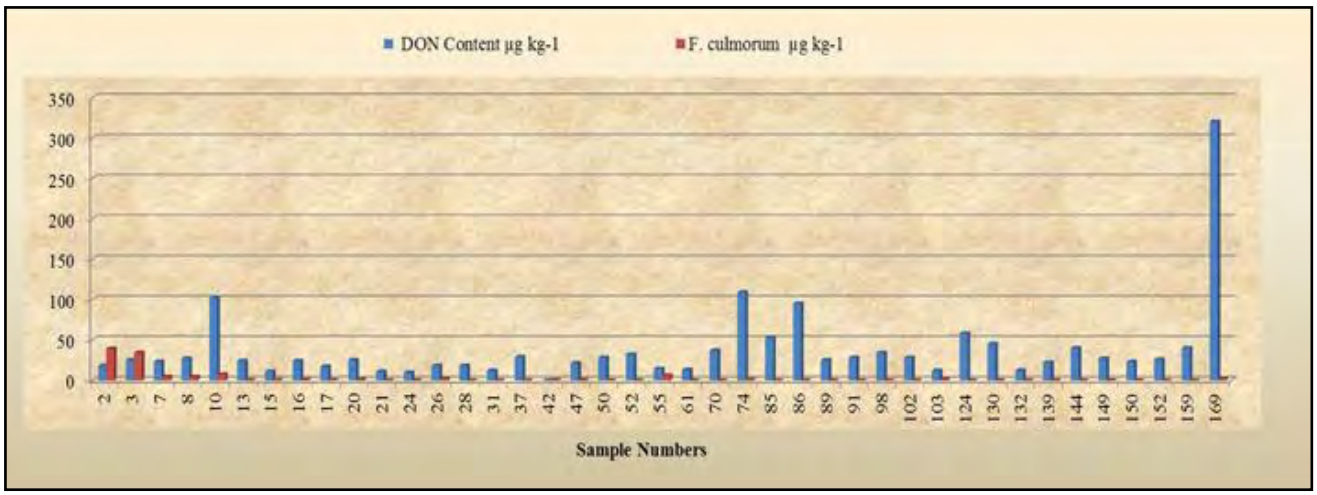
5. Severity of *F.graminearum*and *F.culmorum* infection over Bavaria

The whole picture of DON contents in 172 infected wheat samples in 2005 was highlighted in figure 8 in comparsion with the DON established threshold by FDA organization and European Union. This figure showed that only two samples of all harvested wheat kernel in 2005 lay above the FDA DON advisory level of 1 ppm, and only one sample is higher than the EU threshold of 1.25 ppm.

Discussion:

1. Identification of trichothecene-producing *Fusarium* spp. by PCR

Fusarium species are considered as a potential trichothecene-producing species (Edwards et *al.*, 2001). The conserved region of Tri-5 genehas been detected in *F. culmorum* (snijders and krechting, 1992), *F. graminearum* (Moschini and Fortugno, 1996), *F. poae* (Van Eeuwijk et *al.*, 1995), *F. sporotrichioides* (Hart et *al.*, 1984), and *F. sambucinum* (Hart et *al.*, 1998). There was a direct relationship between Tri-5 gene expression and the increase in deoxynivalenol production (Fernando et *al.*, 1997; Doohanat et *al.*, 1999). Tri-5 primers were designed from highly conserved regions of the Tri-5 gene of *Fusarium* spp. (McMullen et *al.*, 1997c). A Tri5-specific PCR assayhas been developed to detect trichothecene-producing *Fusarium* species in contaminated wheat samples (Neissen and Vogel, 1997). In our study, we used the qualitative Tri-5 specific PCR assay to detect trichothecene-producing *Fusarium* species in contaminated wheat kernel samples (172 samples) collected from south Germany. 59 % (101 samples) of 172 tested samples were positive in the Tri-5 PCR assay and showed intensive bright DNA bands on agarosegels and were highly infected with one or more of *Fusarium* species containing Tri-5 gene. Correspondingly, this result was significantly associated with the results of DON content where the same Tri-5 DNA intensive bright samples had simultaneously the highest amount of DON ranged from 101 to 2990µg kg^{-1}.



