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

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Abstract

This study was carried out on 172 samples of winter wheat. The samples consisted of various cultivars that had been randomly collected from farmers' fields in different areas of Bavaria, South Germany. The objectives of this study were detecting the presence of *Tri-5* gene producing fungus that generates trichothecene mycotoxins, especially Deoxynivalenol (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* infection by Real-Time PCR and estimating the 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 µg kg^{-1}). This study demonstrated that *F. graminearum* is the predominant species associated with *Fusarium* head blight (FHB) and was considered as the predominant trichothecene producer 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-5* gene; Trichothecene DON; Conventional; 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 [1]. Head blight or scab of wheat caused epidemics in many wheat area worldwide [2,3]. The International Maize and Wheat Improvement Centre (CIMMYT) have considered FHB as a major factor limiting wheat production in many parts of the world [2]. FHB is also known as "tombstone" kernels of wheat because of the chalky and lifeless appearance of the infected kernels [4]. It has the capacity to destroy a potentially high-yielding crop within few weeks [5]. FHB is a significant disease of small-grain cereals throughout Europe [3], United States [6], Canada [7], South America [8], Asia [9] and Australia [10]. 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 [11]. It has increased worldwide [12] and it was considered as a major threat to wheat and barley during the early years of the twentieth century [13,14].

FHB is caused by a number of different fungal species of the genus *Fusarium* (*Fusarium* spp). However, *F. avenaceum*, *F. culmorum, F. graminearum* (teleomorph, *Gibberella zeae*), *F. poae*, and *Microdochium nivale* (teleomorph, *Monographella nivalis*) are the species which are most commonly associated with the FHB disease [15].

The threat posed 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 [9]. In addition, grain quality factors such as protein content and germination can be severely affected by the pathogen [16]. 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 [17], while other species are not [18]. DON is the predominant Trichothecenes found in Europe and North America [19]. Trichothecene produced by this fungus pose a serious hazard to human and animal health [10] because these toxic materials are potent inhibitors of eukaryotic protein biosynthesis [20,21]. 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 [22]. 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 [23]. 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 [24,25].

Several genes of *Fusarium* are involved in the biosynthesis of trichothecene and most of them are localized in a *Tri* gene cluster. The *Tri-5* gene encodes the enzyme trichodiene synthase [26], 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 [27]. The nucleotides sequence of the *Tri-5* gene has been characterized in several *Fusarium* species [28,29].

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

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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. culmorum* infection 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

Fungal reference material

50 ng of extracted DNA from *F. graminearum* isolates were applied in tenfold serial dilutions (10^{-1} to 10^{-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, 50 ng of DNA of *F. culmorum* strains were used in tenfold serial dilution also as a quantitative standard for RT-PCR using SYBR Green®1.

Plant material

At harvest time, 172 winter wheat ears 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.

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 mM Tris, 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 were placed 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 4°C. The supernatant was discarded, and the SV Minicolumns were washed with ethanol many times as described by (30). 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 [30].

PCR assay

Two *Tri-5* specific primers have been used to detect the presence of *Tri-5* gene in *F.* 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-5* F: (5'-AGCGACTACAGGCTTCCCTC-3') and reverse primer *Tri-5* R: (5'-AAACCATCCAGTTCTCCATCTG-3'). These primers were derived from the conserved region of *Tri-5* gene in *Fusarium* spp. *Tri-5* primers (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 with 1.5 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 1x Tris-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 ethidium bromide for 100 ml of 1X TAE buffer) and photographed under UV light using a camera and a photo print image visualizer.

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

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′-GTCTCGACAGCAATGGTGTT-3′) and reverse primer FGtubr: (5′-GCTTGTGTTTTTCGTGGCAGT-3′) specifically amplified a 111 bp fragment of the beta-tubulin gene of *F. graminearum* which was quantified by the TaqMan probe FGtubTM (FAM-5′ACAACGGAACGGCACCTCTGAGCTCCAGC3′-TAMRA). PCR was monitored on a Real-Time 7000 Sequence Detection System. PCR Master Mix contained: Hot Start Taq 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, with 1:10 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 as the 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.

