Establishment and application of real-time PCR-based methods to study the epidemiology of Fusarium Head Blight

Etablierung und Anwendung der Real-time PCR
für epidemiologische Untersuchungen zu Ährenfusariosen

Dissertation
zur Erlangung des Doktorgrades
der Fakultät für Agrarwissenschaften
der Georg-August-Universität Göttingen

vorgelegt von
Christoph Brandfaß
geboren in Northeim

Göttingen, im Juli 2006

Contents

Chapter 1: General introduction

Fusarium head blight (FHB) is one of the most destructive diseases of wheat and barley worldwide. High economic losses caused by epidemics of FHB were reported especially from the United States, Canada and China (Shaner, 2003).

The infection of heads of small grain cereals and maize plants with *Fusarium* spp. impairs both grain yield and quality (Parry et al., 1995) by adversely effecting grain size, weight, germination rate, protein content, baking quality of the flour, and other techno-logical parameters through fungal colonization of the ear and cutting off the supply of nutrients to the upper spikelets. Irrespective of those impairments, the most serious consequence of FHB is the contamination of grain and cereal products with *Fusarium* mycotoxins (Pieters et al., 2002; Peraica et al., 1999). Mycotoxins are secondary metabolites produced by fungi that are capable of causing disease and death in humans and animals (Bennett and Klich, 2003).

Because grains of low quality are used in feedstuff production rather than in human foods, livestock health, welfare and productivity may be severely compromised by consumption of *Fusarium* mycotoxins. Safety of some animal products may also be at risk (D'Mello et al., 1999). The effects of low doses of *Fusarium* mycotoxins consumed over prolonged time periods on humans are not fully understood (Pestka and Smolinski, 2005; Rocha et al., 2005). Therefore, fungal contamination has become a key concern in food and feed safety assessments, and many countries have set legal limits for *Fusarium* toxin concentrations in food and feed (Anonymous, 2004; Anonymous, 1999; D'Mello et al., 1999).

The most frequently encountered *Fusarium* mycotoxins in FHB in Europe has proved to be deoxynivalenol (DON) and zearalenone produced by *F. graminearum* and *F. culmorum* (Bottalico and Perrone, 2002). DON is reported to be virulence factor of *F. graminearum* in wheat (Langevin et al., 2004; Bai et al., 2002; Proctor et al., 1995), emphasizing the importance of DON for the fungus. Although DON is one of the least acutely toxic trichothecenes, it rises an important food safety issue because it is a very common contaminant of grain (Rotter et al., 1996). The gross toxic effects of DON on animals include growth retardation, reproductive disorders, immunodeficiency, feed refusal and vomiting. The phytotoxic effects of deoxynivalenol on plants can be

summarized as growth retardation and inhibition of seedling and green plant regeneration (Rocha et al., 2005).

A particular problematic fact is that trichothecenes survive processes employed in the production of food of cereal origin. The fate of trichothecenes during processing is particularly affected by their relatively high heat stability and water solubility. For dry-milled products, the most highly contaminated fractions are those that contain the outer portions of the grain. For wet milling, the trichothecenes are primarily transferred to the aqueous fractions. The most contaminated streams enter the animal food chain, although there is no evidence of a significant trichothecenes transmission into animal products. However, the effects of major steps in grain processing used in European food production, e.g. baking and brewing, are not fully understood (Hazel and Patel, 2004). The Scientific Committee for Food have set Tolerable Daily Intakes (TDIs) for DON (1 µg per kg bodyweight per day) and nivalenol (NIV, 0.7 µg per kg bodyweight per day), trichothecenes of the type B produced by *F. graminearum* and *F. culmorum*. Wheat and wheat containing products (like bread and pasta) represent the major source of intake for these trichothecenes by the population of EU member states. The mean intake for DON is below the TDI, however for the young children groups the mean intakes are sometimes close to the TDI. For NIV, the intake is far below the TDI (Schothorst and van Egmond, 2004).

Several PCR assays were developed for genes involved in the toxin biosynthesis that would permit the early detection of *Fusarium* species producing toxins within a food or feed product (Mule et al., 2005). A common feature of these assays is the incapacity to identify the causal agent, which is important for epidemiological questions. The level of DON and the amount of *F. culmorum* and *F. graminearum* DNA in grain correlate well, indicating that these two species, which are major DON producing *Fusarium* spp., produce quite constant amounts of DON in relation to their biomass. The correlation coefficient for DON and DNA content were reported to be 0.68 (Nicholson et al., 2003) and 0.82 (Waalwijk et al., 2004) for the sum of *F. culmorum* and *F. graminearum* DNA, and 0.96 in artificially inoculated wheat samples for *Fusarium* species containing biosynthetic genes for DON (Schnerr et al., 2002). Additionally, a strong correlation of disease severity of FHB with DON levels after artificial inoculation with *Fusarium* spp. was found (Haidukowski et al., 2005; Mesterhazy et al., 2005). However, these data do not answer the question which of these two fungi is the causing agent of DON contamination.

Epidemiology of FHB

F. graminearum and *F. culmorum* attack a wide range of plant species including members of families Fabaceae, Cucurbitaceae and a wide range of Poaceae (Shaner, 2003), including all cereals produced under moderate climate conditions. A key pre-requisite for FHB reduction through crop management is to understand the epidemiology of the disease.

Many toxigenic *Fusarium* species have been associated with FHB infected grains (Nicholson et al., 2003; Bottalico and Perrone, 2002; Parry et al., 1995), but the pre-dominant pathogens found worldwide are *F. graminearum* and *F. culmorum.* These fungi cause FHB in small grains and Gibberella ear rot in maize (Edwards et al., 2002), whereas *F. graminearum* is supposed to be the dominant species in hotter central Europe, and *F. culmorum* tends to predominate in cooler maritime regions of northwest Europe (Bottalico and Perrone, 2002; Parry et al., 1995). The taxonomical status of *F. graminearum* has recently been challenged. O'Donnell et al. proposed the existence of nine phylogenetically distinct species within the *F. graminearum* clade (O'Donnell et al., 2004). In Europe and America only lineage 7 has so far been reported (Ward et al., 2002; O'Donnell et al., 2000). Therefore, epidemiological studies in those regions may be focused on this lineage.

Possible sources of inoculum for FHB were reported to be crop debris, alternative hosts and Fusarium seedling blight and foot rot of cereals (Parry et al., 1995). For *Fusarium graminearum*, propagules from crop debris are the main source of initial inoculum (Xu, 2003). The spore dispersal of the two *Fusarium* spp. differs. The sexual stage of *F. graminearum, Gibberella zeae,* is capable of producing ascospores formed in asci on plant residues on the soil surface at temperatures between 15 and 25°C, but not below 15°C or above 30°C (Gilbert and Fernando, 2004). These perithecia-derived ascospores are forcibly discharged during the evening in response to rising relative humidity and can be transported by wind (Fernando et al., 1997), but also by rain splashes (Paul et al., 2004). The disease foci deriving of infected plots inoculated with *G. zeae*-colonized corn kernels were limited to several meters (Fernando et al., 1997), making FHB mainly a field-specific problem. In addition to this quite limited transport, a long-distance transport of ascospores via the planetary boundary layer (50 m to nearly 1 km above soil surface) also occurs (Maldonado-Ramirez et al., 2005). This enables *G. zeae* to cause an infection without primary inoculum being present on a field (Schmale et al., 2005).

Weather conditions favorable for ascospore production are closely related to rainfall events, although the precise relationship is not clear (Xu, 2003).

F. culmorum does not possess a sexual cycle. Conidia of *F. culmorum* presumably reach the ears by traversing from one leave to the next in rain splashes. This is also valid for the vegetative conidia of *F. graminearum*. Both conidia and ascospores have been shown to be important for causing FHB (Xu, 2003), dependent on the weather conditions. Infection of wheat ears was shown to occur mainly during anthesis. Warm and moist conditions during anthesis are the key factors for FHB development (Xu, 2003).

Jansen et al. described the infection pathway of *F. graminearum* on wheat spikes in detail. After germination of the fungal spore on the spike surface, the hyphae travel along the epicarp and infect the developing kernel through the epicarp. Successively, the layers of the fruit coat and finally the starch and protein accumulating endosperm are destroyed. Hyphae reaching the rachis proceed to apically located developing kernels. In the absence of trichothecenes, the fungus is blocked by the development of heavy cell wall thickenings in the rachis node of wheat, a defense inhibited by the mycotoxin (Jansen et al., 2005). This observation shows that trichothecenes are virulence factors affecting the spread and the disease in Triticeae. This is not necessarily the case in other host species (Langevin et al., 2004). The disruption of the nutrient supply to the upper spike in combination with the effect of mycotoxins causes shriveled kernels (tombstones) or total degeneration of the seeds. The upper part of the spike becomes bleached resulting in the typical head blight symptoms. The kernels downward the infection site are developed in full size, but contain variable amounts of mycotoxins.

Several empirical models were derived from the field data and proposed for use in disease forecasting (De Wolf et al., 2003; Rossi et al., 2003; Hooker et al., 2002). However, these models may not be applicable to a broader range of areas because of the nature of the field data used (Xu, 2003). The model of De Wolf et al. (2003) is based on within-season weather data in the United States and mainly describes the risk of infection, but not the development of the disease after flowering. Hooker et al. (2002) try to predict deoxynivalenol content in winter wheat using weather variables pre- and post-heading. This model is only based on data from southern and eastern Ontario limiting the applicability of the results. A third model developed by Rossi et al. (2003) assumes that, in a wheat-growing area, inoculum is always present for all included *Fusarium* spp. in equal doses. This is in contradiction to the effect of pre-crop and tillage system to the extent of FHB reviewed repeatedly (Champeil et al., 2004). Therefore, Xu (2003) called

for future research focusing on the development of more generally applicable forecasting models and on understanding the relationships between disease severity, fungal biomass and the production of associated mycotoxins.

Influence of agricultural practices

The influence of agricultural practices on *Fusarium* infection of cereals and subsequent contamination of grain by trichothecene mycotoxins have been reviewed repeatedly (Champeil et al., 2004; Edwards, 2004; Gilbert and Fernando, 2004; Pirgozliev et al., 2003). The effectiveness of cultural, biological and chemical control strategies have been investigated for the control of FHB. The qualitative effects of these practices on the severity of the disease and the production of toxins are in the process of being elucidated but, in many cases, detailed studies have not yet been carried out or conflicting results have been obtained. Most importantly, these effects have seldom been evaluated quantitatively in relation to the biomass of those *Fusarium* spp., which are the cause of FHB symptoms and mycotoxins. Three different cultural practices are considered to be of prime importance for combating this disease and the production of mycotoxins: deep tillage, the choice of the preceding crop in the rotation and the choice of appropriate cultivar, as varietal effects exist (Champeil et al., 2004).

The effects of previous crop residues and tillage practices on FHB of wheat were examined by Dill-Macky and Jones (2000). Fusarium head blight incidence and severity were greatest when wheat followed corn and least when wheat followed soybeans, emphasizing the critical role of the pre-crop maize for the occurrence of FHB. Incidence and severity were lower in moldboard-plowed plots than in either chisel-plowed or no-till plots, although differences among chisel-plow and no-till treatments were not apparent. The higher FHB severity was combined with lower yield and higher DON content of the harvested grain. Hence, the move toward soil conservation and reduced-till systems contributes to an increased risk of FHB. On the other hand, these tillage systems are highly desirable in the context of erosion prevention. Dill-Macky and Jones also found that differences in the type and quantity of crop residues in small plots affected disease development. Therefore, it is likely that local sources of inoculum, such as those within a grower's field, contribute directly to the inoculum load and disease potential (Dill-Macky and Jones, 2000). Thereby, it should be taken in consideration that *F. graminearum* is capable of producing ascospores on wheat residues even after 23 months, especially on residues remaining on the soil surface, although the biomass of the residues decreased

significantly (Pereyra et al., 2004). In contrast to these effective control strategies, the adaptation of nitrogen fertilization represents no relevant tool in managing FHB in practical wheat cultivation (Lemmens et al., 2004).

Concerning the choice of appropriate cultivar, much effort has gone into breeding resistant wheat varieties and into improving our understanding of the possible mechanisms and genetic basis of resistance (Parry et al., 1995). Head blight resistance in wheat is not specific for either *F. graminearum* or *F. culmorum*. Resistance components include resistance to penetration, resistance to colonization and mechanisms that influence kernel DON content. The resistance to *Fusarium* in wheat is a quantitative trait with relative high heritability, controlled by a few genes with major effects. A major quantitative trait locus (QTL) for head blight resistance from the Chinese variety Sumai 3 has been identified. Research is now directed at identifying additional QTLs to make accumulation of resistance genes in elite wheat lines possible. This breeding progress addresses the urgent need for head blight resistance in future wheat varieties (Snijders, 2004). However, resistant cultivars available are reducing FHB severity, but they do not prevent mycotoxin contamination.

A range of fungicides has been identified with good activity against FHB pathogens. Unfortunately, the efficacy of these fungicides is significantly influenced by the time of application and the dose rate used (Pirgozliev et al., 2003). In the context of a good agricultural practice, the use of fungicides to combat FHB is a critical point, as the necessity of a treatment is not foreseeable at the time of application due to the lack of symptoms. In addition, the effect of fungicide treatment is variable and can even cause an increase in mycotoxin contamination (Edwards, 2004).

FHB Diagnostics

The relative contribution of each *Fusarium* species to the disease in a particular situation depends upon a range of variables, which might also affect the amount of mycotoxins produced. It is desirable to know the identity and amount of each pathogen within the host tissues (Nicholson et al., 2003). For example, quantitative analysis of a large number of samples in a species-specific manner is required for the evaluation of the relationship between disease severity, fungal biomass and DON contamination. To elucidate the influence of different agricultural practices and weather conditions on the FHB incidence and severity, it is necessary to track the life cycle of the relevant *Fusarium* spp. in the field, including the plant residues. Plating surface-sterilized plant material or grain on

agar media does not allow the quantification of the growing fungi, and ELISA methods are not capable of the differentiation between different *Fusarium* species, which is important because of significant epidemiological and toxicological differences within this genus.

PCR is the method of choice for species-specific detection of fungi in plant material (Martin and Rygiewicz, 2005). Amplification products are traditionally separated by electrophoresis, which also does not allow for the quantitative analysis of template DNA (McCartney et al., 2003). Quantification of DNA template can be achieved by competitive PCR based on internal standards, which are amplified with the same primer pair as the investigated DNA. The ratio of the intensities of the two products serves as a measure of the amount of the template (Nicholson et al., 1998). However, competitive PCR was never established as a routine method in epidemiology because it is labor-intensive and has a limited throughput.

PCR-based assays to determine the type of toxin produced, especially trichothecenes, have also been developed to be applied for example at the quality control of cereals (Mule et al., 2005; Bluhm et al., 2004; Schnerr et al., 2001). As the toxin producing capacity is not species specific, these assays are not suitable to answer questions related to the involvement of different species.

During the last decade, species specific PCR primers for all FHB-relevant pathogens were developed by several groups, as reviewed by Nicholson et al. (2003). These assays have a common feature of being based on electrophoretic detection in agarose gels, which limits their throughput. Therefore, the applicability for epidemiological studies, which typically include a high number of samples, is limited. The drawbacks of the traditional PCR are reduced by the development of real-time PCR-based methods (Wilhelm and Pingoud, 2003), which allow fast, accurate detection and quantification and provide new tools to monitor the exposure of a crop to pathogen inoculum that are more reliable and faster than conventional methods (McCartney et al., 2003). Real-time PCR combines the sensitivity of conventional PCR with the generation of a specific fluorescent signal, providing real-time analysis of the reaction kinetics and allowing quantification of specific DNA targets (Gachon et al., 2004; Schena et al., 2004). The fluorescent signal eliminates the requirement for post-amplification processing steps, such as gel electrophoresis and ethidium bromide staining. This significantly reduces time and labor required for the analysis and greatly increases the throughput of PCR, making it suitable for large-scale analyses (Schena et al., 2004). Known primers specific

for *F. graminearum* and *F. culmorum* (Nicholson et al., 1998; Schilling et al., 1996) served us as a basis for the development of real-time PCR assays to detect and quantify these pathogens in complex matrices like plant debris or wheat flour.

Aims of the study

The relative contribution of each *Fusarium* species to the disease in a particular situation depends upon a range of variables, which might also affect the amount of mycotoxins produced. Real-time PCR methods should serve to determine the identity and amount of *F. graminearum* and *F. culmorum* within host tissues.

Firstly, the qualitative detection should be established to be able to identify the two pathogens within rachides of infected wheat spikes, enabling the determination of the causing agent of FHB symptoms. A suitable high-throughput DNA extraction method for wheat rachides is required for a fast, inexpensive, qualitative PCR assay for *F. culmorum* and *F. graminearum*.

