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Real-time PCR for quantification of toxigenic *Fusarium* species in barley and malt

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Abstract

A real-time PCR technique was applied for the quantification of trichothecene-producing *Fusarium* species (TMTRI assay) as well as the highly toxigenic *Fusarium graminearum* (TMFg12 assay) present in barley grain and malt. PCR results were compared to the amounts of trichothecenes detected in the samples to find out if the PCR assays can be used for trichothecene screening instead of expensive and laborious chemical analyses. DNA was extracted from ground kernels using a commercial DNA extraction kit and analysed in a LightCycler® system using specific primers and fluorogenic TaqMan probes. Both naturally and artificially contaminated grains were analysed. The TMTRI assay and the TMFg12 assay enabled the quantification of trichothecene-producing *Fusarium* DNA and *F. graminearum* DNA present in barley grain and malt samples, respectively. Both TaqMan assays were considered to be sensitive and reproducible. Linearity of the assays was 4–5 log units when pure *Fusarium* DNAs were tested. The amount of *Fusarium* DNA analysed with the TMTRI-trichothecene assay could be used for estimation of the deoxynivalenol (DON) content in barley grain. Furthermore, the TMFg12 assay for *F. graminearum* gave a good estimation of the DON content in north American barley and malt samples, whilst the correlation was poor among Finnish samples. DON content and the level of *F. graminearum* DNA were found to be naturally low in Finnish barleys.

Introduction

Fusarium species are potential mycotoxin producers in cereals (Desjardins et al., 1993). The predominant *Fusarium* mycotoxins found in cereals are trichothecenes, a group of closely related sesquiterpenoids, among which deoxynivalenol (DON) is the most frequently detected world-wide (Langseth et al., 1999). In the near future, the European Union will apply maximum levels for *Fusarium* toxins in unprocessed cereals and cereal products. According to Commission Regulation (EC) No 856/2005 (2005), the maximum level for DON will be 1250 ppb in unprocessed cereals other than durum wheat, oats and maize.

Chemical mycotoxin analyses of cereals are often expensive and time-consuming. Moreover, the mycotoxin content of raw cereals may change during processing. For example, *Fusarium* fungi are able to proliferate and produce mycotoxins during malting (Schwarz et al., 1995; Munar and Sebree 1997; Sarlin et al., 2005). Currently, fusaria in malting barley are mainly assessed by analyzing the percentage of kernels contaminated (EBC Analytica Microbiologica, 2005). This plating method is laborious and time-consuming, and no

information is obtained concerning toxigenic species. Hence, a rapid and simple quantification method for toxigenic *Fusarium* species is needed in order to be able to evaluate the mycotoxin risk in cereals used in the cereal-based industry.

PCR offers a sensitive, specific, fast and high-throughput method to detect, identify and quantify fungal species in grains. PCR assays developed for *Fusarium* species were reviewed by Nicholson et al. (2003). More recently, real-time quantitative PCR assays with fluorogenic sequence-specific probes for fusaria have been developed (Bluhm et al., 2004; Reischer et al., 2004; Waalwijk et al., 2004; Klemsdal et al., 2006; Yli-Mattila et al., 2006). Real-time PCR provides a means for detection and quantification of DNA targets by monitoring PCR product accumulation during the thermal cycling as indicated by increased fluorescence. Real-time PCR enables simpler and more rapid analysis of data, and has higher sensitivity and a wider dynamic range compared to end-point PCR (McKillip and Drake, 2004). Furthermore, the use of fluorogenic sequence-specific probes enhances specificity. In this study, real-time PCR assays with TaqMan probes were applied for quantification of trichothecene-producing *Fusarium* species as well as *F. graminearum* in barley grain and malt. *Fusarium* DNA levels were compared to mycotoxin content in order to assess the ability of the real-time PCR assays to identify grain samples with a high risk of exceeding the maximum DON limit proposed by the EC.