Quantitation of *F. culmorum* by Real-Time PCR using DNA binding dye assay: Two specific primers were used for the detection of *F. culmorum* by amplifying 140bp fragment of *F. culmorum*. Forward primer sequence Fc03: (5'-TTCTTGCTAGGGTTGAGGATG-3') and reverse primer sequence Fc02: (5'-GACCTTGACTTTGAGCTTCTTG-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

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 kernels were analyzed using *Tri-5* gene primers in 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-5* gene while 14% of samples didn't show any amplification product (Figure 1).

The same wheat samples were used to estimate the content of DON

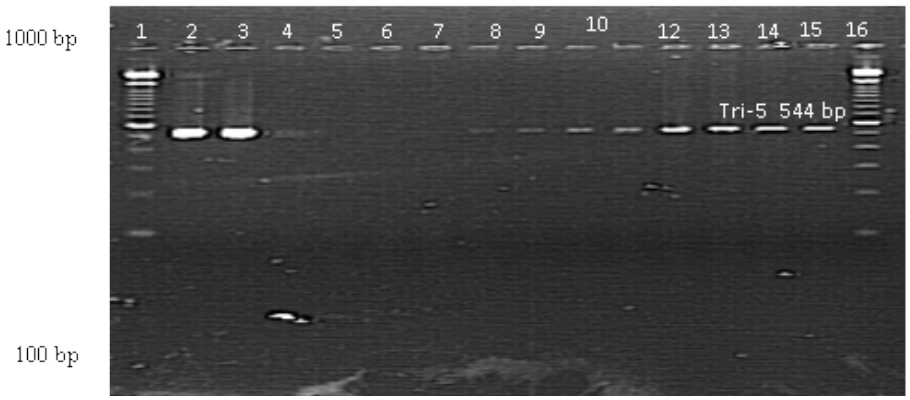

Figure 1: Analysis of DNA samples with specific primers (*Tri-5* gene) on 2% agarose gel.

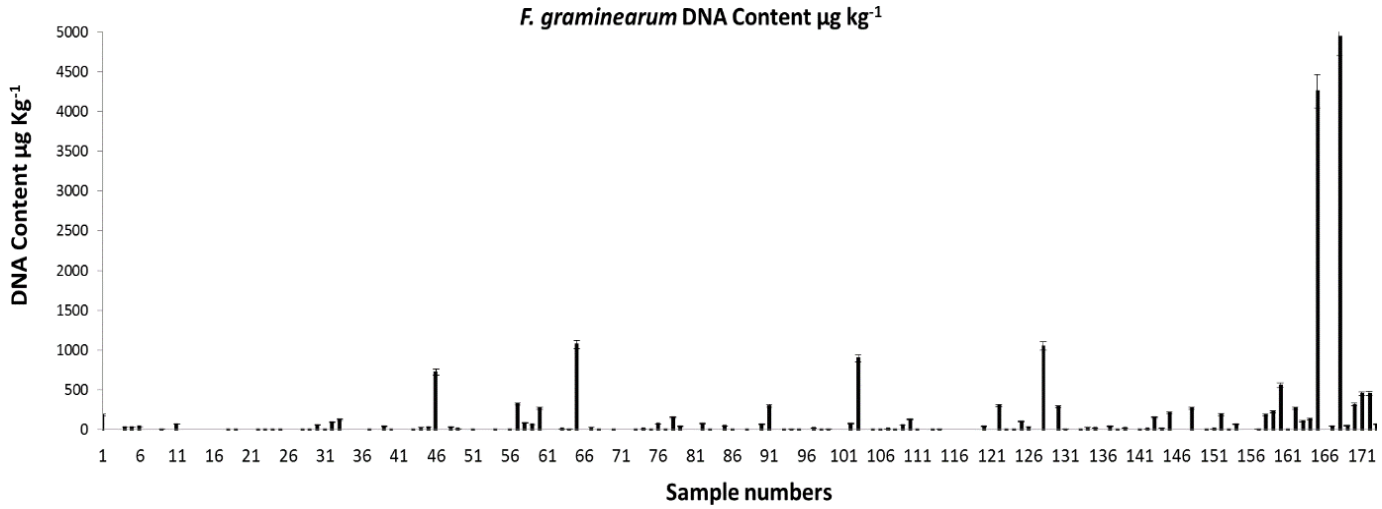

Figure 2: Infestation by *F. graminearum* in wheat samples harvest by Real-Time TaqMan probe. Samples were tested in triplicates.