The second aim was the quantitative determination of both fungi in complex plant material like wheat flour and plant debris. Therefore, a DNA extraction protocol suitable to analyze inhomogeneously colonized plant material in order to reduce the sampling error had to be combined with inexpensive real-time PCR assays for *F. culmorum* and *F. graminearum*. For quantification purpose, a suitable standard curve had to be established to relate the PCR results to the fungal DNA amount per sample. A prerequisite for both assays, besides their performance in a high-throughput environment, was the specificity for *F. graminearum* and *F. culmorum,* which had also to be confirmed.

The third aim of the study was the identification of the causing agent of FHB in wheat rachides and the determination of the relative importance of *F. graminearum* and *F. culmorum* in relation to the DON content of wheat. The collected data should serve to adjust quantitative parameters of a risk assessment model for FHB, which should be the final result of the cooperative project funded by the Federal Ministry of Food, Agriculture and Consumer Protection (BMELV). The construction of the model is not a part of this study.

The goal of the BMELV-project is the development of a computer based decision support system minimizing the fusaria infection risk and toxin contamination in wheat. The project partners are the Central Institution for Decision Support Systems in Crop Protection (ZEPP) and the Dienstleistungszentrum Ländlicher Raum, Rheinhessen-Nahe-

Hunsrück in Bad Kreuznach, the Federal Plant Protection Extension Services, and the Federal Agriculture Research Centre (FAL) in Braunschweig.

The main aim of the model called FUSOPT is the reduction of toxin contamination. The model rates and combines agronomical risk factors and simulates biological processes of spore production, dispersal and infection of wheat dependent of weather conditions before and during the flowering of wheat. FUSOPT shall calculate field-specific threshold for plant protection and identify fields and regions with high risk of a FHB infection. The use of fungicides against FHB also shall be directed, while the priority is given to the other agronomical factors to reduce FHB in the context of the good agricultural practice. The forecasting function of the model will show the effect of agronomical risk constellations and demonstrate options to reduce FHB by adapting agronomical practices. Thus, the model FUSOPT shall become an essential part of good agricultural practice to reduce FHB and toxin contamination in wheat.

References

Anonymous. 1999. Verordnung über Höchstmengen an Mykotoxinen in Lebensmitteln (Mykotoxin-Höchstmengenverordnung – MHmV), p. 1248-1251 Bundesgesetz-blatt, Vol. 1. Bundesanzeiger Verlagsges.m.b.H., Bonn.

Anonymous. 2004. Verordnung zur Änderung der Mykotoxin-Höchstmengenverordnung und der Diätverordnung, p. 151-152 Bundesgesetzblatt, Vol. 1. Bundesanzeiger Verlagsges.m.b.H., Bonn.

Bai, G.H., A.E. Desjardins, and R.D. Plattner. 2002. Deoxynivalenol-nonproducing *Fusarium graminearum* causes initial infection, but does not cause disease spread in wheat spikes. Mycopathologia 153: 91-98.

Bennett, J.W., and M. Klich. 2003. Mycotoxins. Clinical Microbiology Reviews 16: 497-516.

Bluhm, B.H., M.A. Cousin, and C.P. Woloshuk. 2004. Multiplex real-time PCR detection of fumonisin-producing and trichothecene-producing groups of *Fusarium* species. Journal of Food Protection 67: 536-543.

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

Champeil, A., T. Dore, and J.F. Fourbet. 2004. Fusarium head blight: epidemiological origin of the effects of cultural practices on head blight attacks and the production of mycotoxins by *Fusarium* in wheat grains. Plant Science 166: 1389-1415.

De Wolf, E.D., L.V. Madden, and P.E. Lipps. 2003. Risk assessment models for wheat Fusarium head blight epidemics based on within-season weather data. Phytopathology 93: 428-435.

Dill-Macky, R., and R.K. Jones. 2000. The effect of previous crop residues and tillage on Fusarium head blight of wheat. Plant Disease 84: 71-76.

D'Mello, J.P.F., C.M. Placinta, and A.M.C. Macdonald. 1999. *Fusarium* mycotoxins: a review of global implications for animal health, welfare and productivity. Animal Feed Science & Technology 80: 183-205.

Edwards, S.G. 2004. Influence of agricultural practices on fusarium infection of cereals and subsequent contamination of grain by trichothecene mycotoxins. Toxicology Letters 153: 29-35.

Edwards, S.G., J. O'Callaghan, and A.D.W. Dobson. 2002. PCR-based detection and quantification of mycotoxigenic fungi. Mycological Research 106: 1005-1025.

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

Gachon, C., A. Mingam, and B. Charrier. 2004. Real-time PCR: what relevance to plant studies? Journal of Experimental Botany 55: 1445-1454.

Gilbert, J., and W.G.D. Fernando. 2004. Epidemiology and biological control of *Gibberella zeae Fusarium graminearum*. Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie 26: 464-472.

Haidukowski, M., M. Pascale, G. Perrone, D. Pancaldi, C. Campagna, and A. Visconti. 2005. Effect of fungicides on the development of Fusarium head blight, yield and deoxynivalenol accumulation in wheat inoculated under field conditions with *Fusarium graminearum* and *Fusarium culmorum*. Journal of the Science of Food and Agriculture 85: 191-198.

Hazel, C.M., and S. Patel. 2004. Influence of processing on trichothecene levels. Toxicology Letters 153: 51-59.

Hooker, D.C., A.W. Schaafsma, and L. Tamburic-Ilincic. 2002. Using weather variables pre- and post-heading to predict deoxynivalenol content in winter wheat. Plant Disease 86: 611-619.

Jansen, C., D. von Wettstein, W. Schafer, K.H. Kogel, A. Felk, and F.J. Maier. 2005. Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. Proceedings of the National Academy of Sciences of the United States of America 102: 16892-16897.

Langevin, F., F. Eudes, and A. Comeau. 2004. Effect of trichothecenes produced by *Fusarium graminearum* during Fusarium head blight development in six cereal species. European Journal of Plant Pathology 110: 735-746.

Lemmens, M., K. Haim, H. Lew, and P. Ruckenbauer. 2004. The effect of nitrogen fertilization on Fusarium head blight development and deoxynivalenol contamination in wheat. Journal of Phytopathology 152: 1-8.

Maldonado-Ramirez, S.L., D.G. Schmale, E.J. Shields, and G.C. Bergstrom. 2005. The relative abundance of viable spores of *Gibberella zeae* in the planetary boundary layer suggests the role of long-distance transport in regional epidemics of Fusarium head blight. Agricultural and Forest Meteorology 132: 20-27.

Martin, K.J., and P.T. Rygiewicz. 2005. Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. BMC Microbiology 5: 28.

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

Mesterhazy, A., T. Bartok, G. Kaszonyi, M. Varga, B. Toth, and J. Varga. 2005. Common resistance to different *Fusarium* spp. causing Fusarium head blight in wheat. European Journal of Plant Pathology 112: 267-281.

Mule, G., M.T. Gonzalez-Jaen, L. Hornok, P. Nicholson, and C. Waalwijk. 2005. Advances in molecular diagnosis of toxigenic *Fusarium* species: A review. Food Additives and Contaminants 22: 316-323.

Nicholson, P., D.R. Simpson, G. Weston, H.N. Rezanoor, A.K. Lees, D.W. Parry, and D. Joyce. 1998. Detection and quantification of *Fusarium culmorum* and *Fusarium graminearum* in cereals using PCR assays. Physiological and Molecular Plant Pathology 53: 17-37.

Nicholson, P., E. Chandler, R.C. Draeger, N.E. Gosman, D.R. Simpson, M. Thomsett, and A.H. Wilson. 2003. Molecular tools to study epidemiology and toxicology of Fusarium head blight of cereals. European Journal of Plant Pathology 109: 691-703.

O'Donnell, K., H.C. Kistler, B.K. Tacke, and H.H. Casper. 2000. Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum,* the fungus causing wheat scab. Proceedings of the National Academy of Sciences of the United States of America 97: 7905-7910.

O'Donnell, K., T.J. Ward, D.M. Geiser, H.C. Kistler, and T. Aoki. 2004. Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. Fungal Genetics and Biology 41: 600-623.

Parry, D.W., P. Jenkinson, and L. McLeod. 1995. Fusarium ear blight (scab) in small grain cereals -- A review. Plant Pathology 44: 207-238.

Paul, P.A., S.M. El-Allaf, P.E. Lipps, and L.V. Madden. 2004. Rain splash dispersal of *Gibberella zeae* within wheat canopies in Ohio. Phytopathology 94: 1342-1349.

Peraica, M., B. Radic, A. Lucic, and M. Pavlovic. 1999. Toxic effects of mycotoxins in humans. Bulletin of the World Health Organization 77: 754-766.

Pereyra, S.A., R. Dill-Macky, and A.L. Sims. 2004. Survival and inoculum production of *Gibberella zeae* in wheat residue. Plant Disease 88: 724-730.

Pestka, J.J., and A.T. Smolinski. 2005. Deoxynivalenol: Toxicology and potential effects on humans. Journal of Toxicology and Environmental Health-Part B-Critical Reviews 8: 39-69.

Pieters, M.N., J. Freijer, B.J. Baars, D.C.M. Fiolet, J. van Klaveren, and W. Slob. 2002. Risk assessment of deoxynivalenol in food: Concentration limits, exposure and effects, p. 235-248 Mycotoxins and Food Safety, Vol. 504. KLUWER ACADEMIC/PLENUM PUBL, New York.

Pirgozliev, S.R., S.G. Edwards, M.C. Hare, and P. Jenkinson. 2003. Strategies for the control of Fusarium head blight in cereals. European Journal of Plant Pathology 109: 731-742.

Proctor, R.H., T.M. Hohn, and S.P. McCormick. 1995. Reduced Virulence of *Gibberella zeae* Caused by Disruption of a Trichothecene Toxin Biosynthetic Gene. Molecular Plant-Microbe Interactions 8: 593-601.

Rocha, O., K. Ansari, and F.M. Doohan. 2005. Effects of trichothecene mycotoxins on eukaryotic cells: A review. Food Additives and Contaminants 22: 369-378.

Rossi, V., S.G. , E.P. , F.S. , and A.D. Vecchio. 2003. A model estimating the risk of Fusarium head blight on wheat. OEPP/EPPO Bulletin 33: 431-435.

Rotter, B.A., D.B. Prelusky, and J.J. Pestka. 1996. TOXICOLOGY OF DEOXYNIVALENOL (VOMITOXIN) [Review]. Journal of Toxicology & Environmental Health 48: 1-34.

Schena, L., F. Nigro, A. Ippolito, and D. Gallitelli. 2004. Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. European Journal of Plant Pathology 110: 893-908.

Schilling, A.G., E.M. Moller, and H.H. Geiger. 1996. Polymerase chain reaction-based assays for species-specific detection of *Fusarium culmorum*, *F. graminearum*, and *F. avenaceum*. Phytopathology 86: 515-522.

Schmale, D.G., D.A. Shah, and G.C. Bergstrom. 2005. Spatial patterns of viable spore deposition of *Gibberella zeae* in wheat fields. Phytopathology 95: 472-479.

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

Schnerr, H., R.F. Vogel, and L. Niessen. 2002. Correlation between DNA of trichothecene-producing *Fusarium* species and deoxynivalenol concentrations in wheat-samples. Letters in Applied Microbiology 35: 121-125.

Schothorst, R.C., and H.P. van Egmond. 2004. Report from SCOOP task 3.2.10 "collection of occurrence data of *Fusarium* toxins in food and assessment of dietary intake by the population of EU member states" - Subtask: trichothecenes. Toxicology Letters 153: 133-143.

Shaner, G. 2003. Epidemiology of Fusarium Head Blight of Small Grain Cereals in North America, p. 512, *In* K. J. Leonard and W. R. Bushnell, eds. Fusarium Head Blight of Wheat and Barley. The American Phytopathological Society, St. Paul, Minnesota, USA.

Snijders, C.H.A. 2004. Resistance in wheat to *Fusarium* infection and trichothecene formation. Toxicology Letters 153: 37-46.

Waalwijk, C., R. van der Heide, I. de Vries, T. van der Lee, C. Schoen, G. Costrel-de Corainville, I. Hauser-Hahn, P. Kastelein, J. Kohl, P. Lonnet, T. Demarquet, and G.H.J. Kema. 2004. Quantitative detection of *Fusarium* species in wheat using TaqMan. European Journal of Plant Pathology 110: 481-494.

Ward, T.J., J.P. Bielawski, H.C. Kistler, E. Sullivan, and K. O'Donnell. 2002. Ancestral polymorphism and adaptive evolution in the trichothecene mycotoxin gene cluster

of phytopathogenic *Fusarium*. Proceedings of the National Academy of Sciences of the United States of America 99: 9278-9283.

Wilhelm, J., and A. Pingoud. 2003. Real-time polymerase chain reaction. Chembiochem 4: 1120-1128.

Xu, X.M. 2003. Effects of environmental conditions on the development of Fusarium ear blight. European Journal of Plant Pathology 109: 683-689.

BMC Microbiology

Methodology article

Simultaneous detection of *Fusarium culmorum* and *F. graminearum* in plant material by duplex PCR with melting curve analysis

Christoph Brandfass and Petr Karlovsky*

Address: University of Göttingen, Institute of Plant Pathology, Grisebachstraβe 6, 37077 Göttingen, Germany

Email: Christoph Brandfass - cbrandf@gwdg.de; Petr Karlovsky* - Karlovsky@web.de

* Corresponding author

Abstract

Background: Fusarium head blight (FHB) is a disease of cereal crops, which has a severe impact on wheat and barley production worldwide. Apart from reducing the yield and impairing grain quality, FHB leads to contamination of grain with toxic secondary metabolites (mycotoxins), which pose a health risk to humans and livestock. The *Fusarium* species primarily involved in FHB are *F. graminearum* and *F. culmorum*. A key prerequisite for a reduction in the incidence of FHB is an understanding of its epidemiology.

Results: We describe a duplex-PCR-based method for the simultaneous detection of *F. culmorum* and *F. graminearum* in plant material. Species-specific PCR products are identified by melting curve analysis performed in a real-time thermocycler in the presence of the fluorescent dye SYBR Green I. In contrast to multiplex real-time PCR assays, the method does not use doubly labeled hybridization probes.

Conclusion: PCR with product differentiation by melting curve analysis offers a cost-effective means of qualitative analysis for the presence of *F. culmorum* and *F. graminearum* in plant material. This method is particularly suitable for epidemiological studies involving a large number of samples.

Background

Fusarium head blight (FHB) is a disease of cereal crops, which has a severe impact on wheat and barley production worldwide. The infection of heads of small grain cereals and maize plants with *Fusarium* spp. impairs grain yield and quality [1]. Apart from adversely effecting grain size, weight, protein content, baking quality of the flour, and other technological parameters, the contamination of grain and cereal products with *Fusarium* mycotoxins is the most serious consequence of FHB [2,3]. The consumption of commodities contaminated with mycotoxins poses a health risk to both humans and farm animals, making fungal contamination a key concern in food and feed safety assessments. Because grains of low quality are used in feedstuff production rather than in human foods, health and productivity impairment in farm animals caused by mycotoxin contamination of feeds have regularly been reported in the last decades from Europe [4]. Our understanding of the effects of low doses of *Fusarium* mycotoxins consumed over prolonged time periods (possibly the whole life span) on humans is poor because of technical difficulties in addressing these issues by doing epidemiological studies. While the EU is still in the process of developing legal limits for *Fusarium* mycotoxins in grains, food and feeds, some European countries have already established national limits [5,6].

