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# Quantitative detection of *Fusarium* species in wheat using TaqMan

Cees Waalwijk[1], Ruth van der Heide[1,4], Ineke de Vries[1], Theo van der Lee[1], Cor Schoen[1], Guillaume Costrel-de Corainville[2], Isolde Häuser-Hahn[2], Pieter Kastelein[1], Jürgen Köhl[1], Philippe Lonnet[3], Thierry Demarquet[3] and Gert H.J. Kema[1]

[1]*Plant Research International BV, Droevendaalsesteeg 1, P.O. Box 16, 6700 AA Wageningen, The Netherlands (Fax: +31317418094; E-mail: cees.waalwijk@wur.nl);* [2]*Bayer CropScience AG Research, Global Biology Fungicides, 40789 Monheim, Germany;* [3]*Florimond Desprez, B.P. 41, F-59242 Capelle-en-Pévèle, France;* [4]*Present address: Keygene BV, Wageningen, The Netherlands*

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**Abstract**

*Fusarium* head blight (FHB) of wheat and other small-grain cereals is a disease complex caused by several fungal species. To monitor and quantify the major species in the FHB complex during the growing season, real-time PCR was developed. TaqMan primers and probes were designed that showed high specificity for *Fusarium avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae* and *Microdochium nivale* var. *majus*. Inclusion of an internal PCR control and serial dilutions of pure genomic DNAs allowed accurate determination of the concentration of fungal DNA for each of these species in leaves, ears as well as harvested grains of winter wheat. The DNA concentration of *F. graminearum* in grain samples correlated ($r^2 = 0.7917$) with the incidence of this species on the grain as determined by isolation from individual kernels. Application of the TaqMan technology to field samples collected in 40 wheat crops in the Netherlands during the growing season of 2001 revealed that *M. nivale* var. *majus* predominated on leaves early in the season (GS 45-65). Ears and harvested grains from the same fields, however, showed *F. graminearum* as the major species. In 2002, grain samples from 40 Dutch fields showed a much wider range of species, whereas in ears from 29 wheat crops in France, *F. graminearum* was the predominant species. The concentration of DON correlated equally well with the incidence of the DON-producing species *F. culmorum* and *F. graminearum* in the grain samples ($r^2 = 0.8232$) as well as with total DNA of both these species ($r^2 = 0.8259$). The *Fusarium* TaqMan technology is an important tool to quantify and monitor the dynamics of individual species of the complex causing FHB in cereals during the growing season. This versatile tool has been applied in a comparison of different genotypes, but can also be applied to other disease management systems, e.g. fungicide treatments.

## Introduction

*Fusarium* head blight (FHB), also known as Fusarium scab of small grain cereals, is a disease with huge economic impact in many wheat and barley growing areas around the world and can reduce yield by 30–70% (Bai and Shaner, 1994). The major concern regarding FHB arises from the ability of the majority of species in the complex to produce mycotoxins. The United States Department of Agriculture (USDA) ranks FHB as the worst plant disease to hit the nation since the stem rust epidemics in the 1950s (Windels, 2000). Estimates of the direct and secondary economic impacts of FHB infestations during 1998–2000 indicate that FHB is a major problem for US wheat and barley growers. The cumulative direct economic costs for spring wheat, winter wheat, durum wheat and barley amount to $870 million. Combined direct and secondary economic losses were estimated at $2.7 billion (Nganje et al., 2001).

FHB is a disease complex in which several species co-exist. In Europe, Parry et al. (1995) have

described at least 17 different species of *Fusarium* and in China 18 *Fusarium* species associated with diseased wheat have been identified (CWSCG, 1984; Wang, 1997). Worldwide, the predominant pathogen in the FHB complex is *F. graminearum*, which was shown to be a species complex on its own, consisting of at least seven different lineages. Lineage 7 shows pandemic distribution and is the causal organism in the recent FHB outbreaks in the northern hemisphere (O'Donnell et al., 2000).

The predominant *Fusarium* species associated with FHB in small-grain cereals in Europe are *F. graminearum*, *F. culmorum*, *F. avenaceum* and *F. poae* (Bottalico and Perrone, 2002). Regional differences may occur but cropping systems and climatic conditions, in particular temperature and rainfall at flowering, influence the species profile. These toxigenic species frequently co-occur with *Microdochium nivale* (formerly known as *Fusarium nivale*), which is non-toxigenic, and is predominantly involved in snow mould and necrosis of higher leaf layers. Because the fungal species involved in FHB differ in pathogenicity, toxigenicity and fungicide sensitivity, correct identification and quantification of each species is an essential prerequisite for studies on epidemiology and chemical control as well as for risk assessment. Recently, a multiplex PCR was developed in order to identify field strains after their isolation from different plant tissues (Waalwijk et al., 2003). Surveys performed in 2000 and 2001 in the Netherlands (Waalwijk et al., 2003) showed a strong increase in the frequency of *F. graminearum* compared to the early 1990s, which is in agreement with reports from other countries in Western Europe (Parry et al., 1994). PCR has several advantages over classical identification based on morphology, which requires taxonomical expertise and is also time consuming. Moreover, a morphological approach is not feasible to quantify pathogen levels in different plant tissues during the growing season or in commodities after harvest, since large numbers need to be analysed. In addition, isolation and enumeration may introduce a bias in favour of faster-growing species.

Quantitative technologies circumvent these disadvantageous aspects of qualitative assays and would facilitate appropriate statistics on the size and dynamics of populations of individual *Fusarium* species in the FHB complex over time and space. Competitive PCR and real-time PCR are the two methods currently applied as quantitative molecular diagnostics. Competitive PCR is an endpoint measurement which relies on internal standards that react with the same primers as the target DNA, but generates an amplicon that differs in size. Several species-specific competitive PCR assays have been developed for members of the FHB complex. Competitive PCR assays were developed for *M. nivale* var. *majus* and *M. nivale* var. *nivale* (Nicholson et al., 1996) and for *F. graminearum* and *F. culmorum* (Nicholson et al., 1998) based on RAPD fragments that proved to be specific for each of these species. Doohan et al. (1999) and Edwards et al. (2001) used the *tri5* gene, encoding trichodiene synthase, the first and committing step in the synthesis of trichothecene mycotoxins, as a target to quantify the biomass of these species in field samples. Using this single gene assay, generic primers that amplify all trichothecene producers as well as species-specific primers can be designed (Schnerr et al., 2001, 2002). Competitive assays have some degree of quantification, but suffer from putative exhaustion of reaction components. Real-time PCR, on the other hand, monitors the accumulation of PCR products before reaction components may become limiting. This paper reports the successful development and application of TaqMan real-time PCR to quantify *F. avenaceum*, *F. culmorum*, *F. graminearum, F. poae* and *M. nivale* var. *majus*. Inclusion of an internal control allowed the accurate quantification of the pathogens in the FHB complex, which enabled the monitoring of these pathogens in different plant parts of a range of wheat crops during several growing seasons.