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-5* gene primers and the DON contents demonstrated that the samples free of *Tri-5* gene 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 11-226 µg 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 (101-2990 µg kg^{-1}) and possessing an intensive DNA band on agarose gels, while 14% of them (27% of all samples) were considered DON low-producing strains (10-99 µg kg^{-1}) and showing a faint DNA band at 544bp.

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.

Quantification of *F. graminearum* and *F. culmorum* using 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 (Figure 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 (Figure 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

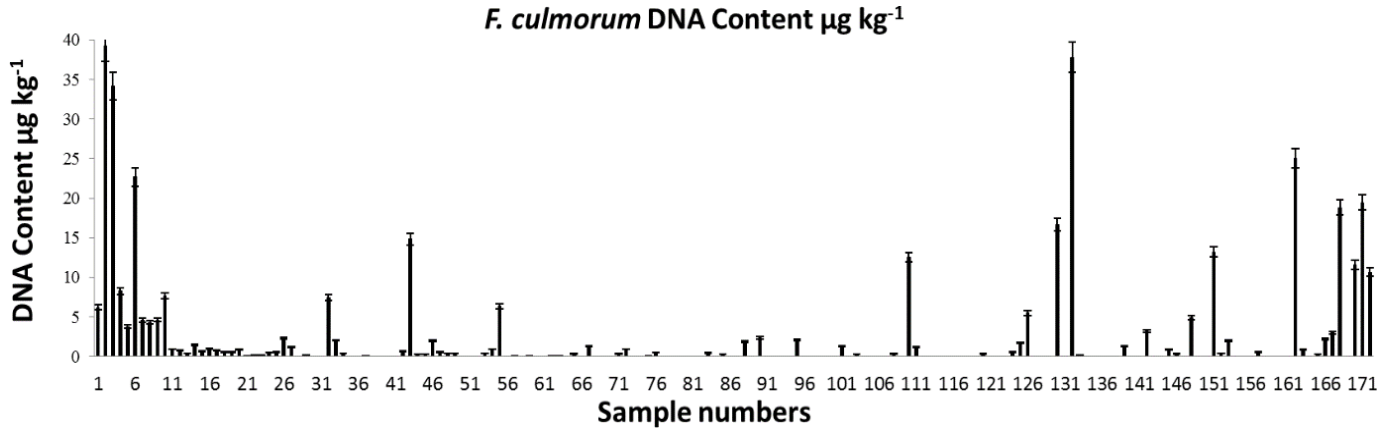

Figure 3: Infestation by *F. culmorum* in wheat samples harvest by Real-Time SYBR Green 1 dye. Samples were tested in triplicates.