Materials and methods

Fungal strains

The fungal isolates used in this study were obtained from the VTT Culture Collection and are shown in Table 1. The strains belonged to the genera *Fusarium*, *Alternaria, Aspergillus*, *Cochliobolus* and *Penicillium*, which are common fungal contaminants in barley.

Grain samples

Both naturally and artificially *Fusarium*-contaminated barley and malt samples were analysed. Natural Finnish malting barley samples ($n = 20$) from the crop year 2003 were kindly provided by Polttimo Companies Ltd, Finland and Raisio Nutrition Ltd., Raisio Malt, Finland. Finnish barley samples from the crop years 2002 ($n = 5$) and 2003 ($n = 10$) were also obtained from the

Table 1. Specificities of TaqMan real-time PCR assays for the detection of trichothecene-producing *Fusarium* species (TMTRI) and *F. graminearum* (TMFg12)

Species	Strain	Trichothecene-producer	TMTRI	TMFg12
F. culmorum	VTT D-80148	Yes	+	–
F. graminearum	VTT D-82082	Yes	+	+
F. graminearum	VTT D-82086	Yes	+	+
F. graminearum	VTT D-82169	Yes	+	+
F. graminearum	VTT D-95472	Yes	+	+
F. poae	VTT D-76038	Yes	+	–
F. poae	VTT D-82182	Yes	+	–
F. sporotrichioides	VTT D-72014	Yes	+	–
F. equiseti	VTT D-82087	Some reports exist	–	–
F. sambucinum	VTT D-77056	Some reports exist	–	–
F. solani	VTT D-77057	Some reports exist	–	–
F. avenaceum	VTT D-80141	No	–	–
F. oxysporum	VTT D-80134	No	–	–
F. oxysporum	VTT D-98690	No	–	–
F. tricinctum	VTT D-96600	No	–	–
Cochliobolus sativus	VTT D-76039	No	–	–
Alternaria alternata	VTT D-76024	No	–	–
Aspergillus ochraceus	VTT D-00808	No	–	–
Penicillium chrysogenum	VTT D-96661	No	–	–

+ : <35 cycles required for threshold value.
–: No amplification or ≥35 cycles required for threshold value.

Plant Production Inspection Centre (KTTK), Finland. North American barley and malt samples ($n = 12$) were obtained from the USA and Canada. In addition, artificial inoculation was performed as described by Pekkarinen et al. (2003) by spraying field-grown barley with spores of *Fusarium culmorum* VTT D-80148, *F. graminearum* (teleom. *Gibberella zeae*) VTT D-95470 or *F. poae* VTT D-82182 during the heading stage of barley ($n_{total} = 16$).

DNA extraction

All the fungal strains were grown on potato dextrose broth (Difco Laboratories, Detroit, USA) at 25 °C for 3–4 days. Mycelium for genomic DNA extraction was harvested by filtration and subsequently freeze-dried. Grain samples (10–20 g) were ground with a Universal Laboratory Disc Mill DLFU (Bühler-Miag GmbH, Braunschweig, Germany). DNA was extracted from freeze-dried mycelia and ground kernels (0.1 g) using FastDNA® Spin Kit for Soil (Qbiogene, Carlsbad, USA) according to the manufacturer's instructions but using a modified lysing step. The lysing step was performed in a 2 ml tube with Lysing Matrix A supplemented with an extra ceramic sphere (FastDNA® Kit, Qbiogene) using a FastPrep® Cell Disrupter, model FP120 (Qbiogene) for 2 min at 6.0 m s^{-1}. To evaluate the repeatability of the DNA extraction method, the DNA extraction from nine selected grain samples was repeated.