## Materials and methods

### *Fungal strains*

The fungal isolates used have been described (Waalwijk et al., 2003). These include *Fusarium avenaceum* IPO 92-3, *F. culmorum* PD90-283, *F. graminearum* PD88-790 (lineage 7, O'Donnell et al., 2000; Waalwijk et al., 2003), *F. poae* PD93-1780 and *M. nivale* var. *majus* IPO 1.21. Absence of cross-reactivity in TaqMan reactions was tested on a series of fungal species, that are either saprophytic on wheat or putative candidates for bio-

logical control including *Clonostachys rosea* isolates 16 and 1457, *Clonostachys rosea* f. sp. *catenulata* isolate 17, *Alternaria alternata* isolate 312, *Ulocladium chartarum* isolate 351, *Ulocladium atrum* isolates 385, 738 and 745, *Stemphylium* spp. isolate 462, *Epicoccum nigrum* isolate 515, *Botrytis cinerea* isolate 700 and *Cladosporium cladosporioides* isolate 761. Moreover, several *Fusarium* spp. were also included: *F. aquaeductuum* isolate 832, *F. equiseti* isolates G2, 1166, 1168 and 1204, *F. oxysporum* isolates G7 and G14, *F. flocciferum* isolate G6 and *F. sambicinum* isolate W3.

### *Field samples*

In 2001, 35 commercial and five experimental fields located in different regions of the Netherlands were sampled. In a first round (GS 45-65, Zadoks et al., 1974), dead lower leaves of 50 randomly picked plants were pooled per sample. In a second round (GS 78-89), each sample comprised of 50 randomly collected ears per field. Additionally, grain samples were obtained at harvest from 35 of these 40 fields. In 2002, grain samples from 40 different experimental fields in the Netherlands were analysed. In addition, ears from 29 plots were sampled at three localities in France, planted with different genotypes of wheat. These fields were located either in the north (Cappelle-en-Pévèlle; $n = 18$), the central (Chartres; $n = 10$) or the south part of the country (Lectour; $n = 1$).

### *DNA isolations*

Genomic DNA was isolated using the Puregene extraction protocol (Kema et al., 2002). This high quality DNA was used to determine the specificity of primers and probes. To facilitate accurate measurement of the DNA concentrations using spectrophotometry, additional RNase treatment and DNA precipitation steps were included. Dilution series of these DNAs (from 0.9 to 9000 pg) were included as standards in every TaqMan experiment.

### *Design of primers and probes*

Amplifications of genomic DNA of *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae* or *Microdochium nivale* var. *majus* with species-specific primers generated unique DNA fragments for each of these species (Nicholson et al., 1996, 1998; Waalwijk et al., 2003). These amplicons were sequenced using BigDye chemistry. From the sequences of these fragments, primers and probes (Table 1) were designed using the Primer Express program Version 1.5 (Applied Biosystems). The probes to detect the different species were all labelled with FAM (6-carboxy-fluorescein) and all probes contained 6-carboxy-tetramethyl rhodamine (TAMRA) as quencher. The specificity of the primers was tested by analyses of PCR products on gel and the specificity of the fluorescent probes was determined in TaqMan PCRs. In some combinations, cross-reactivity was observed e.g. between the *F. culmorum* primer/probe combination and genomic DNA of *F. graminearum* (data not shown). Therefore, novel oligonucleotides were designed including Minor Groove Binder (MGB) ligands (Table 1). Due to the nature of these ligands, these primers and probes exhibit a higher specificity compared to classical primers of the same length (Afonina et al., 2002).

An internal positive control for the PCR reaction was based on the sequence of the Potato Leaf Roll Virus genome (GenBank accession Y07496). The corresponding probe was labelled with VIC. In each multiplex PCR reaction 100 pg of PLRV template was included to discriminate between uninfected samples and possible PCR inhibition.

### *TaqMan reactions*

TaqMan reactions were performed in 30 µl, using 15 µl TaqMan universal PCR master mix (art. 4304437 Applied Biosystems) and 83 nM of FAM-labelled target probe, 83 nM of VIC-labelled PLRV probe and 333 nM of each forward and reverse primer for both the target *Fusarium* species as well as the internal positive control. Thermal cycling conditions consisted of a single cycle of 2 min at 50 °C to degrade uracil containing DNA and 10 min at 95 °C to inactivate uracil-*N*-glycosidase, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

TaqMan reactions were performed on 3 µl DNA preparations from field samples in a 96 well format in an ABI Prism 7700 apparatus. In every TaqMan experiment a serial dilution series (0.9, 9,

*Table 1.* Primers and probes used in this study

| Species | Primer/probe[a] | Sequence |
|---|---|---|
| *F. avenaceum* | *avenaceum* MGB-F | CCATCGCCGTGGCTTTC |
| | *avenaceum* MGB-R | CAAGCCCACAGACACGTTGT |
| | *avenaceum* MGB probe | ACGCAATTGACTATTGC |
| *F. culmorum* | *culmorum* MGB-F | TCACCCAAGACGGGAATGA |
| | *culmorum* MGB-R | GAACGCTGCCCTCAAGCTT |
| | *culmorum* MGB probe | CACTTGGATATATTTCC |
| *F. graminearum* | *graminearum* MGB-F | GGCGCTTCTCGTGAACACA |
| | *graminearum* MGB-R | TGGCTAAACAGCACGAATGC |
| | *graminearum* MGB probe | AGATATGTCTCTTCAAGTCT |
| *M. nivale* var. *majus* | *nivale* 2-F | CGCCAAGGACTCCTCCAGTAG |
| | *nivale* 2-R | GCCGACGAATGGATATTAAGAACT |
| | *nivale* probe | TCCCGCCTTCACGGTGGAAAGC |
| *F. poae* | *poae* 1-F | AAATCGGCGTATAGGGTTGAGATA |
| | *poae* 1-R | GCTCACACAGAGTAACCGAAACCT |
| | *poae* probe | CAAAATCACCCAACCGACCCTTTC |
| PLRV | PLRV-F (3639-3659)[b] | AAGAGGCGAAGAAGGCAATCC |
| | PLRV-R (4103-4124) | GCACTGATCCTCAGAAGAATCG |
| | PLRV probe (3726-3749) | CGAAGACGCAGAAGAGGAGGCAA |

[a] *Fusarium* spp. probes were labelled with the reporter dye FAM (6-carboxy-fluorecein, emission 518 nm) on the 5′-end and the internal standard PLRV probe was labelled with VIC. All these molecules contained TAMRA (6-carboxy-tetramethyl-rhodamine, 582 nm) as a quencher at the 3′-end.
[b] Numbers reflect the position of each of the oligonucleotides in the genomic sequence of Potato Leaf Roll Virus (GenBank accession #Y07496).

90, 900 and 9000 pg of DNA obtained from pure cultures) of the corresponding pathogen was included.