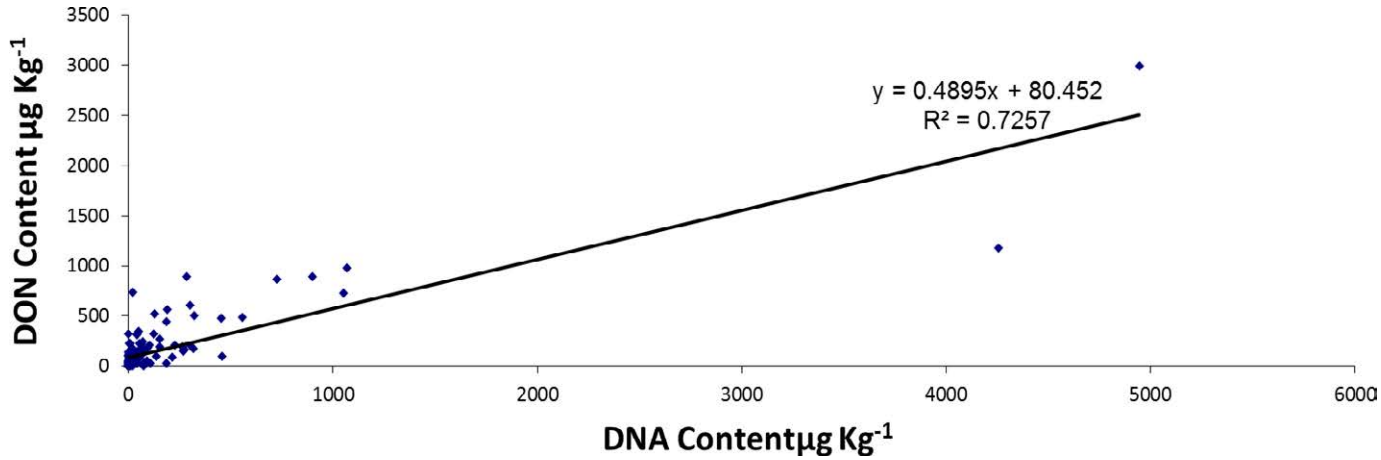

Figure 4: Relationship between DON and *F. graminearum* DNA content in all samples.

amount of *F. graminearum* DNA (4945-100.82 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 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 of absence of *Tri-5* gene products on agarose gels but 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}).

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 (Figure 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).

Correlation between *F. culmorum* DNA and DON content

The plot of DON content against *F. culmorum* DNA standard curve (Figure 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} Figure 6).

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

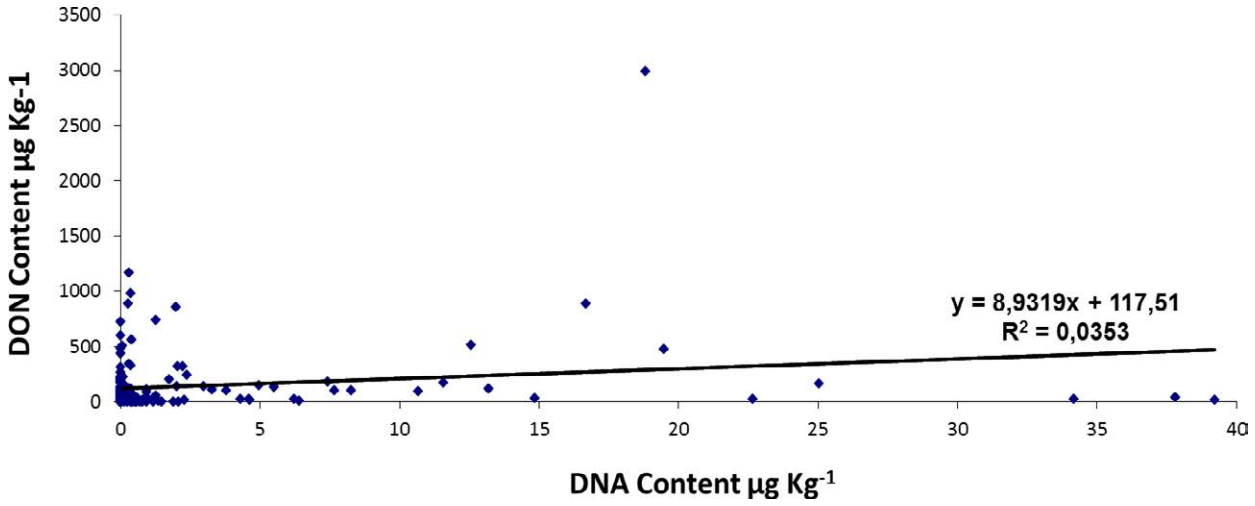

Figure 5: Relationship between DON and *F. culmorum* DNA content in all samples.