.**Fig. 7.** correlation between DON and *F. culmorum* DNA content in all samples

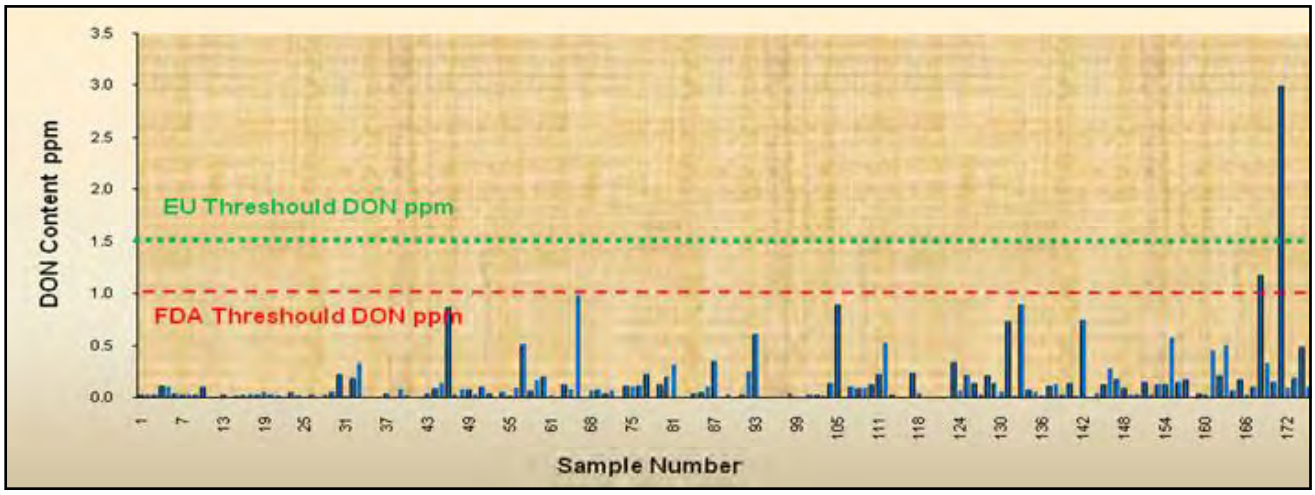


Fig. 8. Relationship between DON content and DON advisory level of EU and FDA organization.

This result was in accordance with some reports on *F. avenaceum* isolates that were positive in the Tri-5 PCR assay and produced DON in culture (Abramson et *al.*, 1993). In addition, the results of *F. graminearum* and *F. culmorum* DNA quantification by RT-PCR showed that the same Tri-5 DNA intensive bright samples contained also the highest amount of *F. graminearum* DNA(4945 - 100.82 µg kg^{-1}) and *F. culmorum* DNA (39.22 -10.67 µg kg^{-1}).Thus, according to our results we could say that there was a significant direct relationship between the density of Tri-5DNA bandson agarose gelsand DON content. The increasing in DON content was also associated with higher concentration of *Fusarium* species, particularly, of *F. graminearum* DNA and slightly with *F. culmorum* DNA quantified by RT-PCR. In other words, we could say the increased density of Tri-5 DNA bands with the highest amounts of DON were related to the presence ofhigh amounts of *F. graminearum* DNA rather than *F. culmorum*. For example, the highest amounts of *F. graminearum* DNA in some samples (4945, 4255, 1069, 1050 µg kg^{-1}) were associated with the largest amounts of DON (2990, 1174, 981, 728 µg kg^{-1}) while the concentration of *F. culmorum* for the same samples were very limited (18.81, 0.30, 0.36, 0.00 µg kg^{-1} respectively Fig. 3-6).On the other hand, faint Tri-5 DNA bands onagarose gels (27% of all tested samples) indicated samples containinga low concentration of Tri-5 gene and as a result, the infection with *Fusarium* spp. was also low. Accordingly, the amount of DON content in these samples wasvery low and ranged from 10 to 99 µg kg^{-1}. These results were in accordance with RT-PCR results, where low amounts of *F. graminearum* DNA (0.04 - 47.15 µg kg^{-1}) and *F. culmorum* DNA (0.07 - 1.99 µg kg^{-1}) were revealed.

Indeed, *Fusarium* spp. infected wheat kernels that showed negative results in the Tri-5 PCR assay and showing absence of DNA bands on agarose gels should be Tri-5 gene free and should not be infected with *Fusarium* spp. containing Tri-5 gene and, accordingly, these samples were DON free. In our results, 7% (12 samples) of all samples were negative in the Tri-5 PCR assay and didn't produce DON and they were approximately free of *F. graminearum* DNA (0.72 µg kg^{-1}) and *F. culmorum* DNA (0.78 µg kg^{-1}).It has been demonstrated that within the same species and in the same cultural conditions toxin production by *Fusarium* strains may vary largely.