*Efficient DNA extraction from complex matrices*

Several DNA extraction protocols were tested to assess TaqMan reactions on wheat samples putatively colonized by *Fusarium* spp. Symptomless leaves and ears as well as leaves and ears inoculated with either *F. culmorum* or *F. graminearum* were subjected to different DNA isolation methods. The quantity and integrity of the DNA (a mixture of plant and fungal DNA) was checked on agarose gels and the quality was analysed by TaqMan reactions for both species. Based on both these criteria, the following method for DNA extraction from these matrices was selected. Freeze-dried material was ground to a very fine powder and sieved to obtain particles of ≤1 mM. Ten milligram was suspended in 200 µl lysis buffer (Mulfinger et al., 2000) and after 5 s of sonification these samples were mixed with 200 µl of buffer AP1 of the plant DNeasy kit (Qiagen, Germany). Subsequently, the samples were processed according to the manufacturers protocol and DNA was finally eluted with 200 µl of elution buffer AE.

*Validation experiments*

The specificity and sensitivity of the primer/probe combinations was tested on genomic DNA of *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae*, *Microdochium nivale* var. *majus* and a series of fungal species commonly found on wheat. Reproducibility was analysed at three different levels: (i) the reproducibility of the DNA extraction procedure was tested on 50 mg aliquots of a field sample that was positive for all five *Fusarium* species ($n = 12$), (ii) multiple real-time PCR reactions were run in parallel on these DNA preparations to evaluate intra-assay reproducibility and (iii) the reproducibility between assays was evaluated by repetitions of the same DNA samples in the different TaqMan experiments.

*Isolation and enumeration*

The Dutch field samples from 2002 ($n = 40$) were also analysed by classical morphological taxonomy. One-hundred kernels from each of the 40 samples were surface sterilised in 4% NaOCl for 4 min, rinsed twice with sterile water for 5 min and plated on potato dextrose agar (3.9%). After 6 days under NUV-light all *Fusarium* colonies grown from these 4000 kernels were analysed and the frequency of the different *Fusarium* species in each sample was determined.

*Mycotoxin analysis*

The Technical Laboratory Rotterdam carried out the deoxynivalenol (DON) analysis using a LC-MS/MS. Twenty-five grams of sample material was ground and mixed with 100 ml acetonitrile and water (80:20) for 2 h. The filtered extracts were diluted 4× with water and 20 µl of the diluted extract was injected into a LC-MS/MS system. The limit of quantification was 100 ppb.

## Results

*TaqMan development*

Primers and probes designed for the major species of the FHB complex, *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae* and *Microdochium nivale* var. *majus*, used in this study (Table 1) are highly specific as shown by TaqMan reactions with genomic DNA of these fungi as illustrated in Figure 1. Each primer/probe combination successfully amplified DNA extracted from pure cultures of the corresponding fungal species, whereas no fluorescence could be measured with any of the other species within the FHB complex (Figure 1). None of the primer/probe sets amplified DNA from putatively uninfected ears and when this wheat DNA was spiked with fungal DNA, amplicons were only detected with the primer/probe set corresponding to the spiked DNA. Nine fungal species known to occur as saprophytes on wheat were tested and all these species did not show cross-reactions (not shown). The same results were obtained with five different species of the genus *Fusarium* (not shown). The absence of cross-reactivity or interference with non-target DNAs that may be present in field samples indicates the high specificity of the method.

The detection limit and dynamic range of the TaqMan reactions were deduced from the standard curves for each of the pathogens using DNA extracted from pure cultures. The primer/probe sets displayed a linear range of at least four orders of magnitude (shown for *F. graminearum* in Figure 2). Similar results were obtained for the other pathogens. The correlation between the CT-value and known DNA quantities was high for *F. graminearum* ($r^2 = 0.9987$) as well as for the other species in the FHB complex: *F. avenaceum* ($r^2 = 0.993$), *F. culmorum* ($r^2 = 0.9834$), *F. poae* ($r^2 = 0.9876$) and *Microdochium nivale* var. *majus* ($r^2 = 0.9793$). All the species in the FHB complex were quantifiable between 0.9 and 9000 pg (Figure 2). The dynamic range of the TaqMan system was at least four orders of magnitude. The data for *F. graminearum* are presented in Figure 2, but similar results were obtained for the other pathogens ($r^2 \geq 0.9793$). The dynamic range for detection of all the pathogens expands beyond the range from 9000 to 0.9 pg. When the subsequent dilution of 0.09 pg was incorporated into the internal standard series CT-values could be obtained in some, but not all experiments. Inclusion of these CT-values in regression analysis did not influence the correlation as the $r^2$-value decreased only slightly to 0.9949. The concentration of 0.09 pg can be used to calculate the absolute presence of *Fusarium* propagules. The genome sequence of *F. graminearum* (Whitehead Institute, 2003) predicts a genome size of 36 Mb, which equals 0.04 pg. Therefore the detection limit of the TaqMan PCR can be calculated to be less than five genome equivalents.

The reproducibility of the DNA extraction and of the real-time PCR were assessed on a field sample that was shown to be positive for each of the five fungi. Quality and concentration of the DNA among 12 sub-samples were highly similar as judged from an agarose gel (not shown). Repetition of TaqMan reactions on different aliquots of these DNA preparations demonstrated that the variation between CT-values of the same sample was considerably less, CT = 24.3 ± 0.4(SD), than among DNA preparations (CT = 25.2 ± 1.2).

With the advent of the genomic sequence of *F. graminearum* (Whitehead Institute, 2003) the sequence of the *F. graminearum* specific amplicon, as well as the copy number of this sequence in the