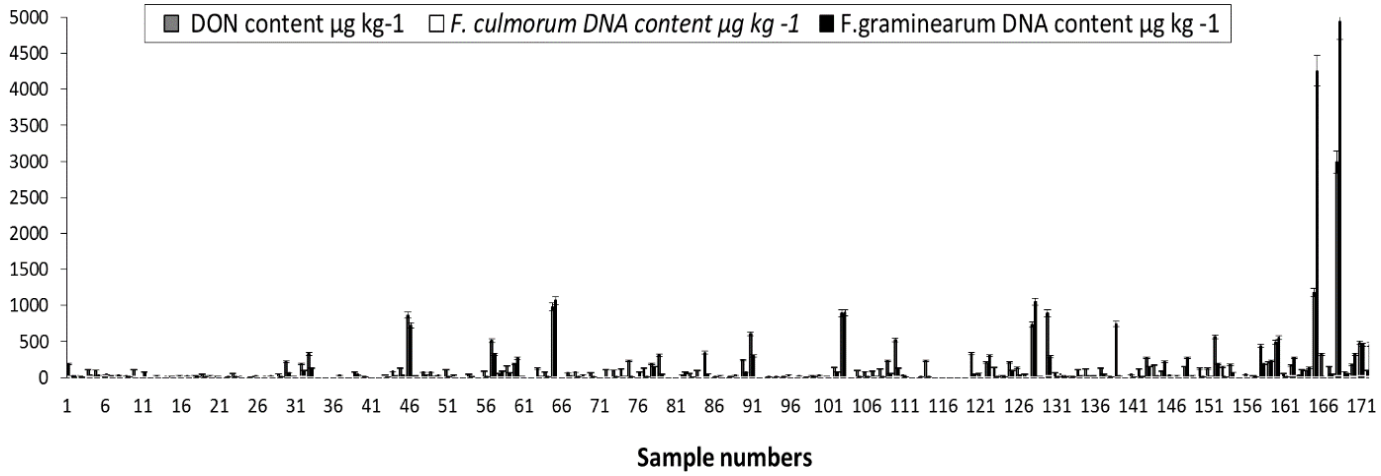

Figure 6: Correlation between DON, *F. graminearum* and *F. culmorum* DNA content in all samples.

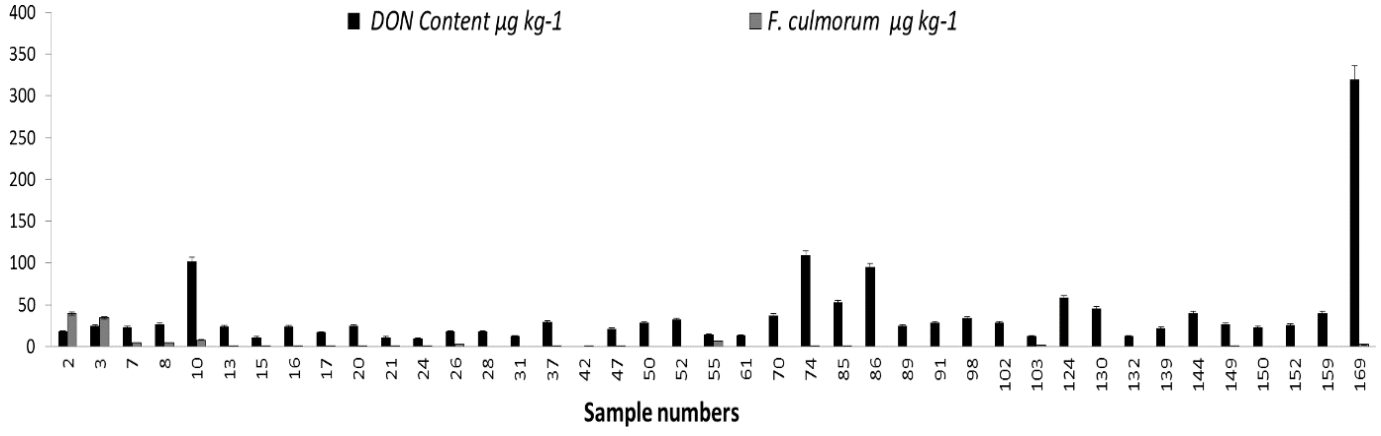

Figure 7: Correlation between DON and *F. culmorum* DNA content in all samples.