Some strains produce large amount of trichothecene, whereas others produce small or undetectable amount of trichothecene (Mesterhazy, 1995; Mesterhazy, 1997; Moschini and Fortugno, 1996; Parry et *al.*, 1995; Schroeder and Christensen, 1963; Snijders and Perkowski, 1990; Strausbaugh and Maloy, 1986; Walker et *al.*, 2001). However, there were other 7% of samples in which no Tri-5 DNA was detected on agarose gels but an amount of DON ranged from 11to 226 µg kg^{-1}, low detectable amounts of *F. graminearum* DNA ranged from 0.66 to 1.83 µg kg^{-1} and an amount of *F. culmorum* DNA (0.08 - 39.22 µg kg^{-1}) were detected. It might be possible that other genes involved in trichothecene biosynthesis have been identified outside the Tri biosynthetic gene cluster including Tri-1 (McCormick et *al.*, 2004) and Tri-101 (Kimura et *al.*, 1998) which requires more investigation in our samples. However, it is possible that the pathogenic isolates producing DON in very small amounts could produce other phytotxins instead of DON in the pathogenesis (Hestbjergat et *al.*, 2002). In our study, the same samples that produced Tri-5 DNA intensive bright bands on agarose gels were containing the highest amount of DON revealed by HPLCanalysis and had also the largest amounts of *F. graminearum* and *F. culmorum* DNA evaluated by RT-PCR.We conclude from the displayed results that there was a positive relationship between the three techniques used in this study as they provided us with similar results for the same samples.

2. Quantification of *F. graminearum* and *F. culmorum*

Molecular diagnostic of plant pathogenic fungi can be highly specific, very sensitive, and relatively fast (McCartney et *al.*, 2003). We used in this study a fast and reliable method for the species-specific identification and absolute quantification of *F. graminearum* and *F. culmorum*. It is a RT-PCR assay using a Taq Man hybridization probe targeting the beta-tubulin gene for *F. graminearum* and SYBR Green 1 for *F. culmorum*. Taq Man method used in this study because of its sensitivity, selectivity, and reduction of faultsignals due to primer-dimer formation (McCartney et *al.*, 2003) and allowed a fast species-specific identification and quantitation of plant infections by *F. graminearum* at very early stages where classical microbiological and toxin analysis methods fail to detect the pathogen (McCartney et *al.*, 2003). The beta-tubulin gene of all non *F. graminearum* isolates failed to be amplified in the reaction while targeting DNA from all isolates yielded product in the PCR assay (Reischer et *al.*, 2004). RT-PCR analysis confirmed that *F. graminearum* was more abundant in the infected grains than *F. culmorum* since the concentration of *F. graminearum* DNA ranged from 0.04 to 4945 µg kg^{-1} while *F. culmorum* DNA content ranged from 0.04 to 39.22 µg kg^{-1}.Consequently, *F. graminearum* infections were severe whilethe severity of *F. culmorum* infection was not high in wheat kernels during the season of 2005. Comparison between the results of Taq Man Real-Time PCR analysis for *F. graminearum* and DON content showed that *F. graminearum* is an efficient DON producer where there was high positive significant correlation (R^2 = 0.7) between DON and *F. graminearum* DNA content. Therefore, *F. graminearum* was considered as the predominant trichothecene associated with FHB and produced the main part of DON in 2005 wheat crop. This is in accordance with former investigations (McMullen et *al.*, 1997c). In contrast, the slight weak correlation (R^2 = 0.03) between DON and *F. culmorum* DNA contentmay reflect that

F. culmorum was the second important speciesin the DON producing *Fusarium* genus. Parry et *al.*, (1995) suggested that *F. culmorum* along with *F. graminearum* were consistently the most pathogenic of the *Fusarium* species infecting cereal ears.