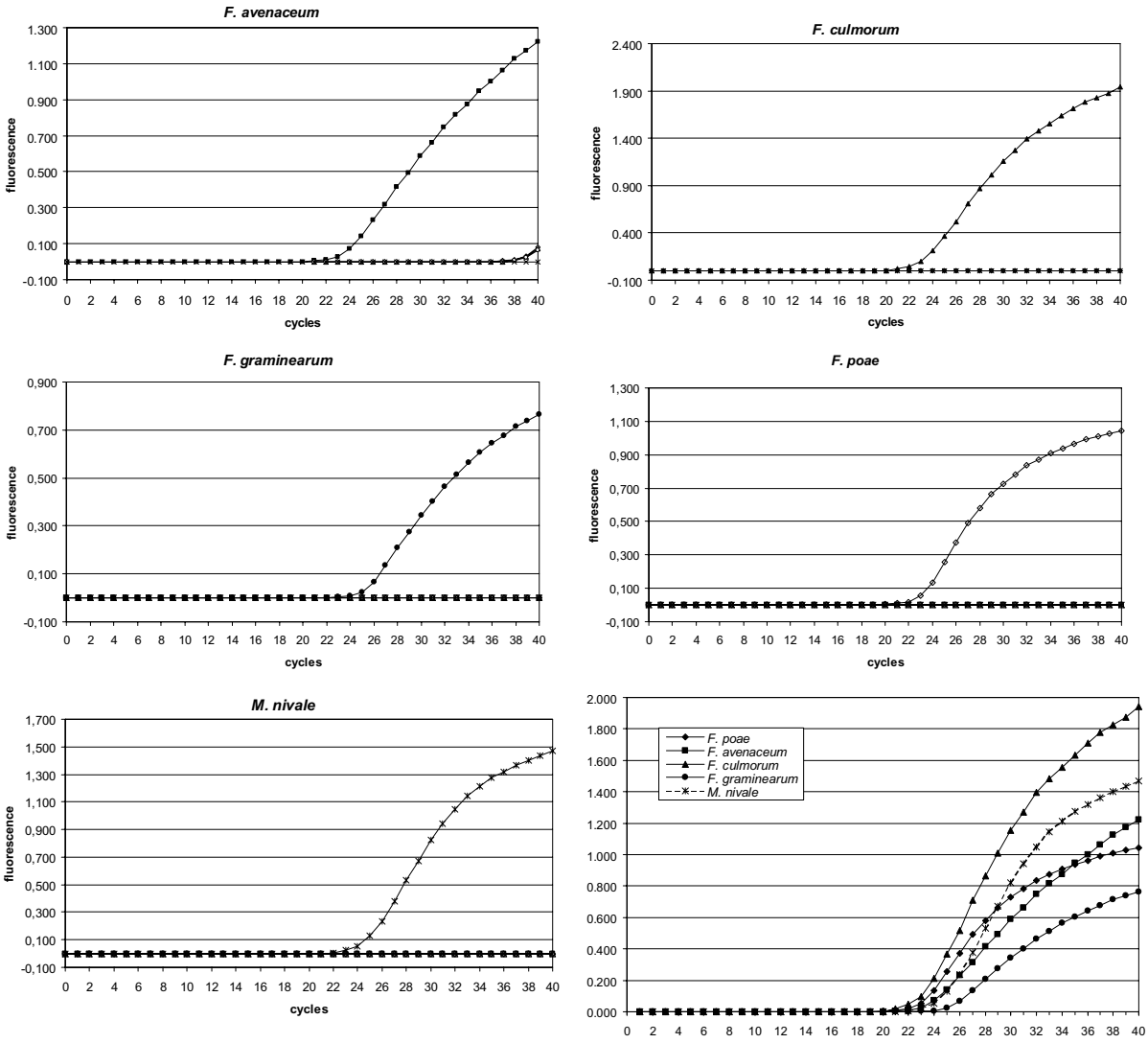


*Figure 1*. Specificity of the TaqMan reactions for five major components of the Fusarium Head Blight complex, *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae* and *Microdochium nivale* var. *majus*. TaqMan reactions performed with the five primer/probe combinations reacted strongly with the corresponding fungal DNA and cross-reactions were not observed until 38 cycles of PCR. (■) *F. avenaceum*; (△) *F. culmorum*; (○) *F. graminearum;* (◇) *F. poae* and (×) *Microdochium nivale* var. *majus*. In most panels the symbols for the non-reacting species are superimposed and appear as filled squarers on the X-axis

genome, were verified. BlastN analyses to the *F. graminearum* genome sequence gave only a single hit that showed perfect homology. BlastN analyses of the sequences for the other species within the FHB complex illustrated that these sequences did not occur in the *F. graminearum* genome (data not shown). The sole exception was the sequence of the *F. culmorum* amplicon that apparently consisted of two halves, one of which showed high homology to the *F. graminearum* genome and the other which did not. Unfortunately the first set of primers and probes designed for *F. culmorum* proved to be located in this region of high homology. This is in agreement with the finding that this *F. culmorum*-specific primer/probe combination showed cross-reactivity with *F. graminearum*. The second set proved to be specific for the sequence not homologous to the *F. graminearum* genome, and this combination did not show cross-reactivity.

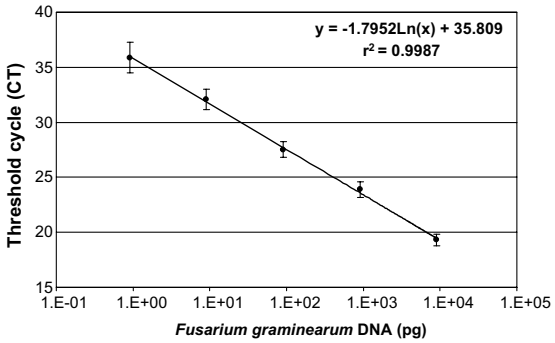


*Figure 2*. Standard curve demonstrating the quantification of *F. graminearum* DNA using TaqMan real-time polymerase chain reaction. Cycle thresholds (CT) were plotted against the log of *F. graminearum* DNA and linear regression equations were calculated for the quantification of unknown samples by interpolation. Similar curves were obtained for the other species within the FHB complex.

*2001 sampling in the Netherlands*

Forty fields throughout the Netherlands (Figure 3(A)) were sampled early in the season for dead lower leaves (GS 45-65), later for wheat heads (GS 78-89) and finally at harvest. The analyses of these samples showed a dramatic change of *Fusarium* spp. populations on the different plant parts (Figure 3(B)–(D)). Virtually the only species encountered on lower leaves was *M. nivale* var. *majus* (Figure 3(B)). This was especially prominent in the southwest and central parts of the country where values frequently exceeded 40 pg $mg^{-1}$ sample (dry weight). In the southeast and east, lower levels were observed ranging between 5 and 20 pg $mg^{-1}$ sample. In contrast, we did not detect *M. nivale* or any other