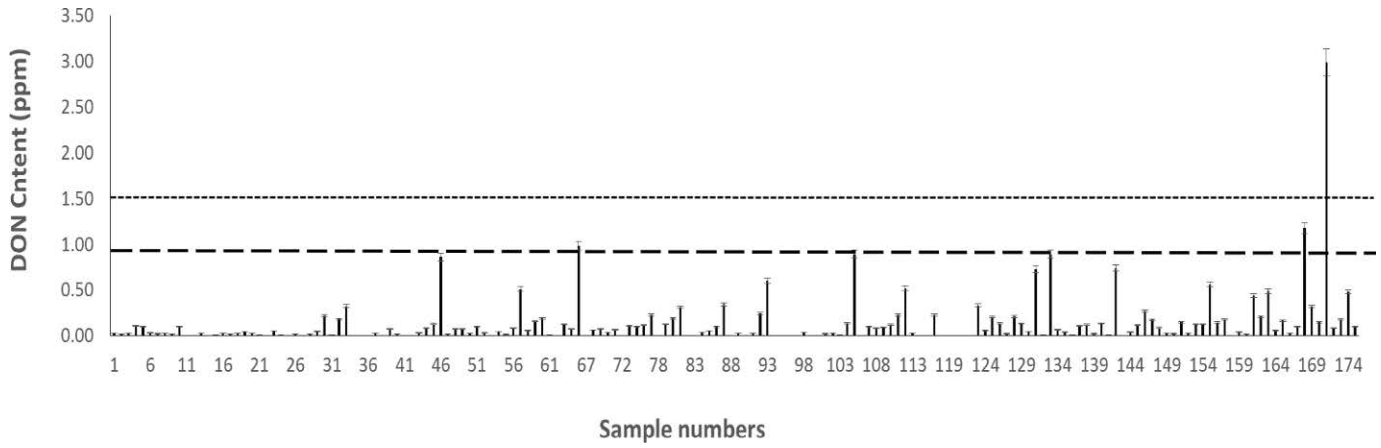

Figure 8: Relationship between DON content and DON advisory level of EU (dotted line) and FDA (broken line) organization.

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

The whole picture of DON contents in 172 infected wheat samples was highlighted in Figures 3-8 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 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

Identification of trichothecene-producing *F.* spp. by PCR

Fusarium species are considered as a potential trichothecene-producing species [15]. The conserved region of *Tri-5* gene has been detected in *F. culmorum* [30,31], *F. graminearum* [32], *F. poae* [21], *F. sporotrichioides* [33], and *F. sambucinum* [7]. There was a direct relationship between *Tri-5* gene expression and the increase in deoxynivalenol production [34,35]. *Tri-5* primers were designed from highly conserved regions of the *Tri-5* gene of *Fusarium* spp. [17]. A *Tri5*-specific PCR assay has been developed to detect trichothecene-producing *Fusarium* species in contaminated wheat samples [36]. 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 agarose gels 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 2990 µg kg^{-1}. 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 [37]. 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-5* DNA bands on agarose gels and 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 of high 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 Figure 6). On the other hand, faint *Tri-5* DNA bands on agarose gels (27% of all tested samples) indicated samples containing a low concentration of *Tri-5* gene and as a result, the infection with *F.*spp. was also low. Accordingly, the amount of DON content in these samples was very 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, *F.*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 *F.* 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 [38-42]. 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 11 to 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 [43] and *Tri*101 [44] 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 [45]. 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 HPLC analysis 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.

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

Molecular diagnostic of plant pathogenic fungi can be highly specific, very sensitive, and relatively fast [46]. 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 TaqMan hybridization probe targeting the beta-tubulin gene for *F. graminearum* and SYBR Green 1 for *F. culmorum*. TaqMan method used in this study because of its sensitivity, selectivity, and reduction of fault signals due to primer-dimer formation [46] 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 [46]. 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 [47]. 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 while the severity of *F. culmorum* infection was not high in wheat kernels. Comparison between the results of TaqMan 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 wheat crop. This is in accordance with former investigations [17]. In contrast, the slight weak correlation (R^2=0.03) between DON and *F. culmorum* DNA content may reflect that *F. culmorum* was the second important species in the DON producing *Fusarium* genus. Previous study suggested that *F. culmorum* along with *F. graminearum* were consistently the most pathogenic of the *Fusarium* species infecting cereal ears [12].

Moreover, the PCR analysis showed that in 20% of total infected samples, DON was found (10-320 μg 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} Figure 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 ref. [48]. 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 over all Bavaria since the DON content was generally low (10-2990 μg kg^{-1} Figure 6). Indeed, the aggressiveness of *Fusarium* was not so high where only 2% of all harvested wheat kernel laid above the FDA DON advisory level of 1 ppm, and only one sample was higher than the EU threshold of 1.25 ppm. Some authors reported that trichothecenes may play an important role in the aggressiveness of fungi towards plant host [49].