Moreover, thePCR analysis showed that in 20% of total infected samples, DON was found (10-320µg kg^{-1}) where no *F. graminearum* DNA was detected and that was linked with only slight content of *F. culmorum* DNA (0.10 - 39.22 µg kg^{-1} Fig 3 - 7). PCR analysis indicated that the presence of other *Fusarium* species within the field plots may account for the FHB disease and this result was consistent with the observation of (Doohan et *al.*, 1998). In these cases, DON content is probably attributable to the possibility that FHB infection in the samples is caused by a complex of *Fusarium spp*. which release DON mycotoxins, and other DON producing *Fusarium spp*. (like *F. pseudograminearum*, *F. poae* and / or *F. sporotrichoides*) might have been presented, that may require further investigation.

The U.S. Food and Drug Administration (FDA) recommend that DON levels in human foods should not exceed 1 ppm. Higher levels of DON are permitted in feed for poultry and ruminant animals. While the European Community supports the setting of European Union (EU) thresholds of trichothecenes as low as reasonably achievable in order to protect public health. For example, DON levels in human foods should not exceed 1.25 ppm.In general, the aggressiveness of *F. graminearum* and *F. culmorum* was relatively low overall Bavaria in 2005 since the DON content was generally low (10 -2990 µg kg^{-1} Fig 3-6). Indeed, the aggressiveness of *Fusarium* was not so high where only 2% of all harvested wheat kernel in 2005 laid above the FDA DON advisory level of 1 ppm, and only one sample was higher than the EU threshold of 1.25 ppm. Proctor et *al.,* (1995) reported that trichothecenes may play an important role in the aggressiveness of fungi towards plant host.

Acknowledgements:

The financial support of Tempus programme is gratefully appreciated. Dr. Luitgardis Seigner, Head of Phytopathology Diagnosis Department in the Institute of Plant Protection (Bayerische Landesanstaltfür Land wirtschaft Institutfür Pflanzenschutz, LfL) is acknowledged for her supports with all required information. Thanks to Miss Astrid Bauer for the technical assistant.The contribution of Dr. puttner J. Lepschy at LfL in DON analysis by HPLC is well appreciated.

References

Abramson, D., R. M. Clear, and D. M. Smith. 1993. Trichothecene production by *Fusarium* spp. isolated from Manitoba grain. Can. J. Plant Pathol. 15:147-152.

Bai, G., F. L. Kolb, G. Shaner, and L. L. Domier. 1999. Amplified fragment length polymorphism markers linked to a major quantitative trait locus controlling scab resistance in wheat. Phytopathology 89:343-348.

Bauer, A., L. Seigner, P. Büttner, and H. Tischner. 2004. Monitoring of FHB using PCR for qualitative and quantitative detection of Fusariumspp. Proceedings of the 2nd International Symposium on *Fusarium* Head Blight. Vol. 2, p. 553.

Bechtel, D. B., L. A. Kaleikau, R. L. Gaines, and L. M .Seitz. 1985. The effects of *Fusarium graminearum* infection on wheat kernels. Cereal Chem. 62:191-197.

Bennett, J. W., and M. Klich. 2003. Mycotoxins. Clin. Microbiol. Rev. 16:497–516.

Berek, L., I. B. Petri, A. A. Mesterhazy, J. Teren, and J. Molnar. 2001. Effects of mycotoxins on human immune functions in vitro. Toxicol. *In Vitro*. 15:25-30.

Bottalico, A., and G. Perrone. 2002. Toxigenic *Fusarium* species and mycotoxins associated with head blight in small grain cereals in Europe. European Journal of Plant Pathology 108:611-624.

Boyacioglu, D., N. S. Hettiarachchy, and R. W Stack. 1992. Effect of three systemic fungicides on deoxynivalenol (vomitoxin) production by *Fusarium graminearum* in wheat. Can. J. Plant Sci. 72:93-101.

Dickson, J. G., and E. B. Mains. 1929. Scab of wheat and barley and its control. USDA Farmers' Bulletin 1599. : 1-18.

Doohan, M. F., D. W. Parry, P. Jenkinson, and P .Nicholson. 1998. The use of species-specific PCR based assays to analyse *Fusarium* ear blight of wheat.47:19-205.

Doohan, M. F., G. Weston, N.H Rezanoor, W.D Parry, and P. Nicholson .1999. Development and Use of a Reverse Transcription-PCR Assay to Study Expression of Tri-5 by *Fusarium* Species *In Vitro* and In Planta. Applied and Environmental Microbiology, :3850-3854

Dubin, H. J., L. Gilchrist, J. Reeves, and A. McNab . 1997. *Fusarium* head scab: Global status and prospects. CIMMYT, Mexico, DF, Mexico. 130 p.