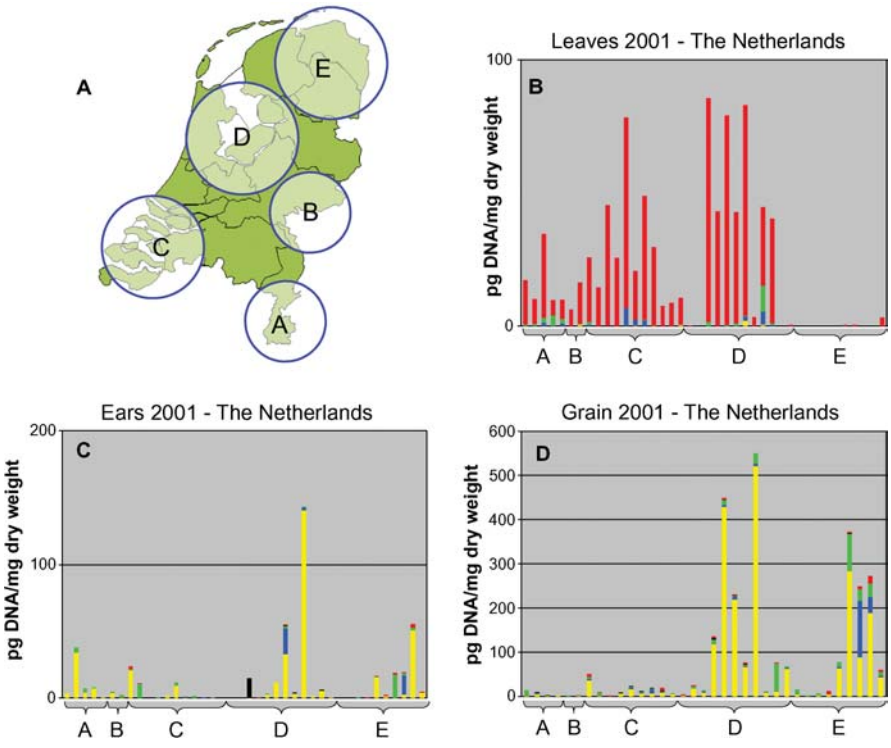


*Figure 3*. Quantification and identification of fungal species causing Fusarium Head Blight in wheat in 2001. (A) Regions in The Netherlands where field samples were collected (40 locations in total). A, Southeast; B, East; C, Southwest; D, Central and E, Northeast part of the Netherlands. (B) Absolute amounts of fungal DNA obtained from leaves; (C) from ears and (D) from grains. Quantities of fungal DNAs are expressed as pg $ml^{-1}$ of dry weight tissue. Note that the scales in (B), (C) and (D) are not the same. For the sake of visualization, scales in the panels have been adapted. *F. avenaceum* (Green), *F. culmorum* (Blue), *F. graminearum* (Yellow), *F. poae* (Black) and *Microdochium nivale* var. *majus* (Red).

species from the FHB complex in fields located in the northeast of the country.

Interestingly, ear samples from the same fields showed completely different results. At this stage of the growing season, *M. nivale* could hardly be detected (<3 pg $mg^{-1}$ sample) and *F. graminearum* was predominant, although some samples also contained significant levels of *F. avenaceum* and/or *F. culmorum*. Only a single sample contained a substantial amount of *F. poae* DNA (15 pg $mg^{-1}$ sample) without detectable levels of any of the other species (Figure 3(C)). Several fields that contained high levels of *M. nivale* on the lower leaves (Figure 3(B)) contained a single *Fusarium* population, or a mixture of several *Fusarium* species in the ears. Samples of ears from some of the fields located in the northeast, that were virtually uninfested at the lower leaf-stage (Figure 3(B)), contained *F. graminearum* DNA up to concentrations of 50 pg $mg^{-1}$ sample (Figure 3(C)). At harvest, the preponderance of *F. graminearum* was even more striking with values occasionally exceeding 300 pg $mg^{-1}$ sample (Figure 3(D)).

Analyses of fungal infestations in harvested grains were repeated in 2002 on samples from 40 other plots throughout the Netherlands. In this year, the weather conditions were much more conducive to FHB and the TaqMan results demonstrated higher levels of fungal DNAs for the pathogens of the FHB complex (Figure 4(A)). Moreover, multiple infections by several pathogen species were common, with several samples containing all five species with cumulative levels above 1000 pg $mg^{-1}$ sample. In 2002, we also examined ear samples from 29 plots located in different regions in France. In contrast to the samples from the Netherlands, primarily *F. graminearum* was present at very high levels, ranging from 1600 pg to more than 20,000 pg $mg^{-1}$ sample. These high levels are irrespective of the origin of sampling in France and only a few samples contained minor quantities of any of the other species (Figure 4(B)).

In the mycological analysis of the field samples in 2002 from the Netherlands, the most important *Fusarium* species was *F. graminearum* (Table 2). This was especially prominent in the northeast region of the Netherlands. Occasionally, a field sample with an incidence of *F. avenaceum* (up to 24%) or *M. nivale* (33%) was observed. Besides the *Fusarium* species detected by TaqMan, *F. tricinctum*, *F. equiseti* and *F. sporotrichioides* were observed, but these species never exceeded a 5% incidence. Some of the samples showed a high incidence by enumeration, but with TaqMan only low levels of the corresponding species were detected (e.g. *F. avenaceum* in samples 2 and 3 or *M. nivale* in 10 and 11). On the other hand, samples in which none of the kernels exhibited outgrowth may well contain large quantities of fungal DNA. This was especially prominent in sample 31 with 597 pg *M. nivale* DNA/mg sample but no outgrowth of the fungus among 100 kernels. Likewise, sample 33 contained 130 pg of *F. graminearum* but no mycelium could be obtained among 100 plated kernels.

The 2002 samples from the Netherlands were also analysed for the presence of the mycotoxin deoxynivalenol (DON). The regression analyses between the DON concentration in the sample and the total number of *F. culmorum* and *F. graminearum* colonies identified among 100 infested kernels was high ($r^2 = 0.8232$; Figure 5(A)). The correlation between the cumulative amount of genomic fungal DNA of these two DON-producing fungi as measured using TaqMan technology and the DON concentration resulted in a similar correlation coefficient ($r^2 = 0.8259$; Figure 5(B)).

## Discussion

Diagnostics for plant pathogens are important to identify fungal contaminants in crops and commodities and are invaluable for quarantine purposes. However, quantitative diagnosis is essential in research on the ecology and population dynamics of plant pathogens and on the epidemiology of diseases, because they enable pathologists to monitor fungal populations over time and space. In particular, when plant diseases are caused by species complexes, quantitative diagnosis of individual populations of the comprising fungal species becomes more important. In the case of FHB, this is highly relevant since some species are able to produce mycotoxins. Specific quantification of individual species is important to study the contribution of each species to the disease as well as the interaction between fungal species in the FHB complex in relation to population dynamics, disease development and mycotoxin formation.