TaqMan assays

The primers and probes were derived from DNA fragments specific either to trichothecene-producing *Fusarium* species (TMTRI, Klemsdal et al., 2006) or to *F. graminearum* (TMFg12, Yli-Mattila et al., 2006). The fluorogenic, sequence-specific TaqMan probes were used to generate the fluorescence signal in a LightCycler® system (Roche Diagnostics GmbH, Germany). The probes were labelled at the 5′ end with the reporter dye FAM (6–carboxy-fluorescein) and at the 3′ end with the quencher dye TAMRA (6-carboxy-tetramethyl rhodamine). The primers and probes were purchased from Sigma-Genosys. For each reaction 2 μl of DNA sample was mixed with 18 μl of PCR reaction mix containing 3 mM $MgCl_2$, 0.5 μM primers, 0.2 μM probe and 2 μl of LightCycler – FastStart DNA Master Hybridization Probes mix (Roche Diagnostics GmbH). The thermal cycling protocol was as follows: initial denaturation 95 °C, 10 min, amplification $45 \times$ (95 °C, 15 s; 60 °C, 60 s), cooling 40 °C, 30 s. For quantification, a standard curve was generated using a serial dilution of *F. poae* D-82182 DNA (TMTRI assay) or *F. graminearum* D-95470 DNA (TMFg12 assay) extracted from pure fungal cultures and quantified spectrophotometrically. The barley and malt samples were run in duplicate. The specificity of the PCR assays was tested against genomic DNA of 19 fungal strains representing 14 different species including also non-trichothecene producers used as negative controls (Table 1). In addition, DNA extracted from barley tissue grown under sterile condition was used as a negative control. Unspecific amplification may occur during the late PCR cycles. In order to minimise wrong positive results, a PCR reaction was considered positive only if the CT-value was <35. To determine the sensitivity and linear range of the PCR assays, tenfold dilutions of genomic DNA from *Fusarium culmorum* VTT D-80148, *F. graminearum* VTT D-95470, *F. poae* VTT D-82182 and *F. sporotrichioides* VTT D-72014 were analysed in triplicate. Besides PCR grade water *F. graminearum* DNA was also serially diluted in barley DNA extracted from kernels with low *Fusarium* contamination.

Mycological analyses

The number of *Fusarium*-infected kernels in barley samples was assessed by plating 100 kernels on a wet filter paper (EBC Analytica Microbiologica, 2005). The results were reported as the percentage of kernels infected with fusaria. *Fusarium* contamination rate was also assessed by analyzing *Fusarium* counts (colony forming units, CFU) in barley kernels by plating homogenized sample slurries on yeast malt agar plates (Difco, Detroit, USA) supplemented with 0.01% chloramphenicol, 0.01% chlorotetracycline and 0.02% Triton X. Colonies were counted after 7 days of incubation at 25 °C.

Mycotoxin analyses

Trichothecenes deoxynivalenol (DON), 3-acetyl deoxynivalenol (3-AcDON), nivalenol (NIV), diacetoxyscirpenol (DAS), fusarenon-X (FX), T-2 and HT-2 toxins were analyzed by gas

chromatography–mass spectrometry (GC-MS) with electron impact ionization as described by Eskola et al. (2001). The limit of quantification for DON, 3AcDON, FX and DAS was 10 ppb, for NIV 30 ppb and for T-2 and HT-2 toxins 20 ppb. In addition, DON was determined using the EZ-Quant High Sensitivity Deoxynivalenol (DON) Plate Kit (Diagnostix, Canada) according to the manufacturer's instructions. The detection limit of the DON kit was 25 ppb.

Statistical analysis

For statistical analysis of the correlation between *Fusarium* DNA and DON levels the Microsoft Excel data analysis tool, Regression, was used to calculate a square of the correlation coefficient (r^2) and a significance of correlation (*P*-value).