Edwards, S. G., S. R. Pirgozliev, M. C. Hare, and P. Jenkinson. 2001. Quantification of trichothecene-producing *Fusarium* species in harvested grain by competitive PCR to determine efficacies of fungicides against *Fusarium* head blight of winter wheat. American Society for Microbiology. 67(4): 1575–1580.

Fekete, C., A. Logrieco, G. Giczey, and L. Hornok . 1997. Screening of fungi for the presence of the trichodiene synthase encoding sequence by hybridization to the Tri-5 gene cloned from *Fusarium poae*. Mycopathologia 138:91-97.

Fernando, W. G. D., T. C. Paulitz, W. L. Seaman, P. Dutilleul, and J. D. Miller . 1997. Head blight gradients caused by Gibberellazeae from area sources of inoculum in wheat field plots. Phytopathology 87:414-421.

Hanson, E. W., E. R. Ausemus, and E. C. Stakman . 1950. Varietal resistance of spring wheats to fusarial head blight. Phytopathology 40:902-914.

Hart, L. P., J. J. Pestka, and M. T. Liu . 1984. Effect of kernel development and wet periods on production of deoxynivalenol in wheat infected with Gibberellazeae. Phytopathology 74:1415-1418.

Hart, L.P., R. Ward, R. Bafus, and K. Bedford. 1998. Return of an Old Problem: *Fusarium* Head Blight of Small Grains. Proceedings of the National *Fusarium* Head Blight Forum. Michigan State Univ., E. LansingThe American Phytopathological Society.

Hestbjerg, H., G. Felding, and S. Elmholt . 2002. *Fusarium culmorum* infection of barley seedling: correlation between aggressiveness and deoxynivalenol content. Journal of Phytopathology. Vol. 150 (4): 308-312.

Hohn, T., and A. E. Desjardins. 1992. Isolation and gene disruption of the tox-5 gene encoding trichodiene synthase in Gibberellapulicaris. mol. Plant-Microbe Interact. 5: 249-256.

Kimura, M., I. Kaneko, M. Komiyama, A .Takatsuki, H . Koshino, K. Yoneyama, and I. Yamaguchi . 1998. Trichothecene 3-O-acetyltransferase protects both the producing organism and transformed yeast from related mycotoxins. Cloning and characterization of Tri-101. J. Biol. Chem. 273: 1654–1661.

Lindsay, J. A. 1997. Chronic sequelae of food borne disease. Emerg.Infect.Dis. 3:443-452.

Liu, Z. Z., and Z. Y. Wang. 1990. Improved scab resistance in China: Sources of resistance and problems : 178-188. IN: D.A.Saunders, ed., Wheat for the Non-traditional Warm Areas. Proc. Int. Conf., CIMMYT, Mexico, D.F.

Marasas,W. F. O., P. E. Nelson, and T. A. Toussoun. 1984. Toxigenic *Fusarium* species: identify and mycotoxicology.Pennsylvania State University Press, University Park.Pa.

Mathre, D. E. 1997. Compendium of barley diseases. 2nd ed. The Am. Phytopathological Soc. Press, St. Paul, MN.

McCartney, H. A., S. J. Foster, B. A. Fraaije, and E. Ward .2003. Molecular diagnostic for fungal plant pathogens. Pest Manag.Sci.59:129-142.

McCormick, S. P., L. J. Harris, N. J. Alexander, T. Ouellet, A. Saparno, S. Allard, and A. E. Desjardins. 2004. Tri-1 in *Fusarium graminearum* encodes a P450 oxygenase. Appl. Environ. Microbiol. 70: 2044-2051.

McMullen, M. P., J. Enz, J. Lukach, and R. Stover. 1997a. Environmental conditions associated with *Fusarium* head blight epidemics of wheat and barley in the Northern Great Plains, North America. Cereal Res. Commun. 25(3/2) :777-778.

McMullen, M. P., R. Jones, and D. Gallenberg . 1997b. Scab of wheat and barley: A re-emerging disease of devastating impact. Plant Dis. 81:1340-1348.

McMullen, M. P., B. Schatz , R. Stover, and T. Gregoire .1997c. Studies of fungicide efficacy, application timing, and application technologies to reduce *Fusarum* head blight and deoxynivalenol. Cereal Res. Commun. 25(3/2): 779-780.