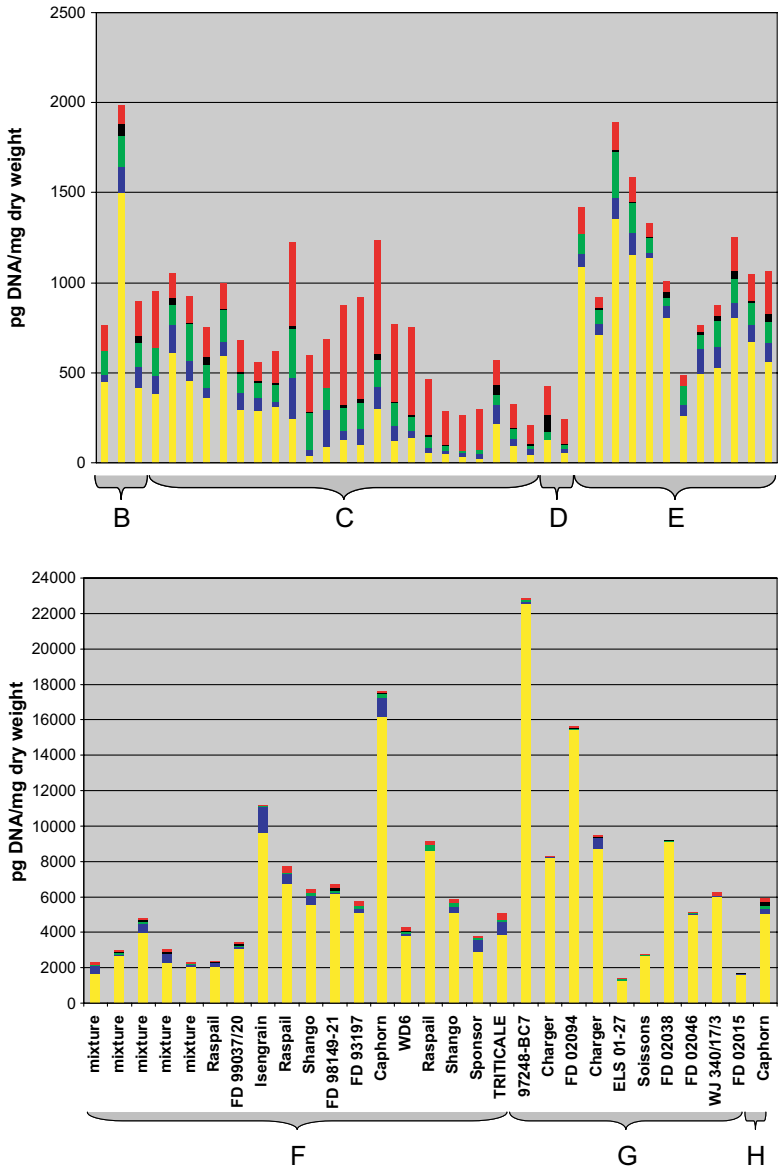


*Figure 4*. Quantification and identification of fungal species causing Fusarium Head Blight in wheat in 2002. (A) Analyses of harvested grain from 40 locations in the Netherlands. Regions B, C, D and E correspond with those in Figure 3. In 2002, no samples were collected in the southeast of the country, e.g. region A. (B) Analyses of ears collected at 29 plots in three localities in France. F represents (Cappelle-en-Pévèle) in the north; G, the central region (Chartres) and H, the south of the country.

Quantitative real-time PCR relies on the efficiency of extraction of DNA from a complex substratum as well as on the accuracy of amplification of template DNA. This can be achieved by inclusion of an internal or an external standard (Gruber et al., 2001). Specific DNA sequences from the host may serve this purpose, as was shown by Winton et al. (2002) to quantify simultaneously host and pathogen in Swiss needle cast of Douglas-fir. In *Fusarium* head blight, wheat-specific sequences could serve as an internal standard, but we intended to apply the methodology to samples from different tissues and different growth stages. It was anticipated that the extraction efficiency of DNA from different tissues would not be identical: e.g. leaf tissue would be more amenable

*Table 2.* Incidence of *Fusarium* head blight fungi among 100 kernels

| | *F. graminearum* | *F. culmorum* | *F. avenaceum* | *F. poae* | *M. nivale* |
|---|---|---|---|---|---|
| 1 | 9 | 4 | 9 | 1 | 10 |
| 2 | 24 | 6 | 20 | | 7 |
| 3 | 16 | 1 | 24 | 1 | 11 |
| 4 | 52 | 3 | 15 | 1 | 7 |
| 5 | 67 | 2 | 3 | | 1 |
| 6 | 63 | | 5 | 1 | 2 |
| 7 | 39 | 2 | 12 | | 4 |
| 8 | 43 | | 12 | 2 | 5 |
| 9 | 51 | 5 | 6 | 3 | 4 |
| 10 | 2 | 1 | 6 | | 31 |
| 11 | | | 1 | 2 | 33 |
| 12 | 8 | 2 | 10 | 2 | 11 |
| 13 | 25 | 7 | 7 | 4 | 5 |
| 14 | 20 | 10 | 11 | 3 | 6 |
| 15 | 32 | 8 | 18 | 1 | 6 |
| 16 | 43 | 7 | 11 | 3 | 5 |
| 17 | 42 | 2 | 12 | 3 | |
| 18 | 23 | | 15 | 2 | |
| 19 | 27 | | 13 | 1 | |
| 20 | 30 | | 11 | | |
| 21 | 55 | 1 | 13 | 1 | |
| 22 | 28 | 2 | 4 | 4 | |
| 23 | 3 | 1 | 3 | 1 | |
| 24 | 11 | | 7 | 2 | |
| 25 | 5 | | 4 | 3 | |
| 26 | 13 | 3 | 16 | 2 | |
| 27 | 7 | 2 | 11 | 2 | |
| 28 | 9 | 4 | 10 | 7 | |
| 29 | 8 | 3 | 4 | 2 | |
| 30 | 2 | | 5 | 5 | |
| 31 | 5 | 1 | 2 | | |
| 32 | 3 | 2 | 5 | 1 | |
| 33 | | | 2 | 1 | |
| 34 | 1 | | 1 | 2 | |
| 35 | 4 | 2 | 1 | 1 | |
| 36 | 3 | | 4 | 2 | |
| 37 | 1 | 1 | 1 | 2 | |
| 38 | 2 | 2 | 2 | 4 | |
| 39 | | | 1 | 2 | |
| 40 | 4 | 1 | 3 | | |

to extraction than kernels. Moreover, the amount of host DNA in these different tissues is likely to be highly dissimilar. The fungal contamination in field samples was thus expressed as pg of fungal DNA per mg of dry weight plant tissue. A cloned fragment from the PLRV genome was therefore added to serve as an internal standard for the PCR reaction only. We used *Fusarium* species-specific amplicons to develop real-time PCR technology and applied it to the FHB disease complex (Waalwijk et al., 2003).

A comparison between the samplings from the same plot allowed investigation of the population dynamics of the various species within the FHB complex. The results obtained suggested differences in the inoculum pressure between different regions. In the southeast, east and southwest regions of the Netherlands, the necrotic lower leaves were predominantly colonised by *M. nivale* var. *majus*. However, this non-toxigenic pathogen was almost completely absent in ears of the same crop, where significant levels of toxigenic species, notably *F. graminearum*, were identified. This species

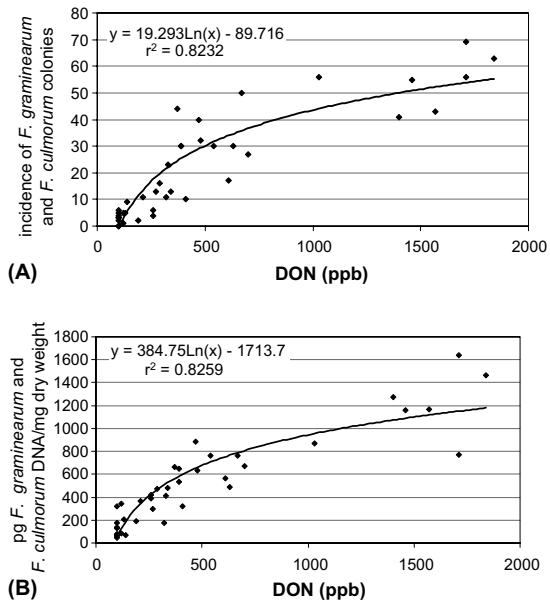


*Figure 5.* Correlation between the concentration of DON and the number of trichothecene producing colonies (*F. culmorum* and *F. graminearum*) among 100 kernels (A) and between the concentration of DON and the total amount of DNA of *F. culmorum* and *F. graminearum* (B).