References

1. Dickson JG, Mains EB (1929) Scab of wheat and barley and its control. USDA Farmers Bulletin 1599. pp: 1-18.
2. Dubin HJ, Gilchrist L, Reeves J, McNab A (1997) Fusarium head scab: Global status and prospects. CIMMYT, Mexico, DF, Mexico, p. 130.
3. McMullen MP, Jones R, Gallenberg D (1997b) Scab of wheat and barley: A re-emerging disease of devastating impact. Plant Dis 81: 1340-1348.
4. Tuite J, Shaner G, Everson RJ (1990) Wheat scab in soft red winter in Indiana in 1986 and its relation to some quality measurements. Plant Dis 74: 959-962.
5. McMullen MP, Enz J, Lukach J, Stover R (1997a) Environmental conditions associated with Fusarium head blight epidemics of wheat and barley in the Northern Great Plains, North America. Cereal Res Commun 25: 777-778.
6. Liu ZZ, Wang ZY (1990) Improved scab resistance in China: Sources of resistance and problems. In: Saunders DA (ed) Wheat for the Non-traditional Warm Areas. Proc Int. Conf., CIMMYT, Mexico, D.F, pp. 178-188.
7. Hart LP, Ward R, Bafus R, Bedford K (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.
8. Hanson EW, Ausemus ER, Stakman EC (1950) Varietal resistance of spring wheats to fusarial head blight. Phytopathology 40: 902-914.
9. Mathre DE (1997) Compendium of barley diseases. (2nded) The Am Phytopathological Soc Press, St. Paul, MN.
10. Bechtel DB, Kaleikau LA, Gaines RL, Seitz LM (1985) The effects of Fusarium graminearum infection on wheat kernels. Cereal Chem 62: 191-197.
11. Wood M, Comis D, Harden D, McGraw L, Stelljes KB (1999) Fighting Fusarium. Agricultural Research. June issue. USDA-ARS, Beltsville, MD.
12. Parry DW, Jenkinson P, McLeod L (1995) Fusarium ear blight (scab) in small grains -a review. Plant Pathol 44: 207-238.
13. Muriuki JG (2001) Deoxynivalenol and nivalenol in pathogenesis of Fusarium head blight in wheat. Thesis, University of Minnesota.
14. Stack RW (2003) History of Fusarium head blight with emphasis on North America. In: Leonard KJ, Bushnell WR (ed) Fusarium head blight of wheat and barley. APS Press, St. Paul, MN, pp: 1-34.
15. Edwards SG, Pirgozliev SR, Hare MC, Jenkinson P (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: 1575-1580.
16. Schwarz PB, Casper HH, Barr JM (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.
17. McMullen MP, Schatz B, Stover R, Gregoire T (1997c) Studies of fungicide efficacy, application timing, and application technologies to reduce Fusarum head blight and deoxynivalenol. Cereal Res. Commun. 25: 779-780.
18. Marasas WFO, Nelson PE, Toussoun TA (1984) Toxigenic Fusarium species: identify and mycotoxicology. Pennsylvania State University Press, University Park. Pa.
19. Bottalico A, Perrone G (2002) Toxigenic Fusarium species and mycotoxins associated with head blight in small grain cereals in Europe. European Journal of Plant Pathology 108: 611-624.
20. Boyacioglu D, Hettiarachchy NS, Stack RW (1992) Effect of three systemic fungicides on deoxynivalenol (vomitoxin) production by Fusarium graminearum in wheat. Can J Plant Sci 72: 93-101.
21. van Eeuwijk FA, Mesterhazy A, Kling CI, Ruckenbauer P, Saur L, et al. (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.
22. Bennett JW, Klich M (2003) Mycotoxins. Clin Microbiol Rev 16: 497-516.

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

24. Berek L, Petri IB, Mesterházy A, Téren J, Molnár J (2001) Effects of mycotoxins on human immune functions in vitro. Toxicol In Vitro 15: 25-30.

25. Lindsay JA (1997) Chronic sequelae of foodborne disease. Emerg Infect Dis 3: 443-452.

26. Bai G, Kolb FL, Shaner G, Domier LL (1999) Amplified fragment length polymorphism markers linked to a major quantitative trait locus controlling scab resistance in wheat. Phytopathology 89: 343-348.

27. Niessen ML, Vogel RF (1998) Group specific PCR-detection of potential trichothecene-producing Fusarium-species in pure cultures and cereal samples. Syst Appl Microbiol 21: 618-631.