Results

Specificity and sensitivity of the TaqMan assays

The specificity of the TaqMan assays was tested against 15 *Fusarium* strains and one strain each of *Alternaria alternata*, *Aspergillus ochraceus*, *Cochliobolus sativus* (SynA *Helminthosporium sativum*, Anam. *Bipolaris sorokiniana*) and *Penicillium chrysogenum*. With the TMFg12 assay only the four *F. graminearum* strains were detected, indicating that the assay was highly specific for *F. graminearum* (Table 1). Strains belonging to the species *F. culmorum*, *F. graminearum*, *F. sporotrichioides* and *F. poae* gave a strong fluorescence signal within 35 cycles in the LightCycler® system when the TMTRI-trichothecene assay was applied (Table 1). No signal or only a weak signal (threshold cycle CT >35) was detected with the other strains. Linear ranges of the TMTRI and the TMFg12 assays were four orders of magnitude (from approx. 6 pg to 60 ng of *F. poae* DNA) and five orders of magnitude (from approx. 0.6 pg to 60 ng of *F. graminearum* DNA), respectively (Figure 1). The correlation between the CT-values and known DNA quantities was high for both assays, r^2 (TMTRI) = 0.9922 and r^2 (TMFg12) = 0.9862, and the coefficient of variation was at most 5% (Figure 1). The slopes of the standard curves in the TMTRI assay were similar when the dilution series were repaired from DNA extracted from *F. culmorum*, *F. graminearum* and *F. sporotrichioides*, although some differences in sensitivity were detected (data not shown). Klemsdal et al. (2006) also reported that the standard curve in the TMTRI assay was similar between *F. culmorum*, *F. graminearum*, *F. langsethiae*, *F. poae* and *F. sporotrichioides* DNA dilution series. When the same diluted *F. graminearum* DNA samples were analysed with both PCR assays, almost identical

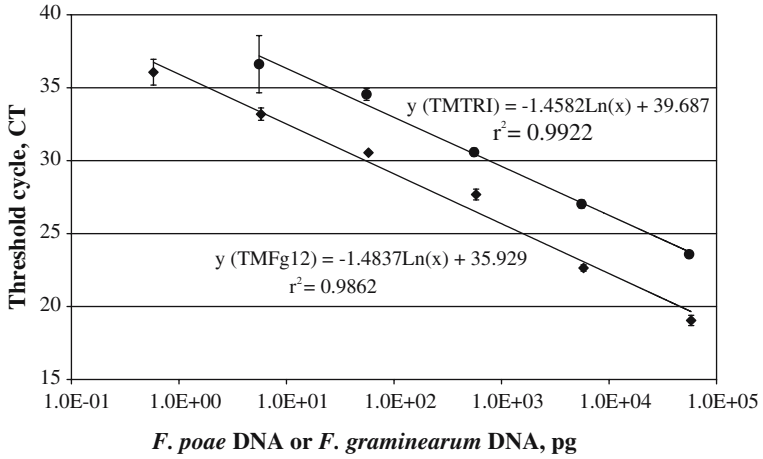

Figure 1. Standard curves of the TMTRI (●) and TMFg12 (♦) assays used for quantification of trichothecene-producing *Fusarium* DNA and *F. graminearum* DNA, respectively. Cycle thresholds (CT) were plotted against the DNA levels of *F. poae* or *F. graminarum* expressed on a logarithmic scale. The standard curves are results of 3 separate PCR analyses. The error bars indicate standard deviations.

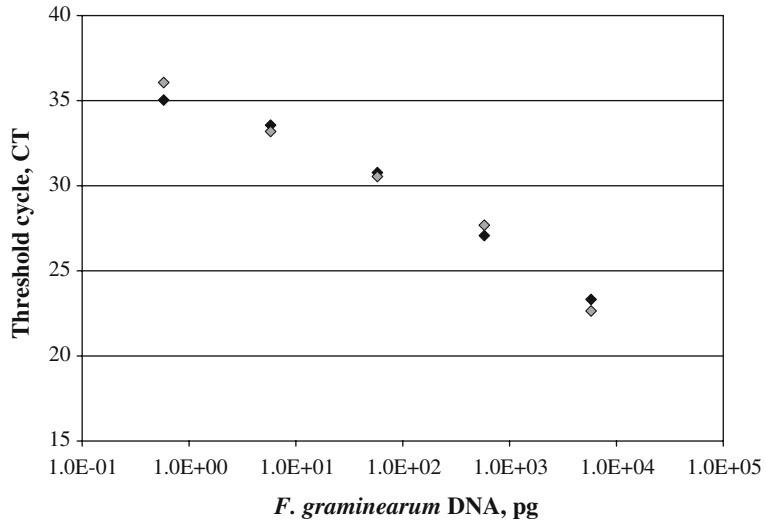

Figure 2. Standard curves obtained from *F. graminearum* DNA samples with (♦) or without (◈) barley DNA analysed by the TMFg12 assay. Cycle thresholds (CT) were plotted against the known amount of *F. graminarum* DNA expressed on a logarithmic scale.