was also predominant, although at moderate levels, in the harvested product. The central region of the Netherlands, encompassing several polders, also showed heavy infestation by *M. nivale* var. *majus* in the lower leaves. In the ears of the same plots, substantial amounts of *F. graminearum* were found, and these levels were even higher in the harvested product. The northeast area showed another pattern of fungal colonisation. Whereas *Fusarium* levels on the lower leaves were minute, several species were detected at substantial levels in the ears, and in the grain *F. graminearum* was present at similar levels as detected in samples from the central region. These results demonstrate that populations early in the season (in the lower leaves) by no means resemble those found on the upper plant parts, later in the season. Leaf-samples did not contain any detectable levels of *F. graminearum*, which suggests that infection of ears is not caused by populations present on the leaves, but rather by propagules that originate from elsewhere within or outside the field. In the case of *F. graminearum* this can easily by explained by the fact that this fungus has a sexual stage, *Gibberella zeae*, enabling the production of large amounts of ascospores that can be transported by wind over long distances (Fernando et al., 1997; Maldonado-Ramirez and Bergstrom, 2000). Obst et al. (2002) have calculated that ascospores can contribute substantially to the inoculum pressure, with two or even more cycles per growing season. Moreover, even *F. graminearum* populations within a small plot show high genotypic variation and isolates with identical fingerprints are rarely found on different heads (Zeller et al., 2003), suggesting an active sexual reproduction. This also indicates the importance of ascospores as a source of infection. Preliminary field experiments with a strain disrupted for the mating type gene, and hence unable to form ascospores, showed that this mutant was significantly less effective than the wild type isolate in reducing yield and in increasing DON levels of harvested seeds (Brown et al., 2001). These lines of evidence confirm that ascospores can play a major role in FHB epidemics of wheat. Thus, blocking ascospore production in *G. zeae* might intervene in the cycle of FHB epidemics in wheat and other cereals, in particular in rotation with maize.

At this stage, it should be noted that the weather conditions in 2001 were not very conducive to FHB. The experiments performed in 2002 allowed us to establish a correlation between fungal DNA and DON content and expand the method to samples from the major wheat growing regions, e.g. France. The concentration of DON correlated equally well with the total number of colonies of *F. culmorum* and *F. graminearum* as well as with the DNA of these species. However, using the TaqMan method, large numbers of samples can be processed allowing a much more intensive monitoring of the population dynamics of the FHB pathogens under different disease management strategies. Moreover, the TaqMan can be easily expanded to include to additional species in the FHB complex, e.g. *F. tricinctum* and *F. equiseti*, or primer/probes that discriminate between DON- and NIV producing strains.

This study confirms earlier results (Waalwijk et al., 2003) which showed a preponderance of *F. graminearum* over *F. culmorum* and other pathogens involved in the FHB complex. The causal factors that augment *F. graminearum* still need to be elucidated, but an increase in maize production has been suggested to play an important role. *Fusarium graminearum*, in contrast to

*F. culmorum*, is well recognised as a major pathogen on maize and, more importantly, has the capacity to survive on maize stubble (Cotton and Munkvold, 1998). Nevertheless, other factors can be put forward to explain the increased occurrence of *F. graminearum*. Climatic changes could favour the growth of *F. graminearum* over *F. culmorum*, although temperature optima of both pathogens seem similar (Brennan et al., 2003; Köhl et al., unpublished). The homothallic nature of *F. graminearum* allows the production of large masses of ascospores that can play a role in the epidemiology, whereas *F. culmorum* has no known sexual stage. Finally, the resistance of cultivars may influence the composition of the FHB complex. The analyses of the French samples clearly showed that *F. graminearum* levels differed significantly on a range of cultivars with low to moderate levels of resistance to this fungus (e.g. WD6 and cv. Soissons). Susceptible cultivars, such as cv. Charger had very high levels of *F. graminearum*. The remarkably low *F. graminearum* observations in the cultivar mixtures might be due to a synergistic effect of different resistances, but this requires further investigation. Although it has generally been accepted that resistance to *F. culmorum* and to *F. graminearum* are indistinguishable (van Eeuwijk et al., 1995), selection is usually performed under natural disease pressure and therefore actual selection efficiency towards the individual species in the complex is hardly possible. The TaqMan approach is an excellent tool to determine precisely the resistance levels in wheat cultivars to different *Fusarium* species.

The TaqMan results and the data obtained by classical microscopy were similar, although some remarkable discrepancies were observed. These incongruities can be explained in several ways. Plating and microscopic examination requires that the organisms under study are easily culturable. It is likely that every kernel will be colonised by a mixture of different fungal species, some of which may belong to the FHB complex. Upon plating, these different species will undergo competition that may result in outgrowth of only a single individual. On the other hand, TaqMan will detect DNA irrespective of whether it stems from live or from dead cells. The quantitative approach presented in this paper is an unprejudiced way to quantify different fungal populations in the FHB complex in different substrates. This will, for the first time, enable detailed studies on population dynamics of main components of the FHB complex. The presence of different pathogen species that may compete, but also may have synergistic interactions, has to be considered when disease management systems are developed. Because ears can be infected by different species causing FHB, selective control of a single pathogen species may not result in disease control. For example, the selective effect of an azole-fungicide, highly effective against *F. graminearum*, but not as effective against *F. sporotrichioides* and *F. tricinctum* (Oerke et al., 2002) may result in shifts between the different populations present in the field. In this situation, quantitative monitoring of FHB species in field samples will support the development of durable application strategies of fungicides.

Quantitative monitoring of fungal species of the FHB complex will also be a powerful tool for the development of preventive measures that are generally aimed at the reduction of pathogen inoculum. For such studies, thorough knowledge on the importance of various inoculum sources of the different pathogens, such as crop residues and alternative hosts including weeds, is essential. This important part of the life-cycle, during which the pathogenic *Fusarium* spp. survive and multiply saprophytically on various substrates, has been studied for a few *Fusarium* spp. separately, e.g. for *F. graminearum* on wheat tissues (Dill-Macky and Jones, 2000; Inch and Gilbert, 2003) and maize stalks (Cotton and Munkvold, 1998). However, studies on the much more complex situation in Western Europe with several different populations of pathogens involved in the FHB complex are rare and incomplete. Understanding interactions between such populations during their saprophytic stage and the effect of preventative measures on the individual populations and their interactions would greatly facilitate the development of preventative measures. Since the inoculum is apparently not derived from the lower leaves, TaqMan technology is currently being deployed to identify other sources of inoculum.

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## References

Afonina IA, Reed MW, Lusby E, Shishkina IG and Belousov YS (2002) Minor Groove Binder-conjugated DNA probes for quantitative DNA detection by hybridization-triggered fluorescence. BioTechniques 32: 940–949.

Bai G and Shaner G (1994) Scab of wheat: Prospects for control. Plant Disease 78: 760–766.

Bottalico A and 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.

Brennan JM, Fagan B, van Maanen A, Cooke BM and Doohan FM (2003) Studies on *in vitro* growth and pathogenicity of European *Fusarium* fungi. European Journal of Plant Pathology 109: 577–587.