Mesterházy, Á. ed.1995. Types and components of resistance to *Fusarium* head blight of wheat. Plant Breeding 114:377-386.

Mesterházy, Á. ed. 1997. Breeding for resistance to *Fusarium* head blight of wheat. Proceedings of the 5th European *Fusarium* Seminar. Cereal Res. Commun. 25(3): 231–866.

Moschini, R. C., and C. Fortugno . 1996. Predicting wheat head blight incidence using models based in meteorological factors in Pergamino, Argentina. Eur. J. Plant Pathol. 102:211-218.

Muriuki, J. G. 2001. Deoxynivalenol and nivalenol in pathogenesis of *Fusarium* head blight in wheat. Thesis, University of Minnesota.

Murphy, M., and D. Armstrong. 1995. Fusariosis in patients with neoplastic disease. Infect. Med. 12:66-67.

Neissen, M.L., and R. F. Vogel. 1997. Amolecular approach to the detection of potential trichothecene producing fungi: 245-249.In A.Mesterhazy (ed), Cereals research communication. Proceeding of the Fifth European *Fusarium* Seminer, Szeged, Hungary-1997.Cereals Reasearch Institute, Szeged, Hungary.

Niessen, M. L., and R. F. Vogel. 1998. Group specific PCR-detection of potential trichothecene-producing *Fusarium*–species in pure culture and cereal samples. Syst. Appl. Microbiol.21:618-631.

Parry, D. W., P. Jenkinson, and L. McLeod. 1995. *Fusarium* ear blight (scab) in small grains -a review. Plant Pathol. 44:207-238.

Proctor, R. H., T. M. Hohn, and S. P. McCormick . 1995. Reduced virulence of Gibberellazeae caused by disruption of a trichothecene toxin biosynthetic gene. Mol. Plant–Microbe Interact. 8: 593–601.

Reischer, G. H., M. Lemmens, A. Farnleitner, A. Adler, and R. L. Mach. 2004. Quantification of *Fusarium graminearum* in infected wheat by species-specific real-time PCR applying a TaqMan probe.

Schroeder, H. W., and J. J. Christensen. 1963. Factors

affecting resistance of wheat to scab caused by Gibberellazeae. Phytopathology 53:831-838.

Schwarz, P. B., H. H. Casper, and J. M . Barr . 1995. Survey of the occurrence of deoxynivalenol (vomitoxin) in barley grown in Minnesota North Dakota and South Dakota during 1993. MBAA Tech. Q.32: 190- 194.

Snijders, C. H. A., and C. F. Krechting .1992. Inhibition of deoxynivalenol translocation and fungal colonization in *Fusarium* head blight resistant wheat. Can. J. Bot. 70: 1570-1576.

Snijders, C. H. A., and J. Perkowski .1990. Effects of head blight caused by *Fusarium culmorum* on toxin content and weight of wheat kernels. Phytopathology 80:566-570.

Stack, R. W. 2003. History of *Fusarium* head blight with emphasis on North America. P: 1-34. In K.J. Leonard and W.R. Bushnell (ed.) *Fusarium* head blight of wheat and barley. APS Press, St. Paul, MN.

Strausbaugh, C. A., O. C. Maloy . 1986. *Fusarium* scab of irrigated wheat in Central Washington. Plant Dis. 70:1104-1106.

Tuite, J., G. Shaner, and R. J. Everson. 1990. Wheat scab in soft red winter in Indiana in 1986 and its relation to some quality measurements. Plant Dis.74:959-962.

Van Eeuwijk, F. A., A. Mesterhazy, C. I. Kling, P. Ruckenbauer, L. Saur, H. Burstmayr, M. Lemmens, L. C. P Keizer, N. Maurin, and C. H. A. Snijders, . 1995. Assessing non-specificity of resistance in wheat to head blight caused by inoculation with European strains of *Fusarium culmorum, F. graminearum*, and *F. nivale* using a multiplicative model for interaction. Theor. Appl. Genet. 90:221-228.

Walker, S., S. Leath., W . Hagler, and J. Murphy . 2001. Variation among isolates of *Fusarium graminearum* associated with *Fusarium* head blight in North Carolina. Plant Dis.85:404-410.

Wood, M., D. Comis, D. Harden, L. McGraw, and K. B. Stelljes. 1999. Fighting *Fusarium*. Agricultural Research. June issue. USDA-ARS, Beltsville, MD.