Table 1: Fungal strains used in the present study. Source code: 1: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; 2: Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; 3: E. Möller, University of Hohenheim, Germany; 4: H. Nirenberg (BBA Berlin, Germany) via E. Möller, University of Hohenheim, Germany; 5: Institute of Plant Pathology, University of Göttingen, Germany; 6: International Center for Agricultural Research in the Dry Areas, Aleppo, Syria; 7: J.F. Leslie (Kansas State University, Manhattan) via E. Möller, University of Hohenheim, Germany; 8: National Institute for Agricultural Research, Paris, France; 9: Th. Miedaner (State Plant Breeding Institute) via E. Möller, University of Hohenheim, Germany

Species	Strain	Source	Origin
Alternaria alternata	A 4.1.1	5	*Triticum aestivum*, leaf, 1983
Cladosporium herbarum	CH 3	3	unknown
Cladosporium herbarum	CH 4	3	unknown
Drechslera sorokiniana	D 3.1	5	*Triticum aestivum*, leaf
Fusarium acuminatum	ICARDA 92090	6	*Hordeum vulgare*, root, Bouider station
Fusarium acuminatum	ICARDA 92099	6	*Hordeum vulgare*, Bouider station
Fusarium acuminatum	ICARDA 93682	6	*Hordeum vulgare*, root
Fusarium acuminatum	ICARDA 93803	6	*Hordeum vulgare*, root, Viransehir
Fusarium acuminatum	ICARDA 93831	6	*Hordeum vulgare*, root, Viransehir
Fusarium avenaceum	DSM 62161	2	*Dianthus caryophyllus*, rotten stem base, Germany
Fusarium avenaceum	FA 95	3	unknown
Fusarium compactum	ICARDA 93823	6	unknown
Fusarium crookwellense	BBA 63558, DSM 8704	4	unknown
Fusarium crookwellense	BBA 64483	4	unknown
Fusarium crookwellense	BBA 64545	4	unknown
Fusarium culmorum	CBS 122.73	1	*Triticum* sp., Great Britain, 1973
Fusarium culmorum	CBS 250.52 (Fc3.3)	1	*Secale cereale*, grain, 1952
Fusarium culmorum	DSM 62184	2	*Zea mays*, grain, 1952
Fusarium culmorum	DSM 62191	2	*Triticum aestivum*, head, Mainz, Germany
Fusarium culmorum	Fc15	9	*Triticum aestivum*, seed, Söllingen, Germany, 1987
Fusarium culmorum	Fc2	4	*Hordeum vulgare*, head, Finland, 1982
Fusarium culmorum	Fc22	9	*Secale cereale*, stem, Bad Schönborn, Germany, 1989
Fusarium culmorum	Fc36	9	*Secale cereale*, stem, Bergen (Celle), Germany
Fusarium culmorum	Fc65	9	*Secale cereale*, Svalöf, Sweden, 1992
Fusarium culmorum	Fc66	3	*Triticum aestivum*, head, Italy, 1992
Fusarium culmorum	Fc67	3	*Triticum aestivum*, head, Italy, 1992
Fusarium culmorum	Fc73	3	soil, Tasmania, Australia
Fusarium equiseti	ICARDA 93002	6	unknown
Fusarium equiseti	ICARDA 93532	6	unknown
Fusarium equiseti	ICARDA 93574	6	unknown
Fusarium equiseti	ICARDA 93715	6	unknown
Fusarium equiseti	ICARDA 93788	6	unknown
Fusarium graminearum	BBA 62048	4	*Triticum aestivum*, Germany, 1973
Fusarium graminearum	CBS 389.62	1	*Triticum aestivum*, East-Flevoland, The Netherlands, 1961
Fusarium graminearum	DSM 1095	2	*Zea mays*, Indiana, USA, 1973
Fusarium graminearum	DSM 4527	2	*Zea mays*, Bavaria, Germany, 1982
Fusarium graminearum	DSM 4528	2	*Zea mays*, Germany, 1982
Fusarium graminearum	DSM 62217	2	*Zea mays*, Ireland, 1968
Fusarium graminearum	DSM 62722	2	*Lolium perenne*, rotting base, Germany
Fusarium graminearum	DSM 62722	2	*Lolium* sp., Kiel, Germany, 1968
Fusarium graminearum	DSM 64848	2	*Triticum durum*, Rottenburg, Germany, 1987
Fusarium graminearum	DSM 67638	2	*Triticum aestivum*, Bavaria, Germany, 1992
Fusarium graminearum	Fg3	4	*Triticum durum*, head, 1985
Fusarium graminearum	Fg5	4	unknown
Fusarium oxysporum	SAGW 124	5	Aalsmeer, The Netherlands, 1987
Fusarium poae	DSM 62376	2	*Avena sativa*, kernel, Germany
Fusarium poae	FP 2	9	unknown
Gibberella fujikuroi	B-00281	7	*Saccharum officinarum*, Taiwan
Gibberella fujikuroi	B-01722	7	unknown
Gibberella fujikuroi	B-03821	7	unknown
Gibberella fujikuroi	B-03828	7	unknown
Gibberella fujikuroi	B-03853	7	unknown
Microdochium nivale var. *majus*	GN 7	9	*Hordeum vulgare*, head, Switzerland
Microdochium nivale var. *nivale*	GN 25	9	*Hordeum vulgare*
Microdochium nivale var. *nivale*	GN 35	9	*Secale cereale*, base, Georgen

Table 1: Fungal strains used in the present study. Source code: 1: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; 2: Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; 3: E. Möller, University of Hohenheim, Germany; 4: H. Nirenberg (BBA Berlin, Germany) via E. Möller, University of Hohenheim, Germany; 5: Institute of Plant Pathology, University of Göttingen, Germany; 6: International Center for Agricultural Research in the Dry Areas, Aleppo, Syria; 7: J.F. Leslie (Kansas State University, Manhattan) via E. Möller, University of Hohenheim, Germany; 8: National Institute for Agricultural Research, Paris, France; 9: Th. Miedaner (State Plant Breeding Institute) via E. Möller, University of Hohenheim, Germany *(Continued)*

Microdochium nivale var. *nivale*	GN 36	9	*Secale cereale*, base, Oli/Schwäbische Alb, Germany
Pseudocercosporella herpotrichoides	INRA 164	8	Ille et Vilaine, France, 1986
Pseudocercosporella herpotrichoides var. *acuformis*	C39A	5	1988
Pseudocercosporella herpotrichoides var. *acuformis*	PHA 20/3	3	*Secale cereale*, 1991
Rhizoctonia cerealis	INRA 161	8	Marne, France, 1981
Rhizoctonia cerealis	SAGW 105	5	*Triticum aestivum*, Loxstedt-Dünenfähr, Germany, 1984
Rhizoctonia cerealis	SAGW 107	5	Oldenburg, Germany, 1985
Septoria nodorum	7n/II/2	5	*Triticum aestivum*, leaf, Göttingen, 1993

In spite of breeding efforts aiming at FHB-resistant cultivars, and despite a recent shift of the priority of chemical protection development towards fungicides targeting FHB, the disease continues to pose a major challenge to grain growers all over the world [7]. A key prerequisite for a reduction in the incidence of FHB through crop management is an understanding of its epidemiology. The *Fusarium* species primarily involved in FHB are *F. graminearum* and *F. culmorum* [8]. The biology and infection mode of these two species are very different from each other: *F. graminearum* reaches cereal heads via ascospores forcibly discharged from asci formed on plant residues on the soil surface. In comparison, *F. culmorum* does not possess a sexual cycle. It is assumed that *F. culmorum* reaches the ears by traversing from one leave to the next in rain splashes [9], but some researchers find this hypothesis unsatisfactory. A speculation that *F. culmorum* and possibly also *F. graminearum* can infect cereal plants systemically and grow in the stem upwards from the root to the ear has been revived repeatedly in recent years, but it has not been proven so far [10].

Another important question in FHB epidemiology is whether species other than from *F. graminearum* and *F. culmorum* also contribute significantly to the symptoms and mycotoxin contamination. A number of fungal species have been isolated from infected or even healthy-looking ears collected in the field, including a plethora of *Fusarium* spp. [11,12], but it is not known whether these isolates actively contribute to the FHB or whether they just grow saprophytically in the dead surface tissues, which are normally removed during grain cleaning and processing.

Current epidemiological studies on FHB are expected to provide answers to these and other questions crucial to disease management. These studies require the analysis of a large number of samples in a species-specific manner. Although the quantitative determination of fungal biomass is required for certain tasks (e.g., virulence assessment for species or isolates), qualitative data are often sufficient in studies in epidemiology. PCR is the method of choice for both purposes, because reliable species-specific primers are known for all FHB-relevant species [12-17]. However, classical PCR is not suitable for epidemiological studies because agarose electrophoresis limits the throughput [18,19]. Here we describe a fast, inexpensive, qualitative duplex PCR assay for *F. culmorum* and *F. graminearum*, which are the key species causing FHB in wheat and barley. The method is based on the melting curve analysis of amplification products generated in a real-time PCR thermocycler.

Results and discussion
Optimization of DNA extraction and PCR conditions
The processing of the wheat rachides posed a challenge, because the small sample size (2–3 segments of the spike axis per sample) and firm texture of the material put high demands on the homogenization of the samples. The high number of samples precluded manual grinding in a mortar; we therefore used a reciprocal mill. Pulverizing wheat rachides in polypropylene tubes with stainless steel or wolfram carbide balls was found to be inefficient, but a large stainless steel ball (32 g, 20 mm) in a stainless steel container crushed the rachides into a fine powder. We optimized the factors affecting the DNA yield and quality in a CTAB-based DNA extraction method (details to be published), the optimized protocol is described in the Methods section.

Our initial PCR experiments, undertaken under the conditions described in the literature for classical PCR with primers Fg16N and OPT18, did not result in the amplification and detection of the expected products by fluorescence [13,14]. Therefore, we optimized the PCR conditions concerning primer concentration, polymerase activity, $MgCl_2$, SYBR Green I concentration and cycling parameters. For this optimization fungal DNA of the strains Fg3, Fg5, Fc2 and CBS 250.52 (Table 1) was used. The improved protocol is described in the Methods section.
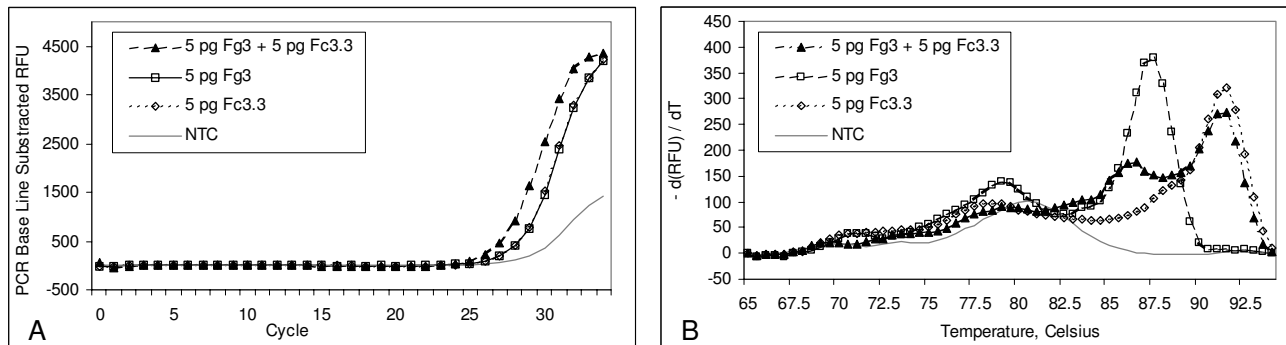
Figure 1
Progress and melting curves of PCR products used for the detection of *F. culmorum* and *F. graminearum*. PCR and melting curve analysis was performed in a real-time thermocycler with primer pairs specific for *F. culmorum* and *F. graminearum* and SYBR Green I fluorescence detection as described in the Methods. The SYBR Green I was diluted to 0.4× the recommended concentration. Genomic DNA from *F. graminearum* (continuous curves with squares), *F. culmorum* (dotted curves with diamonds), a mixture containing DNA from both species (dashed curves with triangles) and no DNA (no-template control, "NTC", gray curve without symbols) were used as templates. A: Progress curves, showing the fluorescence signal during the annealing phase in each reaction cycle. B: Melting curves recorded on PCR products after 35 cycles. The rise of fluorescence in the no-template control produces a melting point maximum at about 80°C, far lower than where *Fusarium* target are detected. Because melting curves presented in Figure 1B were recorded with low template amounts, the unspecific products are also visible, but they do not prevent the detection of *Fusarium* DNA in amounts equal to or higher than the limit of detection.

The specificity of primers we selected for our assay was evaluated in the literature as follows: Fg16N was tested with 21 *F. culmorum* isolates, 24 *F. graminearum* isolates, 20 isolates of other *Fusarium* species and 5 isolates of other fungal species associated with cereals [14]. OPT18 was tested with 69 *F. culmorum* isolates, 34 *F. graminearum* isolates, 25 isolates of other *Fusarium* species and 27 isolates of other fungal species associated with cereals [13]. All tests confirmed the specificity of Fg16N for *F. graminearum* and OPT18 for *F. culmorum*. Because PCR conditions used in these tests were different from the conditions of our assay, we re-evaluated the primer specificity in our duplex assay. We tested a range of fungal species regularly encountered on cereals, especially *Fusarium* spp. (Table 1). All isolates of *F. culmorum* and *F. graminearum* tested positively, giving signals in the expected range of melting temperature. There was no cross-reaction between *F. culmorum* and *F. graminearum*. All other fungal isolates listed in Tab. 1 tested negatively. The amplification of *F. culmorum* and *F. graminearum* was not inhibited by the presence of a large excess of wheat DNA (10 ng wheat DNA in a reaction containing 10 pg of *Fusarium* spp. DNA) nor did pure wheat DNA generate any signal under these conditions.

Duplex PCR with SYBR Green I and melting curve analysis
The fluorescent dye SYBR Green I, which intercalates unspecifically to all double-stranded DNA products, is commonly used in real-time PCR when just one specific amplicon is produced. In our protocol, the dye serves to identify two different products after amplification with the help of a melting curve analysis. In contrast to the real-time PCR mode, this method does not allow for quantification because the fluorescence measured during amplification is the sum of the signals generated by two specific PCR products and possibly unspecific products, too. Particularly, the primer pair Fg16N F/R tends to generate a fluorescent signal even in no-template controls at the end of the amplification (Figure 1).

A melting curve analysis was set up to characterize the amplification products after PCR. Melting points, represented by maxima of the first derivative of the melting curves, were different for the two PCR products in our system. A low heating rate ($0.5°C$ 30 s^{-1}) during melting curve acquisition was essential because the resolution of the melting curves drops with increasing heating rate.

To reduce the fraction of false positives, absolute and relative limits were set for the magnitude of signals represented by peak areas on the first derivative of the melting curves. The absolute peak area and the fraction of the peak area in relation to the total area under the melting curve were used as the absolute and relative thresholds. Their limits were respectively set to 100 and 0.1.

The melting temperature of a PCR product depends mainly on its length and GC content. It is also affected by the nucleotide sequence because of the stacking interactions among adjacent nucleotides. The species-specific

Table 2: Limits of detection for *F. culmorum* and *F. graminearum* in duplex PCR with melting curve analysis.

DNA used as template		Species detected	
F. graminearum	*F. culmorum*	*F. graminearum*	*F. culmorum*
5 pg	5 pg	+	+
100 pg	100 pg	-	+
100 pg	10 pg	+	+
10 pg	100 pg	-	+
5 pg to 5 ng	-	+	-
-	5 pg to 5 ng	-	+

Positive detection (+) was defined as the presence of a peak in the melting profile which fulfills these conditions:
(i) its melting point identified by thermocycler software lies within 91.0 ± 1.0°C for *F. culmorum* and 86.5 ± 1.0°C for *F. graminearum*
(ii) absolute peak area amounts to at least 100 densitometry units
(iii) the relative peak area in relation to the total area under the melting curve amounts to at least 10%.
Identical results were obtained in the presence of an excess (10 ng) of wheat DNA.

PCR product for *F. culmorum* generated with primers OPT18 F/R was 472 bp long and had a melting temperature of 91.0°C ± 1°C in the PCR buffer, whereas the product for *F. graminearum* was 280 bp long and had a melting point of 86.5°C ± 1°C (Figure 1). A third, wide-shaped peak occasionally occurred between 76 and 82°C. This originated from "primer dimers" and other unspecific amplification products. These latter products were found particularly in samples with a low concentration of *Fusarium* DNA. As the result presented in the Figure 1 shows, unspecific products do not prevent the detection of *Fusarium* DNA in amounts equal to or higher than the limit of detection (Table 2). The nonspecific products in the no-template control possess a melting point of about 80°C, which is much lower than the melting temperatures at which *Fusarium* targets are detected.

Experiments with primer pairs used separately revealed that the unspecific products were produced by primers Fg16N F/R, while primers OPT18 F/R did not generate any unspecific products even in controls with no template DNA.

In contrast to the commonly held view that SYBR Green I binds equally well to all double-stranded DNA sequences, we observed that the *F. culmorum* product binds the dye more efficiently than the *F. graminearum* product. Similar differences in the binding affinity of SYBR Green I to different PCR products have been reported by Giglio et al. [20]. Because of this preferential binding of the dye to the *F. culmorum* product, *F. graminearum* could not be detected by melting curve analysis in the presence of 100 pg or more of *F. culmorum* DNA, irrespective of the amount of *F. graminearum* DNA. Attempts to counteract this effect by increasing the SYBR Green I concentration in the PCR reaction mixture were hampered by the inhibition of the PCR by the dye. Since even the addition of more SYBR Green I after the completion of the PCR did not prevent the suppression of the *F. graminearum* signal by the *F. culmorum* DNA (data not shown), we consequently re-optimized the PCR conditions for *F. graminearum* detection in the presence of 5 and 100 pg *F. culmorum* DNA (see Table 2).

During the re-optimization of the PCR conditions we noticed that increasing the SYBR Green I concentration caused an increase in the melting temperature of the PCR products. Table 3 shows the results of a systematic investigation of this phenomenon for the *F. graminearum* product. A shift caused by increasing the SYBR Green I concentration from 0.1× to 0.7× dilution amounted to as much as 2°C.

The detection limits of the melting curve analysis are summarized in Table 2. The assay is used for samples with a low amount of total DNA (e.g., DNA extracted from rachides of cereal ears) or when merely one product was expected (e.g. identification of pure cultures or *Fusarium* spp. isolates). Therefore, we recommend keeping the total amount of *Fusarium* spp. DNA in the assay under 50 pg when the presence of both species is expected. This can be achieved by employing amounts smaller than 1 ng plant DNA in the reaction or by monitoring the C_T value which has to be bigger than 20.