Brown DW, Desjardins AE, Yun SH, Plattner R, Lee T, Dyer R and Turgeon BG (2001) Are *Gibberella zeae* sexual spores the critical inoculum to FHB epidemics? 2001 National *Fusarium* Head Blight Forum Proceedings, (p. 104) Cincinnati, USA.

Cotton TK and Munkvold GP (1998) Survival of *Fusarium moniliforme*, *F. proliferatum*, and *F. subglutinans* in maize stalk residue. Phytopathology 88: 550–555.

CWSCG (1984) *Fusarium* species, distribution and pathogenicity from scabby heads in China. Journal of Shanghai Normal College 3: 69–82.

Dill-Macky R and Jones RK (2000) The effect of previous crop residues and tillage on *Fusarium* head blight of wheat. Plant Disease 84: 71–76.

Doohan FM, Parry DW and Nicholson P (1999) *Fusarium* ear blight of wheat: The use of quantitative PCR and visual disease assessment in studies of disease control. Plant Pathology 48: 209–217.

Edwards SG, Pirgozliev SR, Hare MC and 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. Applied and Environmental Microbiology 67: 1575–1580.

Fernando WGD, Paulitz TC, Seaman WL, Dutilleul P and Miller J (1997) Head blight gradients caused by *Gibberella zeae* from area sources of inoculum in wheat fields plots. Phytopathology 87: 414–421.

Gruber F, Falkner FG, Dorner F and Hämmerle T (2001) Quantification of viral DNA by real-time PCR applying duplex amplification. Internal standardization, and two-color fluorescence detection. Applied and Environmental Microbiology 67: 2837–2839.

Inch S. and J Gilbert (2002). Survival of *Gibberella zeae* in *Fusarium*-damaged wheat kernels. Plant Disease 87: 282–287.

Kema GHJ, Goodwin SB, Hamza S, Verstappen ECP, Cavaletto JR, Van der Lee TAJ, de Weerdt M, Bonants PJM and Waalwijk C (2002) A combined amplified fragment length polymorphism and randomly amplified polymorphism DNA genetic linkage map of *Mycosphaerella graminicola*, the Septoria tritici leaf blotch pathogen of wheat**.** Genetics 161: 1497–1505.

Maldonado-Ramirez SL and Bergstrom GC (2000) Temporal patterns of ascospore discharge by *Gibberella zeae* from colonized corn stalks under natural conditions. 2001 National *Fusarium* Head Blight Forum Proceedings, (pp. 159–162) Cincinnati, USA.

Mulfinger S, Niessen L and Vogel RF (2000) PCR based quality control of toxigenic *Fusarium* spp. in brewing malt using ultrasonication for rapid sample preparation. Advance in Food Sciences 22: 38–46.

Nganje WE, Bangsund DA, Leistritz FL, Wilson WW and Tiapo N (2001) National *Fusarium* Head Blight Forum Proceedings, (pp. 275–281) Cincinnati, USA.

Nicholson P, Lees AK, Maurin N, Parry DW and Rezanoor HN (1996) Development of a PCR assay to identify and quantify *Microdochium nivale* var. *nivale* and *Microdochium nivale* var. *majus* in wheat. Physiological and Molecular Plant Pathology 48: 257–271.

Nicholson P, Simpson DR, Weston G, Rezanoor HN, Lees AK, Parry DW and Joyce D (1998) Detection and quantification of *Fusarium culmorum* and *Fusarium graminearum* in cereals using PCR assays. Physiological and Molecular Plant Pathology 53: 17–37.

Obst A, Günther B, Beck R, Lepschy J and Tischner H (2002) Weather conditions conducive to *Gibberella zeae* and *Fusarium graminearum* head blight of wheat. Journal of Applied Genetics 43A: 185–192.

Oerke E-C, Meier A, Kienemann K, Meyer G, Muthomi J, Schade-Schütze A, Steiner U and Dehne H-W (2002) Incidence and control of *Fusarium* species causing head blight in the Rhineland, Germany. In: Fusarium-Befall und Mykotoxinbelasting von Getreide. Tagungsband der 13. Wissenschaftlichen Fachtagung der Landwirtschaftlichen Fakultät der Universität Bonn, pp. 32–44.

O'Donnell K, Kistler HC, Tacke BK and Casper HH (2000) Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. Proceeding of the National Academy of Sciences USA 97: 7905–7910.

Parry DW, Jenkinson P and MacLeod L (1995) *Fusarium* ear blight (scab) in small grain cereals—a review. Plant Pathology 44: 207–238.

Parry DW, Pettitt TR, Jenkinson P and Lees AK (1994) The cereal *Fusarium* complex. In: Blakeman JP and Williamson B (eds) Ecology of Plant Pathogens (pp. 301–320) CABI, Wallingford.

Schnerr H, Niessen L and Vogel RF (2001) Real time detection of the *tri5* gene in *Fusarium* species by lightcycler-PCR using SYBR Green I for continuous fluorescence monitoring. International Journal of Food Microbiology 71: 53–61.

Schnerr H, Vogel RF and Niessen L (2002) Correlation between DNA of trichothecene-producing *Fusarium* species and

deoxynivalenol concentrations in wheat samples. Letters of Applied Microbiology 35: 121–125.

van Eeuwijk FA, Mesterhazy A, Kling CI, Ruckenbauer P, Saur L, Buerstmayr H, Lemmens M, Keizer LCP, Maurin N and Snijders CHA (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. Theoretical and Applied Genetics 90: 221–228.

Waalwijk C, Kastelein P, de Vries PhM, Kerényi Z, van der Lee TAJ, Hesselink T, Köhl J and Kema GHJ (2003) Major changes in *Fusarium* spp. in wheat in the Netherlands. European Journal of Plant Pathology 109: 743–754.

Wang YZ (1997) Epidemiology and management of wheat scab in China. In: Dubin HJ, Gilchrist L, Reeves J and McNab A (eds) *Fusarium* Head Scab: Global Status and Future Prospects. (pp. 97–105) CYMMIT, Mexico.

Whitehead Institute (2003) The initial release of our high quality draft sequence of the *Fusarium graminearum* genome (http: //www.genome.wi.mit.edu/annotation/fungi/*fusarium*/whatsnew.html).

Windels CE (2000) Economic and social impacts of *Fusarium* head blight: Changing farms and rural communities in the Northern great plains. Phytopathology 90: 17–21.

Winton LM, Stone JK, Watrud LS and Hansen EM (2002) Simultaneous one-tube quantification of host and pathogen DNA with real-time polymerase chain reaction. Phytopathology 92: 112–116.

Zadoks JC, Chang TT and Konzak CF (1974) A decimal code for the growth stages of cereals. Weed Research 14: 415–421.

Zeller KA, Bowden RL and Leslie JF (2003) Diversity of epidemic populations of *Gibberella zeae* from small quadrats in Kansas and North Dakota. Phytopathology 93: 874–880.