standard curves were obtained, indicating that the amplification efficiencies of the assays were equal (data not shown). DNA from barley grain did not affect the sensitivity or linear range of the TMFg12 assay (Figure 2). The result was similar with the TMTRI assay (data not shown). In addition, no amplification occurred when sterile barley DNA was analysed by the assays. When the CT-values of the DNA samples extracted from barley kernels in duplicate were compared, a standard deviation of the analysis was on average ± 0.5 CT (data not shown).

Correlation between the amount of Fusarium *DNA, DON level and* Fusarium *count in barley grain and malt*

A correlation was found between the amount of trichothecene-producing *Fusarium* DNA analysed by the TMTRI assay and the DON level in barley grain and malt samples. The correlation was highly significant among north American barley and malt samples ($r^2 = 0.936$; $P < 0.001$) as well as among artificially *Fusarium*-inoculated barley samples ($r^2 = 0.808$; $P < 0.001$), but was distinctly lower among natural Finnish barley samples ($r^2 = 0.242$; $P < 0.01$) (Figure 3). Most of the natural Finnish samples contained low amount of DON (<100 ppm). The correlation was also significant when the results of all the samples were combined ($r^2 = 0.731$; $P < 0.001$). The results revealed that all samples with DON levels above the EC tentative DON limit, 1250 ppb, contained trichothecene-producing *Fusarium* DNA more than 1000 ng g^{-1} of grain (Figure 3). Only three samples with DON levels under the tentative DON limit contained trichothecene-producing *Fusarium* DNA more than 1000 ng g^{-1} of grain.

The TMFg12 assay provided a good estimation of the DON level in north American barley and malt samples ($r^2 = 0.956$; $P < 0.001$), whereas the correlation was not evident with natural Finnish barley samples ($r^2 = 0.171$; $P < 0.05$) (Figure 4). The results suggested that north American samples having an initial *F. graminearum* DNA concentration above 500 ng g^{-1} of grain have a high risk of containing DON over the proposed EC limit, 1250 ppb (Figure 4). The amount of *F. graminearum* DNA in Finnish barley grain was lower than in the samples from the USA and Canada. When the PCR results of the north American grain samples analysed with both TaqMan assays were compared, the corresponding DNA levels were only slightly higher in the TMTRI assay than in the TMFg12 assay (data not shown). Hence, our result implies that most of the trichothecene-producers present in the north American samples belonged to the species *F. graminearum*.

Trichothecene-producing *Fusarium* DNA levels analysed with the TMTRI assay were compared to

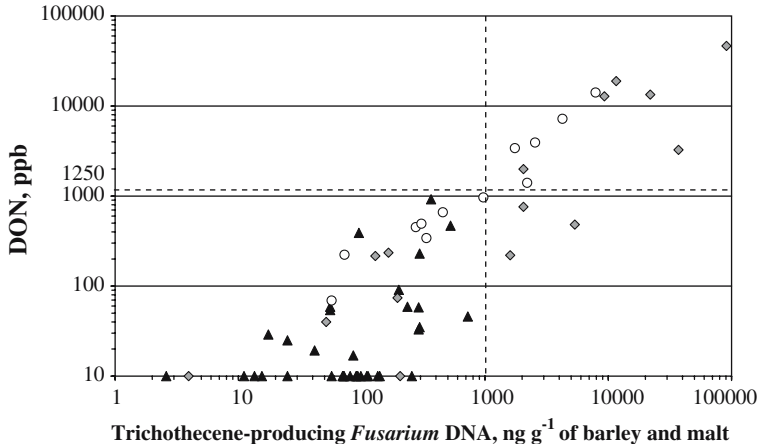

Figure 3. Correlation between DNA levels analysed with the TMTRI assay and DON content in barley and malt expressed on a logarithmic scale. Samples ($n = 63$): Finnish barley grain samples naturally (▲) and artificially (◆) contaminated with fusaria, north American barley and malt samples (o). The maximum level (1250 ppb) for DON in unprocessed cereals proposed by the EC is also shown.