Because the suppression of the *F. graminearum* signal by *F. culmorum* only occurs when the PCR products are characterized by the melting curve analysis, agarose gels can be used to confirm the interpretation of the melting curves when large amounts of *F. culmorum* DNA are detected. This is characterized by a signal (melting curve maximum) specific for *F. culmorum* and a C_T-values of the whole amplicon smaller than 25. In our study of 500 wheat sam-

Table 3: Influence of SYBR Green I concentration on the melting temperature of the PCR product specific for *F. graminearum*. 50 pg of Fg3 DNA were used as a template for PCR with the primer pair Fg16N F/R in addition to different SYBR Green I concentrations and 3 mM MgCl$_2$. The other parameters were set as described in the Methods.

SYBR Green I concentration in the amplification mix	Melting temperature of the 280 bp PCR product
0.1×	84.5°C
0.2×	85.0°C
0.3×	85.5°C
0.4×	86.0°C
0.7×	86.5°C

ples, no *F. graminearum* signal was inhibited by *F. culmorum* DNA (see below).

Data processing

The form in which the results of the melting curve analysis are provided by real-time thermocyclers has not been designed for automated evaluation or a high-throughput environment. To facilitate the interpretation of the results of a multiplex melting-curve-based PCR analysis, we designed a spreadsheet which transformed the output from the thermocycler into a user-friendly, color-enhanced tabular report. In addition to the presentation of the results, the spreadsheet integrates a convenient plate setup (arrangement of samples in the PCR plate) with a pipetting scheme for the reaction mixtures. The data processing and reporting tool consists of eight spreadsheets in Microsoft Excel 2000 (see Additional file 1).

The first sheet "Plate Setup" can be customized according to the user's needs. The content of the plate setup sheet, including identifiers, is used by the interpretation sheet "Data Analysis". The second sheet "Pipetting Scheme" just helps to calculate pipetting, it does not contain any link to the remaining parts of the file. Raw data from the iCycler are copied into the third and fourth sheets: the C$_T$ table

("PCR Quantification" menu) is copied into the third Excel sheet called "CT Table" and the Peak table ("Melt curve" menu) is transferred into the fourth Excel sheet designated "Peak Table". These two sheets, together with the identifiers taken from the plate setup, are the basis for the "Data Analysis" sheet which presents the final results. The auxiliary sheet "Adaptation" contains merely some formatting schemes used by the "Data Analysis" sheet, it does not need to be dealt with by the user. The sixth sheet "Print Sheet" is an empty sheet which can be used for print format adjustments by the user after data from the "Data Analysis" sheet has been copied on to it, because editing options are limited within the "Data Analysis" sheet. The final sheet "Manual" consists of a manual describing the use of the spreadsheet. The spreadsheet file can be downloaded from [21].

The information given for each well in the PCR plate consists of a well identifier, C$_T$ value, melting temperatures, areas under the peaks in the first derivative of the melting curve, and the fraction of the peak area in relation to the total area under the curve (Figure 2). Only these two peaks, corresponding to the products with the highest melting temperatures, are processed. The calculation of the areas under the peaks in the first derivative melting curves is an undocumented feature of the iCycler software:

	A1	5 pg Fg3 + 5 pg Fc3.3	A2	5 pg Fg3	A3	5 pg Fc3.3	A4	negative control
C$_T$	**23.8**	Area / Fraction	**24.3**	Area / Fraction	**24.6**	Area / Fraction	**26.3**	Area / Fraction
1st Peak	91.5	877 / 0.38	87.5	1326 / 0.58	91.5	1354 / 0.57	80.5	623 / 0.85
2nd Peak	86.5	856 / 0.37	79	847 / 0.37	78	1002 / 0.43	73.5	52 / 0.07

Figure 2
Section of the interpretation sheet corresponding to 4 wells. Each result consists of a well position, sample identifier, C$_T$ value, melting temperatures, areas under the melting curve peaks, and fractions of these areas in relation to the total area under the melting curve. The melting data are calculated for the two products with the highest melting temperatures only. Fields are automatically highlighted in a species-specific manner (green: *F. culmorum*, yellow: *F. graminearum*) and a warning is issued by a red coloring of the fields when the peak size does not reach the preset absolute and relative minimal values. In addition, if there is an unexpected third melting-curve maximum at temperatures higher than 80°C (indicating the presence of additional PCR products from *F. culmorum* or *F. graminearum*), the corresponding well will be highlighted in orange to indicate that the user should check the raw data.

these values are not available within iCycler interface, but they are transferred together with other melting curve data when the table is copied to a spreadsheet via the clipboard. To work with other thermocyclers, the spreadsheet presumably will need to be modified according to the format used to export the melting curves.

The interpretation of the results is facilitated by an automatic highlighting of the important fields: the presence of *F. culmorum* (melting temperature between 90.0 and 92.0°C) is indicated by green and *F. graminearum* (melting temperature between 85.5 and 87.5°C) is colored yellow. If the peak area or the fraction of the peak area in relation to the total area under the melting curve does not reach the set quality limits (see previous section), the fields will be colored red to warn of potential false positives. These limits can be adjusted centrally in the head of the interpretation sheet, based on the intensity of the signals produced by the DNA standards.

In some cases, there are more than two melting curve maxima at temperatures higher than 80°C where the PCR products of *F. culmorum* and *F. graminearum* are to be expected. In such a rare case, the corresponding well will be marked orange in the interpretation sheet. This is an indication that the raw data should be checked.

Analysis of field samples

The method was tested by analyzing 500 DNA samples extracted from the rachides of wheat heads with symptoms of FHB sampled throughout Germany in 2003. At least one of the two fungi was identified in all but 21 samples. Both pathogens were detected in 14 rachides. In 398 rachides, only *F. graminearum* could be detected and 67 samples showed only a *F. culmorum* signal. The latter samples were additionally analyzed for the presence of *F. graminearum* by PCR, in which the *F. culmorum*-specific primers were omitted to see whether the *F. graminearum* signals would be suppressed by *F. culmorum* DNA. This single-target analysis confirmed the results of duplex PCR for all 67 samples, demonstrating that the suppression of the *F. graminearum* signals by *F. culmorum* DNA did not occur in any of the 500 samples analyzed.

Therefore, we suggest that a confirmatory analysis (by a single-target PCR or by gel electrophoresis of products of duplex PCR analysis) for samples positive for *F. culmorum* but not *F. graminearum* is unnecessary when working with wheat rachides. However, an extra effort for a confirmatory analysis is rather low (13% of samples in our case). The application of a duplex assay leads to a significant reduction of the sample number as compared to using two separate assays, even when samples positive for *F. culmorum* and negative for *F. graminearum* are analyzed a second time for confirmation.

A standard method for the taxonomic characterization of field samples regarding their colonization with *Fusarium* spp. consists of the isolation of pure cultures on PDA plates, growing the isolates for 7 days on SNA plates or carnation leaf agar and the examination of spores in a microscope. An additional taxonomically relevant marker is the color of fungal colonies on PDA plates. We performed this analysis on 10 rachis samples from ears with FHB symptoms. All samples contained *F. graminearum*, which was confirmed by melting curve analysis-based PCR. In order to verify the PCR method for *F. culmorum*, we selected from a larger set 10 field samples previously identified as containing *F. culmorum* by morphological characters and subjected these samples to melting curve analysis-based PCR. All ten samples tested positive for *F. culmorum*.

To compare both methods, morphological taxonomy requires cultivation for at least 12 days followed by time-consuming microscopic examination. In contrast, the PCR method delivers the results within two days and facilitates parallel processing.

Conclusion

The method developed in this study allows the simultaneous identification of *F. graminearum* and *F. culmorum* in one PCR without the use of doubly labeled hybridization probes or electrophoresis. Species-specific PCR products are differentiated by melting curve analysis. The detection limit for each single species was 5 pg of genomic DNA. An excess of one species impairs the detection limit for the other species due to the competition of the PCR products for binding of the fluorescent dye used for detection. Because *F. culmorum* products bind SYBR Green I better than *F. graminearum* products, large amount of *F. culmorum* DNA impairs the detection of *F. graminearum*. This effect was partially compensated for by increasing the dye concentration. This competition for dye binding does not play a significant role as long as the amount of *F. culmorum* DNA is low. The assay described in this study is suitable for fast and cost-effective analysis of a large number of samples for the presence of *F. culmorum* and *F. graminearum*. It will be particularly useful in epidemiological studies, as exemplified by the investigation of a large collection of rachides isolated from wheat ears exhibiting symptoms of FHB.

Methods

Fungal cultures

The fungal strains used are listed in Table 1. The fungal cultures were maintained at 10°C on SNA medium (0.5 g l⁻¹ MgSO$_4$ × 7 H$_2$O, 1 g l⁻¹ KNO$_3$ (Roth, Karlsruhe, Germany), 0.2 g l⁻¹ sucrose, 0.2 g l⁻¹ glucose, 0.5 g l⁻¹ KCl, 1 g l⁻¹ KH$_2$PO$_4$ and 15 g l⁻¹ agar (Merck, Darmstadt, Germany)). The *Fusarium* spp. cultures for DNA extraction

were grown for 10 days in 100 ml Potato Dextrose Broth (PDB, 24 g l^{-1}; Scharlau, Barcelona, Spain). The fungal mycelium was harvested by filtration and freeze-dried.

DNA isolation from pure fungal cultures grown in liquid media

We used a variant of the CTAB method [22], simplified by others [e.g. [23]], and modified in our laboratory as follows. The lyophilized mycelium (200 mg) was pulverized in a mortar with a small amount of sand (Riedel-de Haen, Hanover, Germany). The ground mycelium was transferred into a 50-ml centrifugation tube containing 10 ml of TES buffer (100 mM Tris (Roth, Karlsruhe, Germany), 20 mM EDTA (Merck, Darmstadt, Germany), 1% (w/v) SDS (Biomol, Hamburg, Germany), pH set to 8.0 with HCL) and 4 mg proteinase K (Merck, Darmstadt, Germany). The lysis mixture was incubated at 45°C for 45 min and the content of the tubes was mixed thoroughly by turning the tubes every 10 min. Subsequently, 3.9 ml of 5 M NaCl (Fluka, Buchs, Switzerland) were added and the sample was mixed before adding 1.4 ml of 10% (w/v) cetyltrimethylammonium bromide (CTAB, Merck, Darmstadt, Germany). The samples were incubated for 10 min at 65°C, cooled in an ice/water bath, and then 10 ml chloroform-isoamyl alcohol (24:1, Roth, Karlsruhe, Germany) were added. After mixing the emulsion thoroughly, the tubes were kept over night in an ice/water-bath. The upper phase (including a small part of the lower phase) was transferred into another centrifugation tube by pipetting and spun for 20 min at 4,000 g (5°C). The watery phase was transferred to a new tube containing 10 ml isopropanol (Roth, Karlsruhe, Germany) at room temperature, mixed thoroughly and centrifuged for 10 min at 4,000 g and room temperature. The supernatant was decanted and the pellet was rinsed with 70% (v/v) ethanol (Roth, Karlsruhe, Germany), dried and dissolved in 4.5 ml TE (10 mM Tris, 1 mM EDTA, pH set to 8.0 with HCl). In spite of the large volume of buffer used to dissolve the DNA, the process took 6 h or longer in some extractions. Dissolving the pellet can be speeded up by heating the tubes to 40°C and then mechanically destroying the pellet. Undissolved material was removed by centrifugation; the DNA was concentrated by ethanol precipitation (1/10 vol. of 5 M ammonium acetate (Merck, Darmstadt, Germany) and 2.5 vol. of 96% (v/v) ethanol) and dissolved in 0.5 ml TE. The quality and quantity of DNA were assessed by electrophoresis in 0.8% (w/v) agarose gels (Cambrex, Rockland, ME, USA) prepared in TAE buffer (40 mM Tris, 1 mM EDTA, pH set to 8.5 with acetic acid, Riedel-de Haen, Hanover, Germany). The electrophoresis was carried out at 4 V cm^{-1} for 60 min. Double-stranded DNA was stained with ethidium (ethidium bromide, 2 mg l^{-1}), (Applichem, Darmstadt, Germany). The gels were documented with the help of a digital imaging system (Vilber Lourmat, Marne la Vallee, France). Densitometry

values were compared with those of Lambda Phage DNA (methylated, from *Escherichia coli* host strain W3110, Sigma, Taufkirchen, Germany). The densitometry was performed using Multi Analyst-Software (BioRad, Hercules, CA, USA).

Isolation of fungi from wheat rachides

To identify the infectious agent causing the FHB, rachides of symptomatic heads were isolated and dissected into pieces spanning 2–3 segments of the spike axis. The rachis samples were rinsed with tap water and surface-sterilized by incubating for 3 min in 3% (v/v) sodium hypochlorite solution (Roth, Karlsruhe, Germany). They were then dried, inserted into agar in PDA-plates (PDB + 1.5% (w/v) agar) and incubated at 20°C for 3–5 days. The mycelia growing out of the rachides were preserved by freezing in 15% (v/v) glycerol (Roth, Karlsruhe, Germany) at -80°C.

DNA extraction from plant material

The rachides were dried at 60°C over night and a single section (2–3 segments) was ground in a ball mill (Mixer Mill MM 200, Retsch, Haan, Germany) in a stainless steel container with a 20-mm, 32-g steel sphere (Retsch, Haan, Germany) for 30 s at maximum speed. The milling container was rinsed with 1.4 ml CTAB-buffer (10 mM Tris, 20 mM EDTA, 0.02 M CTAB, 0.8 M NaCl, 0.03 M N-laurylsarcosine (Fluka, Buchs, Switzerland), 0.13 M sorbitol, 1% (w/v) polyvinylpolypyrolidone, pH set to 8.0 with NaOH (Merck, Darmstadt, Germany). Two µl mercaptoethanol (Fluka, Buchs, Switzerland) and 20 µg proteinase K (from a stock solution 20 mg/ml) were added shortly before use and the mixture was transferred to a 2-ml tube. After an initial incubation period of 10 min at 42°C and a second incubation for 10 min at 65°C, during which the content of the tubes was mixed each 3 min, 0.8 ml of chloroform-isoamyl alcohol (24:1) were added. The samples were then thoroughly emulsified and centrifuged for 10 min at 5,000 g at room temperature. 750 µl of the upper phase were transferred to a 1.5-ml tube containing 500 µl isopropanol, mixed, incubated for 20 min at room temperature and centrifuged for 15 min at 15,000 g at room temperature. The pellet was washed with 70% (v/v) ethanol, dried and dissolved in 200 µl TE. To ensure that the DNA was dissolved completely, the sediment covered by the TE buffer was incubated over night at 4°C. The quality and concentration of DNA were assessed by agarose electrophoresis as described above.

DNA extraction from the mycelia grown on agar plates

To extract the DNA from a mycelium growing on the surface of a small agar plaque, the protocol for plant material (see above) was slightly modified. At the beginning of the procedure, the agar plaque was homogenized with 1 ml CTAB-buffer in a reciprocal mill in a 2-ml tube with 9 wolfram carbide spheres (diameter 3 mm, Retsch, Haan,

Germany). At the end of the procedure, the DNA was dissolved in 100 µl TE-buffer. A 1:100-dilution was used in the PCR.

PCR amplification

The iCycler System (BioRad, Hercules, CA, USA) was used for amplification and melting curve analysis. Primers Fg16N F (ACAGATGACAAGATTCAGGCACA) and Fg16N R (TTCTTTGACATCTGTTCAACCCA) were used to amplify a 280 bp fragment specific for *F. graminearum* [14]. Primers OPT18 F (GATGCCAGACCAAGACGAAG) and OPT18 R (GATGCCAGACGCACTAAGAT) served to multiply a 472 bp fragment specific for *F. culmorum* [13]. All the primers were synthesized by Operon Biotechnologies (Cologne, Germany). Both primer pairs Fg16N F/R and OPT18 F/R were derived from randomly amplified genomic fragments, the function of target sequences is unknown.

The amplification mix consisted of 1× NH_4-reaction buffer (diluted from 10× NH_4-reaction buffer: 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, 0.1% (v/v) Tween-20, pH 8.8 at 25°C, Bioline, Luckenwalde, Germany), 5 mM $MgCl_2$ (Bioline, Luckenwalde, Germany), 0.2 mM of each dATP, dTTP, dCTP and dGTP (Bioline, Luckenwalde, Germany), 0.3 µM of each primer, 0.7 u BIOTaq DNA polymerase (Bioline, Luckenwalde, Germany), 10 nM Fluorescein (BioRad, Hercules, CA, USA, diluted from 1 µM in 1× NH_4-reaction buffer, to collect well factors, specific for the iCycler), 0.4× SYBR Green I solution (Invitrogen, Karlsruhe, Germany), 1 µl of template DNA and ddH_2O up to 25 µl. The detection of amplification products, based on the fluorescence of SYBR Green I, was performed with filters set at 490 ± 10 nm for excitation and 530 ± 15 nm for emission.