28. Fekete C, Logrieco A, Giczey G, Hornok L (1997) Screening of fungi for the presence of the trichodiene synthase encoding sequence by hybridization to the Tri5 gene cloned from Fusarium poae. Mycopathologia 138: 91-97.

29. Hohn TM, Desjardins AE (1992) Isolation and gene disruption of the Tox5 gene encoding trichodiene synthase in Gibberella pulicaris. Mol Plant Microbe Interact 5: 249-256.

30. Bauer A, Seigner L, Battner P, Tischner H (2004) Monitoring of FHB using PCR for qualitative and quantitative detection of Fusarium spp. Proceedings of the 2nd International Symposium on Fusarium Head Blight 2: 553.

31. Snijders CHA, Krechting CF (1992) Inhibition of deoxynivalenol translocation and fungal colonization in Fusarium head blight resistant wheat. Can J Bot 70: 1570-1576.

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

33. Hart LP, Pestka JJ, Liu MT (1984) Effect of kernel development and wet periods on production of deoxynivalenol in wheat infected with Gibberella zeae. Phytopathology 74: 1415-1418.

34. Fernando WG, Paulitz TC, Seaman WL, Dutilleul P, Miller JD (1997) Head Blight Gradients Caused by Gibberella zeae from Area Sources of Inoculum in Wheat Field Plots. Phytopathology 87: 414-421.

35. Doohan FM, Weston G, Rezanoor HN, Parry DW, Nicholson P (1999) Development and use of a reverse transcription-PCR assay to study expression of Tri5 by Fusarium species in vitro and in planta. Appl Environ Microbiol 65: 3850-3854.

36. Neissen ML, Vogel RF (1997) A molecular approach to the detection of potential trichothecene producing fungi. In: Mesterhazy A (ed) Cereals research communication. Proceeding of the Fifth European Fusarium Seminer, Szeged, Hungary-1997.Cereals Reasearch Institute, Szeged, Hungary. pp. 245-249.

37. Abramson D, Clear RM, Smith DM (1993) Trichothecene production by Fusarium spp. isolated from Manitoba grain. Can J Plant Pathol 15: 147-152.

38. Mesterhazy A (1995) Types and components of resistance to Fusarium head blight of wheat. Plant Breeding 114: 377-386.

39. Mesterhzy A (1997) Breeding for resistance to Fusarium head blight of wheat. Proceedings of the 5th European Fusarium Seminar. Cereal Res Commun 25: 231-866.

40. Schroeder HW, Christensen JJ (1963) Factors affecting resistance of wheat to scab caused by Gibberella zeae. Phytopathology 53: 831-838.

41. Snijders CHA, Perkowski J (1990) Effects of head blight caused by Fusarium culmorum on toxin content and weight of wheat kernels. Phytopathology 80: 566-570.

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

43. McCormick SP, Harris LJ, Alexander NJ, Ouellet T, Saparno A, et al. (2004) Tri1 in Fusarium graminearum encodes a P450 oxygenase. Appl Environ Microbiol 70: 2044-2051.

44. Kimura M, Kaneko I, Komiyama M, Takatsuki A, Koshino H, et al. (1998) Trichothecene 3-O-acetyltransferase protects both the producing organism and transformed yeast from related mycotoxins. Cloning and characterization of Tri101. J Biol Chem 273: 1654-1661.

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

46. McCartney HA, Foster SJ, Fraaije BA, Ward E (2003) Molecular diagnostics for fungal plant pathogens. Pest Manag Sci 59: 129-142.

47. Reischer GH, Lemmens M, Farnleitner A, Adler A, Mach RL (2004) Quantification of Fusarium graminearum in infected wheat by species specific real-time PCR applying a TaqMan Probe. J Microbiol Methods 59: 141-146.

48. Doohan M F, Parry DW, Jenkinson P, Nicholson P (1998) The use of species-specific PCR based assays to analyse Fusarium ear blight of wheat 47: 19-205.

49. Proctor RH, Hohn TM, McCormick SP (1995) Reduced virulence of Gibberella zeae caused by disruption of a trichothecene toxin biosynthetic gene. Mol Plant Microbe Interact 8: 593-601.