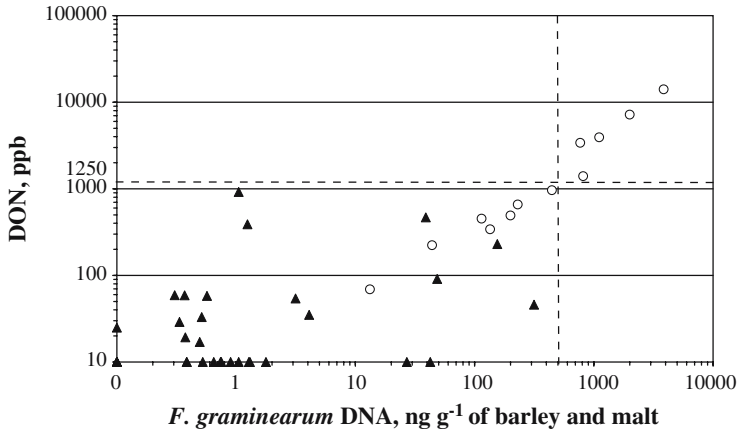

Figure 4. Correlation between *F. graminearum* DNA levels analysed with the TMFg12 assay and DON content in north American barley and malt samples (o), and in Finnish natural barley samples (▲) expressed on a logarithmic scale. The maximum level (1250 ppb) for DON in unprocessed cereals proposed by the EC is also shown.

Fusarium counts (CFU g^{-1}) and contamination rates (%) of the barleys artificially inoculated with *F. culmorum, F. graminearum* and *F. poae*. The correlation between the DNA levels and *Fusarium* counts was evident ($r^2 = 0.868$; $P < 0.001$), whereas there was no correlation between the DNA levels and the percentages of *Fusarium*-contaminated kernels (Figures 5 and 6).

Discussion

The TMFg12 assay was highly specific for *F. graminearum*, amplifying DNA only from the *F. graminearum* strains. The TMTRI assay for trichothecene-producers amplified DNA from the strains belonging to the species *F. culmorum*, *F. graminearum*, *F. sporotrichioides* and *F. poae*. These species are well known trichothecene-producers in cereals (Desjardins et al., 1993). No amplification within 35 cycles occurred from DNA samples of species *F. avenaceum*, *F. equiseti*, *F. oxysporum*, *F. sambucinum*, *F. solani* and *F. tricinctum* as well as from DNA of the other field fungi studied. *Fusarium equiseti*, *F. sambucinum* and *F. solani* have been reported to be able to produce trichothecenes (El-Banna et al., 1984; Bosch and

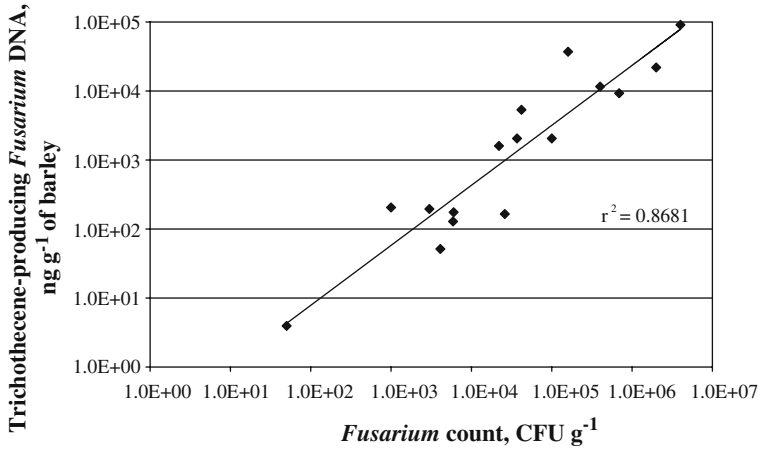

Figure 5. Correlation between DNA levels analysed with the TMTRI assay and *Fusarium* counts (CFU g^{-1}) in barley samples artificially inoculated with *F. culmorum*, *F. graminearum* and *F. poae*. The results are expressed on a logarithmic scale.