The PCR was performed with the following cycling protocol. Initial denaturation for 1.5 min at 95°C (the denaturation time is used by the thermocycler to collect data for the calculation of the well correction factors, which is needed to compensate for differences among wells of the microtitre plate) was followed by 35 cycles with 30 s at 94°C, 45 s at 64°C, and 45 s at 72°C. The final elongation was performed for 5 min at 72°C. During the PCR, the detection of fluorescence was carried out in the annealing step of each cycle. Following amplification, the melting curves were acquired by heating the samples to 95°C for 1 min, cooling to 55°C for one min and then slowly increasing the temperature from 65°C to 95°C at the rate of 0.5°C 30 s-1, with a continuous measurement of the fluorescence.

When an electrophoretic analysis of PCR products was necessary, 4 µl of the reaction mixture were combined with 2 µl of loading buffer (100 mM EDTA, 50% (v/v) Glycerol, 0.025% (w/v) bromphenol-blue, Merck, Darmstadt, Germany) and loaded on a 1.7% (w/v) agarose gel prepared in TAE buffer. The electrophoresis and the documentation were carried out as described above.

Data analysis

Sample identifiers, C_T values (threshold cycles) and the results of the melting curve analysis were exported from the thermocycler into the pre-formatted Excel spreadsheets, which facilitated the automatic data processing and reporting (see Additional file 1). The Excel file can be downloaded from [21], its structure and function is described in detail in the Data Processing paragraph of the Results section.

Authors' contributions

CB carried out the experiments, designed the spreadsheets and drafted parts of the manuscript. PK conceived and guided the study, and wrote parts of the manuscript. Both authors read and approved the final version of the manuscript.

Additional material

Additional File 1
DuplexPCRFusarium. The pre-formatted Excel spreadsheet facilitates automatic data processing and reporting. In short, data concerning the two largest maxima identified on the first derivative of each melting curve are processed as follows: the size of the peaks are checked against pre-set minimal values, the melting temperatures are compared with values expected for both species-specific PCR products, and the final results are presented in a tabular report.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2180-6-4-S1.xls]

References
1. Parry DW, Jenkinson P, McLeod L: **Fusarium ear blight (scab) in small grain cereals – A review.** *Plant Pathology* 1995, **44**:207-238.
2. Pieters MN, Freijer J, Baars BJ, Fiolet DCM, van Klaveren J, Slob W: **Risk assessment of deoxynivalenol in food: Concentration limits, exposure and effects.** In *Mycotoxins and Food Safety Volume 504*. New York: Kluwer Academic/Plenum Publ; 2002:235-248.
3. Peraica M, Radic B, Lucic A, Pavlovic M: **Toxic effects of mycotoxins in humans.** *Bulletin of the World Health Organization* 1999, **77**:754-766.
4. D'Mello JPF, Placinta CM, Macdonald AMC: **Fusarium mycotoxins: a review of global implications for animal health, welfare and productivity.** *Animal Feed Science and Technology* 1999, **80**:183-205.

5. Anonymous: **Verordnung über Höchstmengen an Mykotoxinen in Lebensmitteln (Mykotoxin-Höchstmengenverordnung – MHmV).** In *Bundesgesetzblatt Volume 1*. Bonn: Bundesanzeiger Verlagsges.m.b.H; 1999:1248-1251.
6. Anonymous: **Verordnung zur Änderung der Mykotoxin-Höchstmengenverordnung und der Diätverordnung.** In *Bundesgesetzblatt Volume 1*. Bonn: Bundesanzeiger Verlagsges.m.b.H; 2004:151-152.
7. Pirgozliev SR, Edwards SG, Hare MC, Jenkinson P: **Strategies for the control of Fusarium head blight in cereals.** *European Journal of Plant Pathology* 2003, **109**:731-742.
8. Waalwijk C, Kastelein P, de Vries I, Kerenyi Z, van der Lee T, Hesselink T, Kohl J, Kema G: **Major changes in *Fusarium* spp. in wheat in the Netherlands.** *European Journal of Plant Pathology* 2003, **109**:743-754.
9. Xu XM: **Effects of environmental conditions on the development of Fusarium ear blight.** *European Journal of Plant Pathology* 2003, **109**:683-689.
10. Snijders CHA: **Systemic fungal growth of *Fusarium culmorum* in stems of winter wheat.** *Journal of Phytopathology – Phytopathologische Zeitschrift* 1990, **129**:133-140.
11. Bottalico A, Perrone G: **Toxigenic *Fusarium* species and mycotoxins associated with head blight in small-grain cereals in Europe.** *European Journal of Plant Pathology* 2002, **108**:611-624.
12. Nicholson P, Chandler E, Draeger RC, Gosman NE, Simpson DR, Thomsett M, Wilson AH: **Molecular tools to study epidemiology and toxicology of Fusarium head blight of cereals.** *European Journal of Plant Pathology* 2003, **109**:691-703.
13. Schilling AG, Moller EM, Geiger HH: **Polymerase chain reaction-based assays for species-specific detection of *Fusarium culmorum*, *F. graminearum*, and *F. avenaceum*.** *Phytopathology* 1996, **86**:515-522.
14. Nicholson P, Simpson DR, Weston G, Rezanoor HN, Lees AK, Parry DW, Joyce D: **Detection and quantification of *Fusarium culmorum* and *Fusarium graminearum* in cereals using PCR assays.** *Physiological and Molecular Plant Pathology* 1998, **53**:17-37.
15. Edwards SG, O'Callaghan J, Dobson ADW: **PCR-based detection and quantification of mycotoxigenic fungi.** *Mycological Research* 2002, **106**:1005-1025.
16. Reischer GH, Lemmens M, Farnleitner A, Adler A, Mach RL: **Quantification of *Fusarium graminearum* in infected wheat by species specific real-time PCR applying a TaqMan Probe.** *Journal of Microbiological Methods* 2004, **59**:141-146.
17. Reischer GH, Lemmens M, Farnleitner A, Adler A, Mach RL: **Erratum to "Quantification of *Fusarium graminearum* in infected wheat by species specific real-time PCR applying a TaqMan probe" [J. Microbiol. Methods 59 (2004) 141–146].** *Journal of Microbiological Methods* 2004, **59**:437-437.
18. Schena L, Nigro F, Ippolito A, Gallitelli D: **Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi.** *European Journal of Plant Pathology* 2004, **110**:893-908.
19. McCartney HA, Foster SJ, Fraaije BA, Ward E: **Molecular diagnostics for fungal plant pathogens.** *Pest Management Science* 2003, **59**:129-142.
20. Giglio S, Monis PT, Saint CP: **Demonstration of preferential binding of SYBR Green I to specific DNA fragments in real-time multiplex PCR.** *Nucleic Acids Research* 2003, **31**:.
21. **Downloads Petr Karlovsky group** [http://www.gwdg.de/~inst phyt/karlovsky/downloads/DuplexPCRFusarium.xls]
22. Murray MG, Thompson WF: **Rapid isolation of high molecular-weight plant DNA.** *Nucleic Acids Research* 1980, **8**:4321-4325.
23. Stewart CN, Via LE: **A rapid CTAB DNA isolation technique useful for rapid fingerprinting and other PCR applications.** *Biotechniques* 1993, **14**:748.

Chapter 3: Cost-effective quantification of *Fusarium culmorum* and *F. graminearum* in plant material by real-time PCR

Christoph Brandfass, Petr Karlovsky

Abstract

Brandfass, C., Karlovsky, P. 2006. Cost-effective quantification of *Fusarium culmorum* and *F. graminearum* in plant material by real-time PCR.

We describe a real-time PCR-based method for the quantification of *F. culmorum* and *F. graminearum* in plant material. After a CTAB-based DNA-extraction, species-specific DNA fragments are amplified by PCR. The kinetics of the amplification is monitored with the help of the fluorescent dye SYBR Green I which intercalates into double-stranded DNA. We demonstrate that real-time PCR with product quantification by SYBR Green fluorescence offers a cost-effective alternative to the use of doubly labeled hybridization probes for the quantitative analysis of *F. culmorum* and *F. graminearum* biomass in plant material. This method is particularly suitable for epidemiological studies involving a large number of samples.

Additional keywords: assay, corn, *Gibberella zeae*, ear blight, epidemiology, head blight, maize debris, small-grain cereals, wheat scab.

Introduction

Fusarium head blight (FHB) is a disease of cereal crops with a severe impact on wheat and barley production worldwide. The infection of heads of small grain cereals and maize plants with *Fusarium* spp. impairs both grain yield and quality (Leonard and Bushnell, 2003; Parry et al., 1995). Apart from adversely effecting the grain size, weight, protein content, baking quality of the flour, and other technological parameters, the most serious consequence of FHB is the contamination of grain and cereal products with *Fusarium* mycotoxins (Pieters et al., 2002; Peraica et al., 1999). Because grains of low quality are used in feedstuff production rather than for human foods, health damage in

farm animals and losses in meat production caused by mycotoxin contamination of feeds have regularly been reported in the last decades (D'Mello et al., 1999). Concerning the impact of mycotoxins on human health, our understanding of the effects of low doses consumed over prolonged time periods or the whole life span is poor because of technical difficulties in addressing these issues in epidemiological studies (Pestka and Smolinski, 2005). Two trichothecene mycotoxins produced by *Fusarium* spp. are listed among small-molecular toxins regarded as global bioterrorist threat agents (Ecker et al., 2005). While the EU is still in the process of developing legal limits for *Fusarium* mycotoxins in grains, food and feeds, many countries have already established national limits (Anonymous, 2004; Anonymous, 1999; D'Mello et al., 1999).

In spite of breeding efforts aiming at FHB-resistant cultivars (Bai and Shaner, 2004; Snijders, 2004) and prioritization of fungicide development towards FHB, the disease continues to pose a major challenge to grain growers all over the world (Pirgozliev et al., 2003). A key prerequisite for FHB reduction through crop management is to understand the epidemiology of the disease. *Fusarium* species primarily involved in FHB are *F. graminearum* and *F. culmorum* (Waalwijk et al., 2003). The biology and the infection mode of these two species differ: *F. graminearum* reaches cereal heads via ascospores forcibly discharged from asci formed on plant residues on the soil surface, while *F. culmorum* does not possess a sexual cycle. *F. culmorum* presumably reaches the ears by traversing from one leave to the next in rain splashes (Xu, 2003), but some researchers find this hypothesis unsatisfactory. A speculation that *F. culmorum* and possibly also *F. graminearum* can infect cereal plants systemically and grow within the stem from the root up to the ear has been revived repeatedly in recent years, but it has not been proven so far (Snijders, 1990).

Another important question in FHB epidemiology is whether species other than *F. graminearum* and *F. culmorum* contribute significantly to the symptoms of FHB and mycotoxin contamination. A number of fungal species have been isolated from infected or even healthy-looking ears collected in the field, including a plethora of *Fusarium* spp. (Nicholson et al., 2003; Bottalico and Perrone, 2002; Parry et al., 1995), but it is not known whether all these species actively contribute to the FHB or whether they just grow saprophytically in dead tissues on the ear surface. These are normally removed during grain cleaning and processing.

Deoxynivalenol (DON) and nivalenol (NIV) are the major representatives of trichothecene mycotoxins in grain. The level of trichothecenes and the amount of

F. culmorum and *F. graminearum* DNA correlate well, indicating that these two species are largely responsible for the presence of the trichothecenes. The correlation coefficient for DON and DNA content were reported to be 0.68 (Nicholson et al., 2003) and 0.82 (Waalwijk et al., 2004) for the sum of *F. culmorum* and *F. graminearum* DNA, and 0.96 or 0.75 (depending on the concentration range) for *Fusarium* species containing biosynthetic genes for DON (Schnerr et al., 2002).

The relative contribution of each *Fusarium* species to the disease in a particular situation depends upon a range of variables, which might also affect the amount of mycotoxins produced. It is desirable to know the identity and amount of each pathogen within the host tissues (Nicholson et al., 2003). For example, quantitative analysis of a large number of samples in a species-specific manner is required for the evaluation of the relationship between fungal biomass and DON contamination, and for the assessment of the virulence of fungal species and isolates.

PCR is the method of choice for species-specific detection of fungi in plant material (Martin and Rygiewicz, 2005). Amplification products are traditionally separated by electrophoresis, which does not allow for the quantitative analysis of template DNA (Schena et al., 2004; McCartney et al., 2003). Quantification of DNA template can be achieved by competitive PCR, based on internal standards, which are amplified with the same primer pair as the investigated DNA. The ratio of the intensities of the two products serves as a measure of the amount of the template. Paul Nicholson's group developed competitive PCR assays for several *Fusarium* species (Nicholson et al., 1998) and used them successfully in a series of projects, among others to address the effects of fungicides on DON production. However, competitive PCR never took off as a routine method in epidemiology because it is labor-intensive and has a limited throughput.

Real-time PCR allows species-specific quantification within a range of several orders of magnitude and is capable of processing several hundred samples per instrument per day. Reliable species-specific PCR primers are known for all FHB-relevant species (Reischer et al., 2004a; Reischer et al., 2004b; Nicholson et al., 2003; Edwards et al., 2002; Nicholson et al., 1998; Schilling et al., 1996).

Existing real-time PCR methods for *Fusarium* spp. are based on the TaqMan technology and commercial DNA extraction kits. Here we describe a combination of an upscaled DNA extraction protocol reducing the sampling error with inexpensive real-time PCR assays for *F. culmorum* and *F. graminearum* based on SYBR Green I fluorescence detection. The protocol is particularly useful in epidemiological studies, as we

demonstrated in an investigation of the colonization of a large number of wheat samples by *F. culmorum* and *F. graminearum.*

Materials and Methods

Fungal cultures

The fungal strains we used are listed in Table 1. Cultures were maintained at 10°C on SNA medium (0.5 g l^{-1} $MgSO_4$ x 7 H_2O, 1 g l^{-1} KNO_3, 0.2 g l^{-1} sucrose, 0.2 g l^{-1} glucose, 0.5 g l^{-1} KCl, 1 g l^{-1} KH_2PO_4 and 15 g l^{-1} agar). The *Fusarium* spp. cultures for DNA extraction were grown for 10 days in 100 ml potato dextrose broth (PDB, 24 g l^{-1}); mycelium was harvested by filtration and freeze-dried.

Table 1: Fungal strains used in the present study. Sources and origins of isolates are listed in Brandfass and Karlovsky (2006).

Species	Number of Isolates
Alternaria alternata	1
Cladosporium herbarum	2
Drechslera sorokiniana	1
Fusarium acuminatum	5
Fusarium avenaceum	2
Fusarium compactum	1
Fusarium crookwellense	3
Fusarium culmorum	12
Fusarium equiseti	5
Fusarium graminearum	12
Fusarium oxysporum	1
Fusarium poae	2
Gibberella fujikuroi	5
Microdochium nivale var. *majus*	1
Microdochium nivale var. *nivale*	3
Pseudocercosporella herpotrichoides	1
Pseudocercosporella herpotrichoides var. *acuformis*	2
Rhizoctonia cerealis	3
Stagonospora nodorum	1

DNA isolation from pure fungal cultures grown in liquid media

We used a variant of the CTAB method (Murray and Thompson, 1980), simplified by others (e.g. Stewart and Via, 1993), and modified in our laboratory as follows. The lyophilized mycelium (200 mg) was pulverized in a mortar with a small amount of silica sand. The ground mycelium was transferred into a 50-ml centrifugation tube containing 10 ml of TES buffer (100 mM Tris, 20 mM EDTA, 1% (w/v) SDS, pH set to 8.0 with HCL) and 4 mg proteinase K. The lysis mixture was incubated at 45°C for 45 min and the content of the tubes was mixed by turning the tubes every 10 min. Subsequently, 3.9 ml of 5 M NaCl were added and the sample was mixed before adding 1.4 ml of 10% (w/v) cetyltrimethylammonium bromide (CTAB, Merck, Darmstadt, Germany). The samples were incubated for 10 min at 65°C, cooled in an ice/water bath, and then 10 ml chloroform-isoamyl alcohol (24:1) were added. After mixing the emulsion thoroughly, the tubes were kept over night in an ice/water-bath. The upper phase (including a small part of the lower phase) was transferred into another centrifugation tube by pipetting and spun for 20 min at 4,000 g (5°C). The aqueous phase was transferred to a new tube containing 10 ml isopropanol at room temperature, mixed thoroughly and centrifuged for 10 min at 4,000 g and room temperature. The supernatant was decanted and the pellet was rinsed with 70% (v/v) ethanol, dried and dissolved in 4.5 ml TE (10 mM Tris, 1 mM EDTA, pH set to 8.0 with HCl). In spite of the large volume of buffer used to dissolve the DNA, the process took 6 h or longer in some extractions. Dissolving the pellet can be speeded up by heating the tubes to 40°C and then mechanically destroying the pellet. Undissolved material was removed by centrifugation; the DNA was concentrated by ethanol precipitation (1/10 vol. of 5 M ammonium acetate and 2.5 vol. of 96% (v/v) ethanol) and dissolved in 0.5 ml TE. Quality and quantity of DNA were assessed by electrophoresis in 0.8% (w/v) agarose gels (Cambrex, Rockland, ME, USA) prepared in TAE buffer (40 mM Tris, 1 mM EDTA, pH set to 8.5 with acetic acid). The electro-phoresis was carried out at 4 V cm^{-1} for 60 min. Double-stranded DNA was stained with ethidium (ethidium bromide, 2 mg l^{-1}). Gels were documented with the help of a digital imaging system (Vilber Lourmat, Marne la Vallee, France). Densitometry values were compared with those of lambda phage DNA (methylated, from *Escherichia coli* host strain W3110). The densitometry was performed using Multi Analyst-Software (BioRad, Hercules, CA, USA).