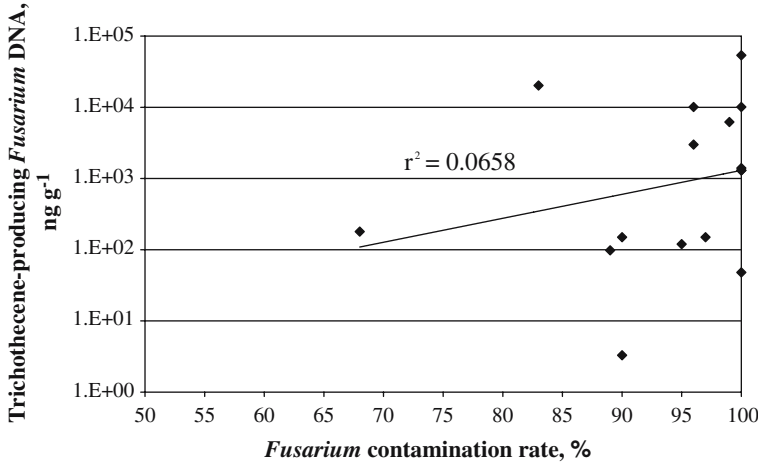

Figure 6. Correlation between DNA levels analysed with the TMTRI assay and *Fusarium* contamination rates (%) in barley samples artificially inoculated with *F. culmorum*, *F. graminearum* and *F. poae*.

Mirocha, 1992; Langseth et al., 1999; Logrieco et al., 2003), but they are not regarded as the main trichothecene-producers in cereals (Bottalico and Perrone, 2002). *Fusarium avenaceum*, *F. oxysporum* as well as *F. tricinctum* are regarded as non-trichothecene-producers (Logrieco et al., 2003).

Real-time PCR with specific primers and TaqMan probes enabled the specific quantification of trichothecene-producing *Fusarium* species (the TMTRI assay) and *F. graminearum* (the TMFg12 assay) present in barley grains. Both TaqMan assays were considered to be sensitive and reproducible. The detection limits and linear ranges of the TMTRI and TMFg12 assays were comparable to those of the *Fusarium* TaqMan assays reported by Klemsdal et al. (2006), Waalwijk et al. (2004) and Yli-Mattila et al. (2006). The use of TaqMan probes for fluorescence generation enhances the specificity and reproducibility of PCR assays compared to the use of SYBR Green chemistry. SYBR Green binds unspecifically to double-stranded DNA, whereas the TaqMan probe binds to its target DNA sequence, allowing more accurate quantification. No PCR inhibitors were detected when known amounts of *Fusarium* DNA were mixed with barley DNA and analysed by the

TMTRI and TMFg12 assays. However, there is a possibility that some grain samples contain substances which inhibit the PCR reaction. To improve the reliability of the assays, an internal positive control could be included in the PCR analyses (Waalwijk et al., 2004).