Preparation of plant samples

Wheat grain samples (88-92% dry matter) were separated from the chaff by airflow and sieve cleaning with a stationary threshing machine, especially to remove rachides and glumes. Subsequently 500 g-portions of grain were ground in a cross hammer mill (Cross Beater Mill SK 1, bottom sieve 2 mm, Retsch, Haan, Germany), the flour was mixed and a 40 g sub-sample was frozen at –20°C.

Maize debris meal was prepared by drying basal stems without leaves (pieces spanning 20 to 30 cm upwards the onset of the adventitious roots) to 88-92% dry matter. Then the samples were chaffed and ground with the cross hammer mill, bottom sieve 2 mm, before sub-samples were frozen at –20°C.

Quantitative DNA extraction from plant material

To extract DNA from ground plant samples, 1 g of wheat flour or 500 mg of maize debris flour were blended in a 50 ml-tube with 10 ml CTAB-buffer (10 mM Tris, 20 mM EDTA, 0.02 M CTAB, 0.8 M NaCl, 0.03 M N-laurylsarcosine, 0.13 M sorbitol, 1% (w/v) polyvinylpolypyrolidone (Merck, Darmstadt, Germany), pH set to 8.0 with NaOH. Twenty microliters mercaptoethanol and 0.2 mg proteinase K (from a stock solution 20 mg ml^{-1}) were added shortly before use. The mixture was treated for 5 sec in an ultrasonic bath (Sonorex RK 100, Bandelin, Berlin, Germany). After an initial incubation period of 10 min at 42ºC and a second incubation for 10 min at 65ºC, during which the content of the tubes was mixed every 3 min, 8 ml of chloroform-isoamyl alcohol (24:1) were added. The samples were then thoroughly emulsified, incubated for 10 min on ice and centrifuged for 10 min at 5,000 g at room temperature. Six hundred microliters of the upper phase were transferred to a 1.5-ml tube containing 194 µl of a 30 % (w/v) PEG (Serva, Heidelberg, Germany) solution and 100 µl 5 M NaCl, mixed, and centrifuged for 15 min at 15,000 g at room temperature. The pellet was washed with 70% (v/v) ethanol, dried and dissolved in 200 µl TE. To ensure that the DNA was dissolved completely, the sediment covered by the TE buffer was incubated over night at 4°C. The quality and concentration of DNA were assessed by agarose electrophoresis as described above. A 1:10-dilution was used in the PCR.

Preparation of standards for quantitative real-time PCR

DNA standards and DNA from unknown samples have to be amplified under identical conditions. Therefore, quantified standard-DNA of *Fusarium* spp. was mixed with DNA extracted from uncontaminated wheat flour to imitate matrix effects. A dilution series

from 0.5 pg to 0.5 ng of *Fusarium* spp. DNA with a dilution factor of 10 was produced separately for *F. culmorum* and *F. graminearum* DNA in the matrix of plant DNA. For each pathogen two standard curves were set up, one for wheat flour and one for maize debris. In addition to the standard curve, two negative controls were processed in each PCR set, one containing no template (water) and one containing 50 pg of the other *Fusarium* species (e.g. *F. graminearum* for *F. culmorum* analysis) mixed with DNA of a healthy plant. In Table 2 the standards for the quantification of *F. culmorum* in wheat flour are shown as an example.

Table 2: Standards for the quantification of *F. culmorum* DNA in wheat flour samples. Fungal DNA of the strains *Fusarium graminearum* CBS 389.62 (Fg7.1) and *Fusarium culmorum* DSM 62191 (Fc1) was used as standards.

Sample type	Negative controls		Standards			
Sample No.	1	2	3-4	5-6	7-8	9-10
DNA content	No template	50 pg Fg7.1 in healthy wheat flour DNA 1:10	0.5 pg Fc1 in healthy wheat flour DNA 1:10	5 pg Fc1 in healthy wheat flour DNA 1:10	50 pg Fc1 in healthy wheat flour DNA 1:10	0.5 ng Fc1 in healthy wheat flour DNA 1:10

PCR amplification

The iCycler System (BioRad, Hercules, CA, USA) was used for the amplification and quantification of *Fusarium* spp. DNA in plant samples. Primers Fg16N F (ACAGAT GACAAGATTCAGGCACA) and Fg16N R (TTCTTTGACATCTGTTCAACCCA) were used to amplify a 280 bp fragment specific for *F. graminearum* (Nicholson et al., 1998). Primers OPT18 F (GATGCCAGACCAAGACGAAG) and OPT18 R (GATGCC AGACGCACTAAGAT) served to multiply a 472 bp fragment specific for *F. culmorum* (Schilling et al., 1996). Both primer pairs were derived from randomly amplified genomic fragments, the function of the target sequences is unknown.

The amplification mix for *F. culmorum*-specific PCR consisted of NH_4-reaction buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, 0.01% (v/v) Tween-20, pH 8.8 at 25°C; Bioline, Luckenwalde, Germany), 4 mM $MgCl_2$, 0.2 mM of each dATP, dTTP, dCTP and dGTP (Bioline, Luckenwalde, Germany), 0.3 µM of primer OPT18 F and OPT18 R, 0.25 u BIOTaq DNA polymerase (Bioline, Luckenwalde, Germany), 10 nM Fluorescein (BioRad, Hercules, CA, USA, diluted from 1 µM in 1x NH_4-reaction buffer, to collect

well factors, which is specific for the iCycler), 0.1x SYBR Green I solution (Invitrogen, Karlsruhe, Germany), 1 μl of template DNA and doubly distilled water (ddH$_2$O) filled to a total volume of 25 μl. The amplification mix for the *F. graminearum*-specific PCR consisted of 1x SYBR Premix Ex Taq (containing TaKaRa Ex Taq HS, dNTP Mixture, Mg^{2+}, and SYBR Green I, Takara Bio, Otsu, Japan), 0.3 μM of primer Fg16N F and Fg16N R, 10 nM Fluorescein, 1 μl of template DNA and ddH$_2$O filled to 25 μl. The detection of amplification products, based on the fluorescence of SYBR Green I, was performed with filters set at 490±10 nm for excitation and 530±15 nm for emission.

The PCR was performed with the following cycling protocol. Initial denaturation for 1.5 min at 95°C (the denaturation time is used by the thermocycler to collect data for the calculation of the well correction factors, which is needed to compensate for differences among wells of the microtiter plate) was followed by 35 cycles with 30 s at 94°C, 45 s at 64°C, and 45 s at 72°C. The final elongation was performed for 5 min at 72°C. During the PCR, the detection of fluorescence was carried out in the annealing step of each cycle. Following amplification, the melting curves were acquired by heating the samples to 95°C for 1 min, cooling to 55°C for one min and then slowly increasing the temperature from 65°C to 95°C at the rate of 0.5°C 10 s^{-1}, with continuous measurement of the fluorescence.

When an electrophoretic analysis of PCR products was necessary (only during the optimization process), 4 μl of the reaction mixture were combined with 2 μl of loading buffer (100 mM EDTA, 50% (v/v) Glycerol, 0.025% (w/v) bromphenol-blue) and loaded on a 1.7% (w/v) agarose gel prepared in TAE buffer. The electrophoresis and the documentation were carried out as described above.

PCR product and *Fusarium* spp. quantification

The quantitative data were exported from the iCycler software using the "Reports" function within the "PCR Standard Curve" sheet of the iCycler program. To relate the DNA quantities per PCR tube to the plant samples, the data had to be multiplied with a factor specific for the DNA extraction method used. These factors are 33.333 for the conversion of pg *Fusarium* spp. DNA per PCR sample to μg *Fusarium* spp. DNA per kg wheat flour and 66.667 to convert the pg *Fusarium* spp. DNA per PCR sample to μg *Fusarium* spp. DNA per kg maize debris flour. Different factors for wheat flour and maize debris are needed because the weights of these kinds of samples as used in the

optimized extraction protocol differ (see Material and Methods, Quantitative DNA extraction from plant material).

Results and Discussion

Optimization of the DNA extraction

Our goal was to optimize DNA extraction for invariable yield and constant PCR efficiency. We started with samples of 100 mg wheat flour in 1 ml CTAB buffer using a protocol developed for agar plaques (Brandfass and Karlovsky, 2006) and experienced a large variation among PCR signal among sample replicas. The same was true for maize stem flour, prepared from maize residues collected in the field after harvest in autumn or in spring of the following year. Furthermore, the high starch and protein content of wheat flour samples interfered with DNA precipitation, resulting in large pellets that were difficult to dissolve.

Mulfinger et al. (2000) reported that sampling technique has a major influence on the analytical results in *Fusarium* contamination studies. The sample size is a critical factor for the quantification of fungal biomass in plant samples, as fungal growth is inhomogeneous within a kernel and variable among kernels. To reduce the effect of this variability on the DNA yield, we upscaled the DNA extraction procedure as follows. For each sample, 500 g of wheat was ground in a hammer mill and one gram of the resulting flour was used for DNA extraction with 10 ml of CTAB buffer. The homogenization in a reciprocal mill used in the original protocol with agar plaques was replaced with a short treatment in an ultrasonic bath. Because wheat flour contains large amounts of starch and protein, which co-precipitate with DNA and hamper the dissolution of the DNA pellet, we prolonged the chloroform-isoamyl extraction to 10 minutes on ice. Furthermore, we found that shortening the PEG precipitation step improved reproducibility of PCR amplification; therefore the centrifugation was started immediately after the DNA extract was vigorously mixed with PEG/NaCl solution. In order to prevent PCR inhibition by substances co-extracted with DNA (Wilson, 1997), DNA dissolved in 200 μl TE still needed to be diluted 10-fold with water for PCR. One microliter of 10-times diluted DNA was used as a template per 25 μl reaction mixture. Increasing the amount of DNA has not improved the detection limit with DNA prepared according to our CTAB protocol nor with DNA extracted with DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Presumably, PCR inhibitors extracted from plant material were not

completely eliminated by the DNA purification protocols used, or the large amount of target DNA inhibited PCR per se. The increase of sample size and the modifications of the DNA purification protocol significantly improved the reproducibility of both DNA yield and PCR efficiency. The reproducibility of the yield in the optimized DNA extraction protocol is demonstrated in Fig. 1, the protocol is described in detail in Materials and Methods. We suppose that other protocols designed for DNA extraction from plant material would also work well with our assay (for example, see Manen et al., 2005).
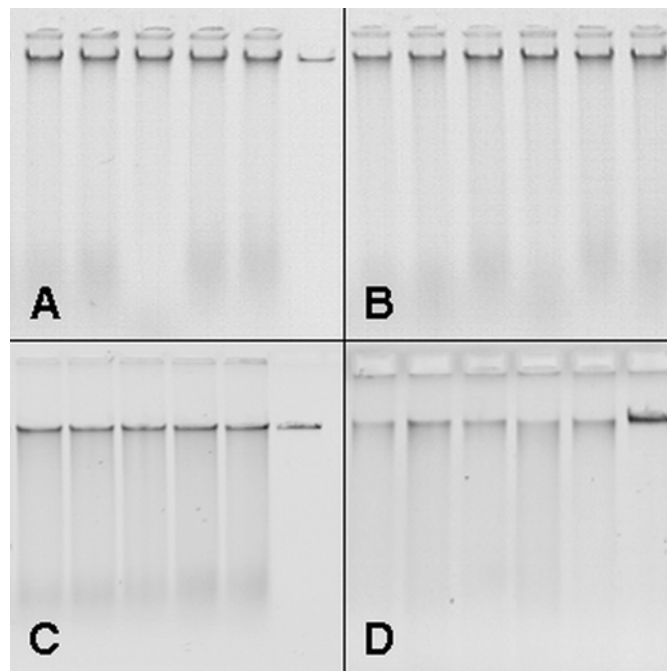
Figure 1: Yield and quality of DNA extracted from wheat flour and maize stem residues. Four microliters (of 200 µl total) of DNA extracted from 1 g of wheat flour or 500 mg of maize debris flour by a modified CTAB protocol (see Material and Methods for details) were loaded on a 0.8% agarose gel and separated at 4 V cm^{-1} for 60 min.
A) Lane 1-5: healthy wheat kernels (five identical samples), lane 6: 25 ng λ-DNA
B) Lane 1-6: infected wheat kernels (six different samples)
C) Lane 1-5: healthy maize stem residues (five identical samples), lane 6: 25 ng λ-DNA
D) Lane 1-5: infected maize stem residues (five different samples), lane 6: 25 ng λ-DNA

Optimization of PCR conditions

Our initial PCR experiments were done under the conditions described in the literature for classical PCR with primers Fg16N and OPT18 (Nicholson et al., 1998; Schilling et al., 1996). Because these conditions led to poor performance in the real-time mode, we re-optimized the primer concentration, polymerase activity, MgCl$_2$, SYBR Green I concentration and cycling parameters, using a native Taq polymerase (BioTaq, Bioline,

Luckenwalde, Germany). The performance of the optimized assay for *F. culmorum* was satisfactory, but the sensitivity of the *F. graminearum*-assay was not sufficient for wheat flour samples. Furthermore, unspecific products were formed in samples with a low concentration of *F. graminearum* DNA. After comparing different commercial kits and ready-made polymerase cocktails, we found that "SYBR Premix Ex Taq" (Takara Bio, Otsu, Japan) secured the best performance for the *F. graminearum*-assay, lowering the detection limit tenfold as compared with our reaction mix based on native Taq polymerase.

The specificity of the primers we selected for our assay was evaluated in the literature as follows: Fg16N was tested with 21 *F. culmorum* isolates, 24 *F. graminearum* isolates, 20 isolates of other *Fusarium* species and 5 isolates of other fungal species associated with cereals (Nicholson et al., 1998). O'Donnell et al. (2004) proposed the existence of nine phylogenetically distinct species within the *F. graminearum* clade. In Europe and America only lineage 7 has so far been reported (Ward et al., 2002; O'Donnell et al., 2000). It is not sure that all the lineages proposed by O'Donnell et al. can be detected with the help of Fg16N F/R primers. These primers amplify a section of the sequence amplified with the Fg16 F/R primer set (Nicholson et al., 1998). According to Waalwijk et al. (2003), *F. graminearum* lineages 1, 2, 6 and 7 were amplified using the Fg16 F/R primer set, whereas no PCR products were detected for lineages 3, 4 and 5.

F. culmorum-specific primers OPT18 were tested in the original publication with 69 *F. culmorum* isolates, 34 *F. graminearum* isolates, 25 isolates of other *Fusarium* species and 27 isolates of other fungal species associated with cereals (Schilling et al., 1996). All tests confirmed the specificity of OPT18 for *F. culmorum*.

Because PCR conditions used in specificity tests reported in the literature were different from the conditions of our assay, we re-evaluated the primer specificity in the real-time PCR. A range of fungal species regularly encountered on cereals was tested (Table 1). All *F. culmorum* and *F. graminearum* (12 isolates of each species) tested positively in the corresponding assays generating PCR products with the expected melting temperatures. There was no cross-reaction between *F. culmorum* and *F. graminearum*. All other fungal isolates listed in Table 1 tested negatively in both assays. The amplification of *F. culmorum* and *F. graminearum* was not inhibited by the presence of a large excess of wheat DNA (10 ng wheat DNA in a reaction containing 10 pg of *Fusarium* spp. DNA) nor did pure wheat DNA generate any signal under these conditions.