Trichothecene-producing *Fusarium* DNA levels analysed with the TMTRI assay correlated with DON contents in barley and malt samples, especially in north American barley and malt samples as well as in artificially *Fusarium*-inoculated barley samples. The correlation was lower in natural Finnish barley grains, which contained relatively low DON levels. We were able to discriminate grain samples with DON levels above the EC tentative limit value based on the amount of trichothecene-producing *Fusarium* DNA measured in the samples. Our results are in accordance with the results of Klemsdal et al. (2006), Waalwijk et al. (2004) and Yli-Mattila et al. (2006), who analysed wheat and oats samples collected from Norway, the Netherlands and Finland with *Fusarium* species-specific PCR assays. We found that the DNA levels analysed with the TMTRI assay also correlated with *Fusarium* counts (CFU g^{-1}), but not with *Fusarium* contamination rates (%) in artificially inoculated barley samples. *Fusarium* contamination rate analysis is a widely used quality control method for grains in the malting and brewing industry (EBC Analytica Microbiologica, 2005). The contamination rate analysis gives the percentage of kernels infected with fusaria, but it does not measure the amount of *Fusarium* biomass present in samples. The plate count method has been found to provide a better assessment of the amount of fusaria in grains compared to the contamination rate analysis (Sarlin et al., 2005). Unlike the TMTRI assay, the plating techniques do not provide information about toxigenic *Fusarium* species without a species identification which is laborious and requires special skills. In addition, the TMTRI assay proved to be more sensitive than the plate count analysis; some samples which contained undetectable amounts of fusaria (<50 CFU g^{-1}) still gave a weak response in the TMTRI assay. This can be partly explained by the fact that PCR also amplifies DNA from dead fungal tissues whereas only living mycelia and spores are detected by plate counting. Ergosterol content, which has been used to estimate the extent of *Fusarium* contamination in artificially inoculated grains, has also been shown to increase with DON concentration (Miedaner et al., 2000). The ergosterol assay measures the total fungal biomass, which limits its use in estimating the amount of a single species.

We observed a correlation between the *F. graminearum* DNA level and the DON content in north American barley and malt samples, whereas the correlation was not evident with Finnish barley samples which had naturally low DON and *F. graminearum* DNA levels. Based on a comparison between TMTRI and TMFg12 results, most of the trichothecene-producers present in the north American grain samples belonged to the species *F. graminearum*. Further analyses are still needed to confirm these observations. In North America, *F. graminearum* has been reported to be the most prevailing trichothecene producer, compared to *F. culmorum* in northern Europe (Mills, 1989; Schwarz et al., 1996; Steffenson, 1998; Bottalico and Perrone, 2002; Yli-Mattila et al., 2002). Recent reports from central Europe have indicated that the incidence of *F. graminearum* in cereals has increased (Bottalico and Perrone, 2002; Waalwijk et al., 2004).

The influence of environmental factors on *Fusarium* species and their mycotoxin production in cereals was recently reviewed by Doohan et al. (2003). These factors include temperature, humidity, presence of other micro-organisms, host plant etc. Temperature and water activity, together with the type and level of *Fusarium* inoculum, mainly affect the mycotoxin content accumulating in a host plant (Doohan et al., 2003). The presence of a toxin gene does not necessarily lead to toxin production. In our study, some natural Finnish barley grain samples contained relatively high amounts of trichothecene-producing *Fusarium* DNA although no DON was detected. This result suggests that the *Fusarium* species present in these samples did not produce trichothecenes in Finnish field conditions, or that they produced other trichothecenes than DON. The latter explanation was true in some cases; instead of DON, nivalenol, T-2 and HT-2 toxins were detected at concentrations up to 63, 34 and 68 ppb, respectively. However, most of the samples which contained relatively high DNA levels but no DON did not have detectable amounts of other trichothecenes. Mycological analyses of these samples revealed the presence of fusaria, which favours the explanation

that some fusaria may not produce trichothecenes in field-grown barley under Finnish climatic conditions although they do have the production ability. This observation, together with the low *F. graminearum* incidence, contributes to the low trichothecene content detected in Finnish barley samples. The effects of environmental factors on mycotoxin formation complicate the estimation of the absolute trichothecene content in cereals based on the *Fusarium* DNA level. On the other hand, we were able to distinguish the samples having a DON concentration close to the EC tentative limit value, which indicates the usefulness of PCR assays for quality control purposes in the cereal-based industry. The process conditions in industry may favour toxin formation, which must be taken into account when the results of the PCR assays are interpreted.

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