Standards for quantitative real-time PCR

As in any indirect analytical method, the quality of the standard curve is crucial for quantitative real-time PCR. Coamplification of specific plant DNA sequences (Winton et al., 2002) cannot serve as an internal standard because plant DNA in maize debris is degraded by saprophytes to a variable degree. Mixing defined amounts of fungal mycelium with uncontaminated plant material is probably the best way of preparing standard curves in *Fusarium* biomass determination (Lüders et al., *in preparation*). Fungal biomass in our plant samples was very low, however, making the preparation of defined mycelium/plant mixtures difficult. A naturally infected maize debris sample from heavily infected material contained about 1 µg *F. graminearum* DNA per gram, the least colonized wheat and maize samples contained *F. graminearum* DNA under the detection limit of the method. Therefore, the calibration curve was constructed with pure *F. graminearum* DNA dissolved in the matrix (DNA extracted from healthy maize or wheat flour, Fig. 2).
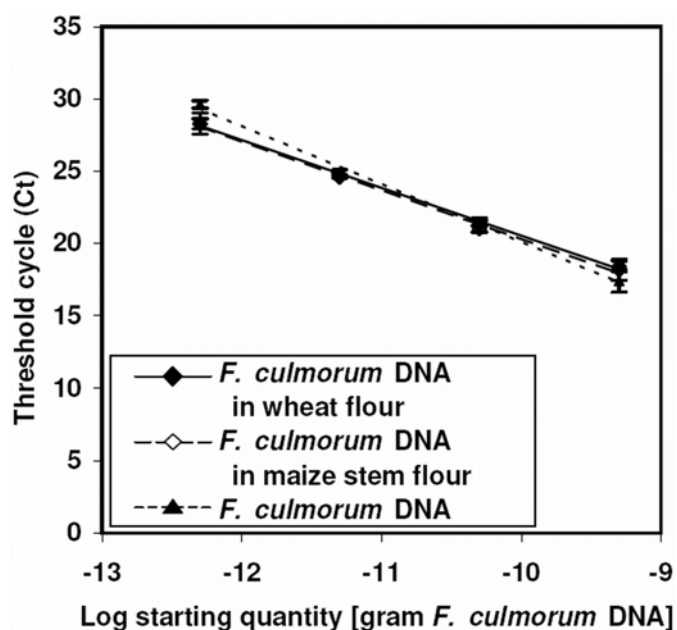
Figure 2: Standard curves used for the quantification of *F. culmorum* by real-time PCR. PCR was performed in a real-time thermocycler with primer pairs specific for *F. culmorum* and SYBR Green I fluorescence detection as described in Materials and Methods. Standard curves consisted of 10-fold serial dilutions from 0.5 pg to 0.5 ng *F. culmorum* DNA in different matrices as specified in the inlet. Threshold cycles (Ct) means and standard deviations of three replicas were plotted against amount of *F. culmorum* DNA (logarithmic scale) and linear regression equations were calculated. R^2 (square of the correlation coefficient) and PCR efficiency for the three matrices were: 0.998/100% for maize stem debris, 0,997/97% for wheat flour and 0.996/77% for water.

In case of wheat flour, the calibration curve represents a range of 16.7 ng to 16.7 μg *Fusarium* spp. DNA per g wheat flour, while the standard curve for maize stem flour ranged from 33.3 ng to 33.3 μg *Fusarium* spp. DNA per g material. The upper limits of the detection lies outside the standard curve, but the amplification of field samples never resulted in Ct values lower than the highest DNA concentration used in the standard curve. For comparison, maize cobs fully colonized by *F. graminearum* after an artificial inoculation contained up to 143 μg *F. graminearum* DNA per gram maize material.

Comparison with other PCR-based methods

Qualitative PCR assays to detect a *Fusarium* species (Demeke et al., 2005; Jurado et al., 2005; Nicolaisen et al., 2005) are suitable for some purposes, but epidemiological questions require the quantification of the infecting agents. Real-time PCR assays available so far for the quantification of toxigenic *Fusarium* spp. in infected plants are costly because they utilize doubly-labeled probes in combination with kit-based DNA extraction (Waalwijk et al., 2004). A recently published competitive microsatellite amplification-based *F. graminearum* detection uses a kit-based DNA extraction, labeled primers and capillary electrophoresis of PCR products of co-amplified microsatellite alleles originating from a target strain and another strain used as a standard (Naef et al., 2006). The method is basically unsuitable for naturally infected material because it requires prior knowledge of microsatellite alleles present in the target strain. Further serious drawbacks are the need to weigh a separate mycelium standard for each sample and the restriction of the quantification range to just two orders of magnitude.

Our method allows the quantification of *F. culmorum* and *F. graminearum* at low costs in a high-throughput environment suitable for epidemiological studies. Concerning costs, the price of a DNA extraction kit for 50-100 mg plant material varies between 2.0 to 2.5 € per sample while the value of chemicals used in our protocol to extract DNA from 1 g material is 0.40 €, the working time is similar for our assay and the DNeasy Plant Kit. The replacement of doubly-labeled hybridization probes by SYBR Green I-based detection further lowers the costs of the assay: a TaqMan probe used up in one reaction costs about 0.1 €, the costs of the SYBR Green dye is about 0.1 cent. The costs of other reaction components and disposables except DNA polymerase are the same for all PCR-based methods. The demand for high-quality DNA polymerases (e.g., hot-start modification) tends to increase with the complexity of the method. For example, both Waalwijk et al. (2004) and Reischer et al. (2004a) use hot start polymerase in their

TaqMan-based assays for *Fusarium* spp. Our assay for *F. graminearum* uses hot-start polymerase as a component of the SYBR Premix Ex Taq, while our *F. culmorum* assay uses a regular native Taq polymerase.

Compared with other published PCR assays for *Fusarium* spp., our method uses larger amounts of material, which reduces the sampling error by improving the representation of inhomogeneous material in the sample (Mulfinger, 2000).

Analysis of field samples

The method has been routinely used in our laboratory in projects on the epidemiology of FHB. Five hundred and seventy wheat flour samples and 310 maize debris samples collected throughout Germany from 2002 to 2005 were analyzed recently, while 2000 samples of wheat stem DNA extracted with a similar protocol were analyzed for the presence of each species in another project.

In accordance with Brandfass and Karlovsky (2006) and Waalwijk et al. (2003), *F. graminearum,* and to a lesser extent *F. culmorum,* play a dominant role in the infection of wheat kernels leading to FHB symptoms. The proportion of *F. graminearum* and *F. culmorum* depends on the growing season and other parameters.

In maize debris samples, *F. culmorum* is found more often than in wheat kernels, probably due to a higher saprophytic fitness of this fungus, but *F. graminearum* is still the dominant species. Detailed data are being prepared for publication.

Conclusions

The method developed in this study is suitable for cost-effective, high throughput quantification of *F. graminearum* and *F. culmorum* in plant material by real-time PCR. Extraction of DNA from 1 g of plant material, rather than from 50-100 mg usually used with commercial kits, substantially reduces sampling error caused by the inhomogeneous distribution of fungal mycelium in the matrix. The quantification limit for each species is 0.5 pg of genomic DNA. The assay is suitable for the analysis of a large number of samples for *F. culmorum* and *F. graminearum,* which is particularly useful in epidemio-logical studies as exemplified by our investigation of a large collection of wheat flour and maize debris samples.

Acknowledgements

Literature Cited

Anonymous. 1999. Verordnung über Höchstmengen an Mykotoxinen in Lebensmitteln (Mykotoxin-Höchstmengenverordnung – MHmV), p. 1248-1251 Bundesgesetzblatt, Vol. 1. Bundesanzeiger Verlagsges.m.b.H., Bonn.

Anonymous. 2004. Verordnung zur Änderung der Mykotoxin-Höchstmengenverordnung und der Diätverordnung, p. 151-152 Bundesgesetzblatt, Vol. 1. Bundesanzeiger Verlagsges.m.b.H., Bonn.

Bai, G.H., and G. Shaner. 2004. Management and resistance in wheat and barley to Fusarium head blight. Annual Review of Phytopathology 42: 135-161.

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

Brandfass, C., and P. Karlovsky. 2006. Simultaneous detection of *Fusarium culmorum* and *F. graminearum* in plant material by duplex PCR with melting curve analysis. BMC Microbiology 6: 4.

Demeke, T., R.M. Clear, S.K. Patrick, and D. Gaba. 2005. Species-specific PCR-based assays for the detection of *Fusarium* species and a comparison with the whole seed agar plate method and trichothecene analysis. International Journal of Food Microbiology 103: 271-284.

D'Mello, J.P.F., C.M. Placinta, and A.M.C. Macdonald. 1999. *Fusarium* mycotoxins: a review of global implications for animal health, welfare and productivity. Animal Feed Science & Technology 80: 183-205.

Ecker, D.J., R. Sampath, P. Willett, J.R. Wyatt, V. Samant, C. Massire, T.A. Hall, K. Hari, J.A. McNeil, C. Buchen-Osmond, and B. Budowle. 2005. The Microbial Rosetta Stone Database: A compilation of global and emerging infectious microorganisms and bioterrorist threat agents. BMC Microbiology 5: 19.

Edwards, S.G., J. O'Callaghan, and A.D.W. Dobson. 2002. PCR-based detection and quantification of mycotoxigenic fungi. Mycological Research 106: 1005-1025.

Jurado, M., C. Vazquez, B. Patino, and M.T. Gonzalez-Jaen. 2005. PCR detection assays for the trichothecene-producing species *Fusarium graminearum*, *Fusarium culmorum*, *Fusarium poae*, *Fusarium equiseti* and *Fusarium sporotrichioides*. Systematic and Applied Microbiology 28: 562-568.

Leonard, K.J., and W.R. Bushnell, (eds.) 2003. Fusarium Head Blight of Wheat and Barley, pp. 1-512. The American Phytopathological Society, St. Paul, Minnesota, USA.

Manen, J.-F., O. Sinitsyna, L. Aeschbach, A. Markov, and A. Sinitsyn. 2005. A fully automatable enzymatic method for DNA extraction from plant tissues. BMC Plant Biology 5: 23.

Martin, K.J., and P.T. Rygiewicz. 2005. Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. BMC Microbiology 5: 28.

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

Mulfinger, S., Niessen, L. and Vogel, R.F. 2000. PCR based quality control of toxigenic *Fusarium* spp. in brewing malt using ultrasonication for rapid sample preparation. Advances in Food Science 22: 38-46.

Murray, M.G., and W.F. Thompson. 1980. Rapid Isolation of High Molecular-Weight Plant DNA. Nucleic Acids Research 8: 4321-4325.

Naef, A., M. Senatore, and G. Defago. 2006. A microsatellite based method for quantification of fungi in decomposing plant material elucidates the role of *Fusarium graminearum* DON production in the saprophytic competition with *Trichoderma atroviride* in maize tissue microcosms. FEMS Microbiology Ecology 55: 211-220.

Nicholson, P., D.R. Simpson, G. Weston, H.N. Rezanoor, A.K. Lees, D.W. Parry, and D. Joyce. 1998. Detection and quantification of *Fusarium culmorum* and *Fusarium graminearum* in cereals using PCR assays. Physiological and Molecular Plant Pathology 53: 17-37.

Nicholson, P., E. Chandler, R.C. Draeger, N.E. Gosman, D.R. Simpson, M. Thomsett, and A.H. Wilson. 2003. Molecular tools to study epidemiology and toxicology of

Fusarium head blight of cereals. European Journal of Plant Pathology 109: 691-703.

Nicolaisen, M., A.F. Justesen, U. Thrane, P. Skouboe, and K. Holmstrom. 2005. An oligonucleotide microarray for the identification and differentiation of trichothecene producing and non-producing *Fusarium* species occurring on cereal grain. Journal of Microbiological Methods 62: 57-69.

O'Donnell, K., H.C. Kistler, B.K. Tacke, and H.H. Casper. 2000. Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. Proceedings of the National Academy of Sciences of the United States of America 97: 7905-7910.

O'Donnell, K., T.J. Ward, D.M. Geiser, H.C. Kistler, and T. Aoki. 2004. Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. Fungal Genetics and Biology 41: 600-623.

Parry, D.W., P. Jenkinson, and L. McLeod. 1995. Fusarium ear blight (scab) in small grain cereals -- A review. Plant Pathology 44: 207-238.

Peraica, M., B. Radic, A. Lucic, and M. Pavlovic. 1999. Toxic effects of mycotoxins in humans. Bulletin of the World Health Organization 77: 754-766.

Pestka, J.J., and A.T. Smolinski. 2005. Deoxynivalenol: Toxicology and potential effects on humans. Journal of Toxicology and Environmental Health-Part B-Critical Reviews 8: 39-69.

Pieters, M.N., J. Freijer, B.J. Baars, D.C.M. Fiolet, J. van Klaveren, and W. Slob. 2002. Risk assessment of deoxynivalenol in food: Concentration limits, exposure and effects, p. 235-248 Mycotoxins and Food Safety, Vol. 504. KLUWER ACADEMIC/PLENUM PUBL, New York.

Pirgozliev, S.R., S.G. Edwards, M.C. Hare, and P. Jenkinson. 2003. Strategies for the control of Fusarium head blight in cereals. European Journal of Plant Pathology 109: 731-742.

Reischer, G.H., M. Lemmens, A. Farnleitner, A. Adler, and R.L. Mach. 2004a. Quantification of *Fusarium graminearum* in infected wheat by species specific real-time PCR applying a TaqMan Probe. Journal of Microbiological Methods 59: 141-146.

Reischer, G.H., M. Lemmens, A. Farnleitner, A. Adler, and R.L. Mach. 2004b. Erratum to "Quantification of *Fusarium graminearum* in infected wheat by species specific real-time PCR applying a TaqMan probe" [J. Microbiol. Methods 59 (2004) 141-146]. Journal of Microbiological Methods 59: 437-437.

Schena, L., F. Nigro, A. Ippolito, and D. Gallitelli. 2004. Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. European Journal of Plant Pathology 110: 893-908.

Schilling, A.G., E.M. Moller, and H.H. Geiger. 1996. Polymerase chain reaction-based assays for species-specific detection of *Fusarium culmorum*, *F. graminearum*, and *F. avenaceum*. Phytopathology 86: 515-522.

Schnerr, H., R.F. Vogel, and L. Niessen. 2002. Correlation between DNA of trichothecene-producing *Fusarium* species and deoxynivalenol concentrations in wheat-samples. Letters in Applied Microbiology 35: 121-125.

Snijders, C.H.A. 1990. Systemic fungal growth of *Fusarium culmorum* in stems of winter wheat. Journal of Phytopathology-Phytopathologische Zeitschrift 129: 133-140.

Snijders, C.H.A. 2004. Resistance in wheat to *Fusarium* infection and trichothecene formation. Toxicology Letters 153: 37-46.

Stewart, C.N., and L.E. Via. 1993. A rapid CTAB DNA isolation technique useful for rapid fingerprinting and other PCR applications. Biotechniques 14: 748-751.

Waalwijk, C., P. Kastelein, I. de Vries, Z. Kerenyi, T. van der Lee, T. Hesselink, J. Kohl, and G. Kema. 2003. Major changes in *Fusarium* spp. in wheat in the Netherlands. European Journal of Plant Pathology 109: 743-754.

Waalwijk, C., R. van der Heide, I. de Vries, T. van der Lee, C. Schoen, G. Costrel-de Corainville, I. Hauser-Hahn, P. Kastelein, J. Kohl, P. Lonnet, T. Demarquet, and G.H.J. Kema. 2004. Quantitative detection of *Fusarium* species in wheat using TaqMan. European Journal of Plant Pathology 110: 481-494.

Ward, T.J., J.P. Bielawski, H.C. Kistler, E. Sullivan, and K. O'Donnell. 2002. Ancestral polymorphism and adaptive evolution in the trichothecene mycotoxin gene cluster of phytopathogenic *Fusarium*. Proceedings of the National Academy of Sciences of the United States of America 99: 9278-9283.

Wilson, I.G. 1997. Inhibition and facilitation of nucleic acid amplification. Applied and Environmental Microbiology 63: 3741-3751.

Winton, L.M., J.K. Stone, L.S. Watrud, and E.M. Hansen. 2002. Simultaneous one-tube quantification of host and pathogen DNA with real-time polymerase chain reaction. Phytopathology 92: 112-116.

Xu, X.M. 2003. Effects of environmental conditions on the development of Fusarium ear blight. European Journal of Plant Pathology 109: 683-689.

Chapter 4: Detection of *Fusarium graminearum* and *F. culmorum* in rachides from wheat ears afflicted with Fusarium head blight and in grain identifies *F. graminearum* as the major cause of FHB and DON contamination

Christoph Brandfass, Joachim Weinert, Petr Karlovsky

Abstract

Brandfass, C., Weinert, J., Karlovsky, P. 2006. Detection of *Fusarium graminearum* and *F. culmorum* in rachides from wheat ears afflicted with Fusarium head blight and in grain identifies *F. graminearum* as the major cause of FHB and DON contamination.

Fusarium head blight (FHB) is a disease of cereal crops, which has a severe impact on wheat production worldwide. Apart from reducing the yield and impairing grain quality, FHB leads to contamination of grain with mycotoxins. Numerous *Fusarium* species have been isolated from ears or kernels of cereal plants exhibiting scab symptoms. While the involvement of *F. graminearum* and *F. culmorum* is well established, the extent to which different *Fusarium* species might contribute to FHB and grain contamination with myco-toxins remains unclear. In order to distinguish between systemic colonization of the ear leading to FHB and infections confined to single-spikelets, we investigated 540 rachides prepared from wheat ears afflicted with FHB, which were collected throughout Germany in 2003. Essentially all rachides contained *F. graminearum, F. culmorum* or both, indicating that these two species were the causal agents of FHB in our samples. Rachides from fields with DON contamination higher than 500 µg DON per kg grain were colonized with *F. graminearum* to a higher extent (94%) as compared to rachides from fields yielding less contaminated grain (71%). To assess the relative importance of *F. graminearum* and *F. culmorum* for the contamination of grain with deoxynivalenol (DON), fungal DNA and DON concentration were determined in 54 samples of grain harvested from the same fields and in additional 334 grain samples collected throughout

Germany in 2003-2005. *F. graminearum* turned out to be the major cause of DON accumulation in wheat grain.

Additional keywords: *Gibberella zeae*, ear blight, wheat scab.

Introduction

Fusarium head blight (FHB) is a disease of cereal crops with a severe impact on wheat and barley production worldwide. The infection of heads of small grain cereals and maize plants with *Fusarium* spp. impairs both grain yield and quality (Leonard and Bushnell, 2003; Parry et al., 1995). The most serious consequence of FHB is the contamination of grain and cereal products with *Fusarium* mycotoxins (Pieters et al., 2002; Peraica et al., 1999), causing health risk in farm animals (Rocha et al., 2005; D'Mello et al., 1999) and humans (Pestka and Smolinski, 2005). Therefore, many countries have established maximum limits for *Fusarium* mycotoxins in grains, food and feeds (Anonymous, 2004; Anonymous, 1999; D'Mello et al., 1999). In spite of breeding efforts aiming at FHB-resistant cultivars (Bai and Shaner, 2004; Snijders, 2004) and the development of fungicides against FHB, the disease continues to pose a major challenge to grain growers all over the world (Aldred and Magan, 2004; Pirgozliev et al., 2003).

A key prerequisite for FHB reduction through crop management is to understand the epidemiology of the disease. In spite of extensive research into FHB worldwide, there are still significant gaps in our understanding of the etiology of the disease. Surprisingly, even the identity of the *Fusarium* species causally involved is not clarified definitely. While most authors maintain that five to seventeen fungal species participate on FHB development (Edwards, 2004; Doohan et al., 2003; Pirgozliev et al., 2003; Xu, 2003; Birzele et al., 2002; Parry et al., 1995), other argue that only *F. graminearum* and *F. culmorum* are causally involved in the disease (Champeil et al., 2004; Waalwijk et al., 2003, Weinert, pers. commun.). *F. graminearum* is supposed to dominate hotter central Europe, whereas *F. culmorum* shall occur mainly in cooler maritime regions of northwest Europe (Parry et al., 1995). The controversy about the number of *Fusarium* species involved in FHB arose from the fact that a number of species have been isolated from infected or even healthy-looking ears collected in the field (Nicholson et al., 2003; Bottalico and Perrone, 2002; Parry et al., 1995), but it is not known which actively contribute to FHB. Many species might just grow saprophytically on the spike surface or within the dead tissue, which is normally removed during grain cleaning and processing.

Some species might infect a single spikelet without spreading along the ear, which is not expected to cause typical FHB symptoms. Therefore, the first goal of our work was to find out to which extent rachides of wheat ears afflicted with FHB are colonized by the DON producers *F. graminearum* or *F. culmorum*. A species-specific duplex PCR assay based on melting curve analysis (without electrophoresis) was used to facilitate processing the large number of samples.

Both *F. graminearum* and *F. culmorum* produce two toxicologically relevant groups of mycotoxins, trichothecenes (nivalenol, deoxynivalenol (DON) and their acetylated derivatives) and zearalenone and its derivatives. DON is known to be a virulence factor of *F graminearum* in wheat (Langevin et al., 2004; Bai et al., 2002; Proctor et al., 1995), thus having a direct role in the disease. The correlation coefficient for DON and DNA content were reported to be 0.68 (Nicholson et al., 2003) and 0.82 (Waalwijk et al., 2004) for the sum of *F. culmorum* and *F. graminearum* DNA, and 0.96 in artificially inoculated wheat samples for *Fusarium* species containing biosynthetic genes for DON (Schnerr et al., 2002). Therefore, the presence of the trichothecene in grain can be explained to a large extent by the activity of these *Fusarium* species. Furthermore, a strong correlation between the severity of FHB and the DON level after artificial inoculation with *Fusarium* spp. was found (Haidukowski et al., 2005; Mesterhazy et al., 2005). In contrast, the relative contribution of *F. graminearum* and *F. culmorum* to the contamination of grain with trichothecenes is not known. Therefore, the second goal of our work was to determine the association between the amount of DON accumulated in grain and the biomass of each fungus, determined by quantitative real-time PCR.

Materials and Methods

Qualitative detection of *F. graminearum* and *F. culmorum* in wheat rachides
The qualitative determination of *F. graminearum* and *F. culmorum* in wheat rachides was done following the protocol of Brandfass and Karlovsky (2006a).

Preparation of wheat grain
Wheat grain samples (88-92% dry matter) were separated from the chaff by airflow and sieve cleaning with a stationary threshing machine, while particular attention was paid to the complete removal of rachides and the glumes. 500 g-portions of cleaned grain were

ground in a cross hammer mill (Cross Beater Mill SK 1, bottom sieve 2 mm, Retsch, Haan, Germany), the flour was mixed and 40 g sub-samples were stored at -20°C.

Quantitative DNA extraction from plant material

To extract DNA from ground wheat flour, 1 g of flour was blended in a 50 ml-tube (Sarstedt, Nümbrecht, Germany) with 10 ml CTAB-buffer (10 mM Tris, 20 mM EDTA, 0.02 M cetyltrimethylammonium bromide (CTAB, Merck, Darmstadt, Germany), 0.8 M NaCl, 0.03 M N-laurylsarcosine (Fluka, Buchs, Switzerland), 0.13 M sorbitol, 1% (w/v) polyvinylpolypyrolidone, pH set to 8.0 with NaOH (Merck, Darmstadt, Germany). 20 µl mercaptoethanol (Fluka, Buchs, Switzerland) and 0.2 mg proteinase K (from a stock solution 20 mg ml^{-1}, Merck, Darmstadt, Germany) were added shortly before use. The mixture was treated for 5 sec in an ultrasonic bath (Sonorex RK 100, Bandelin, Berlin, Germany). After an incubation at 42°C for 10 min, followed by 10 min at 65°C (the content of the tubes was mixed every 3 min), 8 ml of chloroform-isoamyl alcohol (24:1, Roth, Karlsruhe, Germany) were added. The samples were thoroughly emulsified, incubated for 10 min on ice and centrifuged for 10 min at 5,000 g at room temperature. 600 µl of the upper phase were transferred to a 1.5-ml tube containing 194 µl of a 30% (w/v) polyethylene glycol 6000 (Serva, Heidelberg, Germany) solution and 100 µl 5 M NaCl, mixed, and centrifuged for 15 min at 15,000 g at room temperature. The pellet was washed with 70% (v/v) ethanol (Roth, Karlsruhe, Germany), dried and dissolved in 200 µl TE (10 mM Tris, 1 mM EDTA, pH set to 8.0 with HCl). To ensure that the DNA was dissolved completely, the pellet covered by TE buffer was incubated over night at 4°C. The quality and quantity of DNA were assessed by electrophoresis in 0.8% (w/v) agarose gels (Cambrex, Rockland, ME, USA) prepared in TAE buffer (40 mM Tris, 1 mM EDTA, pH set to 8.5 with acetic acid). The electrophoresis was carried out at 4 V cm^{-1} for 60 min. Double-stranded DNA was stained with ethidium (ethidium bromide, 2 mg l^{-1}), (Applichem, Darmstadt, Germany). The gels were documented with the help of a digital imaging system (Vilber Lourmat, Marne la Vallee, France). Densitometry values were compared with those of Lambda Phage DNA (methylated, from *Escherichia coli* host strain W3110, Sigma, Taufkirchen, Germany). The densitometry was performed using Multi Analyst-Software (BioRad, Hercules, CA, USA). A 1:10-dilution of the DNA solution was used in the PCR.

Preparation of standards for quantitative real-time PCR

The fungal cultures were maintained and their DNA was extracted as described in (Brandfass and Karlovsky, 2006a). DNA standards and DNA from unknown samples have to be amplified under identical conditions. Therefore, quantified standard-DNA of *Fusarium* spp. was mixed with DNA extracted from uncontaminated wheat flour to imitate matrix effects. A dilution series from 0.5 pg to 0.5 ng of *Fusarium* spp. DNA with a dilution factor of 10 was produced separately for *F. culmorum* and *F. graminearum* DNA in the matrix of plant DNA. For each pathogen a standard curve for wheat flour was set up. In addition to the standard curve, two negative controls were processed in each PCR set, one containing no template (water) and one containing 50 pg of the other *Fusarium* species (e.g. *F. graminearum* for *F. culmorum* analysis) mixed with DNA of a healthy plant.

PCR amplifikation

The iCycler System (BioRad, Hercules, CA, USA) was used for the amplification and quantification of *Fusarium* spp. DNA in plant samples. Primers Fg16N F (ACAGAT GACAAGATTCAGGCACA) and Fg16N R (TTCTTTGACATCTGTTCAACCCA) were used to amplify a 280 bp fragment specific for *F. graminearum* (Nicholson et al., 1998). Primers OPT18 F (GATGCCAGACCAAGACGAAG) and OPT18 R (GATGCC AGACGCACTAAGAT) served to multiply a 472 bp fragment specific for *F. culmorum* (Schilling et al., 1996). All primers were synthesized by Operon Biotechnologies (Cologne, Germany). Both primer pairs were derived from randomly amplified genomic fragments, the function of the target sequences is unknown.

The amplification mix for *F. culmorum*-specific PCR consisted of NH$_4$-reaction buffer (16 mM (NH$_4$)$_2$SO$_4$, 67 mM Tris-HCl, 0.01% (v/v) Tween-20, pH 8.8 at 25°C; Bioline, Luckenwalde, Germany), 4 mM MgCl$_2$, 0.2 mM of each dATP, dTTP, dCTP and dGTP (Bioline, Luckenwalde, Germany), 0.3 µM of primer OPT18 F and OPT18 R, 0.25 u BIOTaq DNA polymerase (Bioline, Luckenwalde, Germany), 10 nM Fluorescein (BioRad, Hercules, CA, USA, diluted from 1 µM in 1x NH$_4$-reaction buffer, to collect well factors, which is specific for the iCycler), 0.1x SYBR Green I solution (Invitrogen, Karlsruhe, Germany), 1 µl of template DNA and doubly distilled water (ddH$_2$O) filled to a total volume of 25 µl. The amplification mix for the *F. graminearum*-specific PCR consisted of 1x SYBR Premix Ex Taq (contains TaKaRa Ex Taq HS, dNTP Mixture, Mg^{2+}, and SYBR Green I, Takara Bio, Otsu, Japan), 0.3 µM of primer Fg16N F and

Fg16N R, 10 nM Fluorescein, 1 μl of template DNA and ddH$_2$O filled to 25 μl. The detection of amplification products, based on the fluorescence of SYBR Green I, was performed with filters set at 490±10 nm for excitation and 530±15 nm for emission. The PCR was performed with the following cycling protocol. Initial denaturation for 1.5 min at 95°C (the denaturation time is used by the thermocycler to collect data for the calculation of the well correction factors, which is needed to compensate for differences among wells of the microtiter plate) was followed by 35 cycles with 30 s at 94°C, 45 s at 64°C, and 45 s at 72°C. The final elongation was performed for 5 min at 72°C. During the PCR, the detection of fluorescence was carried out in the annealing step of each cycle. Following amplification, the melting curves were acquired by heating the samples to 95°C for 1 min, cooling to 55°C for one min and then slowly increasing the temperature from 65°C to 95°C at the rate of 0.5°C 10 s^{-1}, with a continuous measurement of the fluorescence.

PCR product and *Fusarium* spp. quantification

The quantitative data were exported from the iCycler software using the "Reports" function within the "PCR Standard Curve" sheet of the iCycler program. To relate the DNA quantity per PCR tube (in pg) to DNA content in plant sample (in μg kg^{-1}), the result of PCR analysis was multiplied with a factor 33.3, which reflects dilutions performed during DNA extraction.

Quantitative analysis of deoxynivalenol (DON)

The quantification of deoxynivalenol was performed with the Ridascreen DON enzyme immunoassay (R-Biopharm, Darmstadt, Germany) following the manufacturers instructions with the following modification. Five grams of ground wheat grain were added to 25 ml of distilled water and mixed for 60 min on a horizontal shaker (100 rpm) at 4°C. An aliquot of 1 ml was transferred to a 1.5-ml tube and centrifuged for 10 min at 15,000 g. Fifty microliters of the resulting supernatant were used in the test. The standard curve was supplemented with an additional standard of 66.0 μg kg^{-1} DON and the absorbance of the wells was read at 405 nm (reference 595 nm).

Field samples

Field samples were taken throughout Germany in cooperation with the Federal Plant Protection Extension Services. To identify the infectious agent causing FHB, wheat heads with FHB symptoms were sampled in 2003 at the milk development stage from 54 fields distributed throughout Germany. The rachides were isolated from 10 spikes

selected from each field, dissected into pieces spanning 2-3 segments of the spike axis and surface-sterilized. These segments were used to isolate the colonizing fungi on agar plates containing potato dextrose medium (PDA; Merck, Darmstadt, Germany). Then the segments were used for DNA extraction and determination of the presence of *F. graminearum* and *F. culmorum* as described in Brandfass and Karlovsky (2006a). The DON-content of grain from the same fields was determined after the harvest.

In 388 wheat flour samples collected throughout Germany from 2003 to 2005, DON content was determined by ELISA (see above). Samples with DON levels exceeding 300, 200 and 100 µg kg^{-1} in years 2003, 2004 and 2005, respectively, were selected for the quantification of *F. graminearum* and *F. culmorum* DNA by real-time PCR. These year-specific thresholds were set with consideration of the general level of DON contamination, the available sample number and the PCR detection limit (Brandfass and Karlovsky, 2006b). Further 30, 25 and 14 samples from year 2003, 2004 and 2005, respectively, were analyzed to represent samples with a low DON content. An overview of all samples is given in Table 1. DNA yield was checked by agarose electrophoresis for each sample prior PCR.

Table 1: Number and distribution of wheat rachides and grain samples 2003 – 2005.

|	Number of fields for rachides analysis (10 rachides each)	Number of wheat grain samples		
Federal States	**2003**	**2003**	**2004**	**2005**
Bavaria	4	4	6	7
Brandenburg	3	4	6	10
Lower Saxony	21	51	90	31
Mecklenburg Western Pomerania	6	6	1	
Northrhine-Westphalia	1	2		
Rhineland Palatinate	13	29	44	11
Saarland			1	
Saxony		6	11	17
Schleswig Holstein		2	1	
Thuringia	6	16	14	18
total	**54**	**120**	**174**	**94**

Weather data

Weather data recorded at the meteorological station in Göttingen, Germany were obtained online from the DWD (German Meteorological Service) in Offenbach via the Central Institution for Decision Support Systems in Crop Protection (ZEPP). The data set included daily readings of temperature [°C], relative humidity [%], both recorded 2 m above ground, and precipitation [mm] from 2003 to 2005.

Results

Analysis of wheat rachides for *Fusarium* colonization

Our first goal was to identify the causal agent of FHB and DON contamination in Germany. To differentiate systemic infection spreading along the ear from the infection confined to a single-spikelet and from mycelium and spores transferred to the ears by the wind, we analyzed fungal DNA within the rachides of wheat heads with FHB symptoms rather than extracting DNA from the entire heads. Qualitative duplex PCR with melting curve analysis was used for the detection of *F. graminearum* and *F. culmorum* in DNA extracted from each of the 540 rachides collected from 54 fields in the 2003 season. *F. graminearum* and/or *F. culmorum* DNA was found in 516 samples (96%), indicating that these two species were responsible for the brown coloration of the rachides. The surface-sterilized rachides sections were placed on PDA plates as described in Brandfass and Karlovsky (2006a). With a few exceptions, only one morphologically distinct fungal species grew out of each rachis. The morphology of the colonies was assessed visually and cultures deviating from a typical phenotype of *F. graminearum* and *F. culmorum* were transferred to SNA and identified microscopically. The only *Fusarium* species found apart from *F. graminearum* and *F. culmorum* was *F. poae*, occurring just in 3 instances in rachides, which also contained *F. graminearum* or *F. culmorum*, and in 4 instances in rachides where none of the other two *Fusarium* spp. was detected. Other fungal species occasionally recovered from the rachides were *Alternaria* spp., *Rhizoctonia cerealis* and *Epicoccum* spp.

A North-South gradient in the ratio of *F. graminearum* to *F. culmorum* frequently reported in the literature was not detectable (Fig. 1), because no significant correlation between the latitude of the field, from which the rachides originated, and the proportion of the occurrence of *F. graminearum* or *F. culmorum* was found (Pearson's test of correlation, α = 5%).
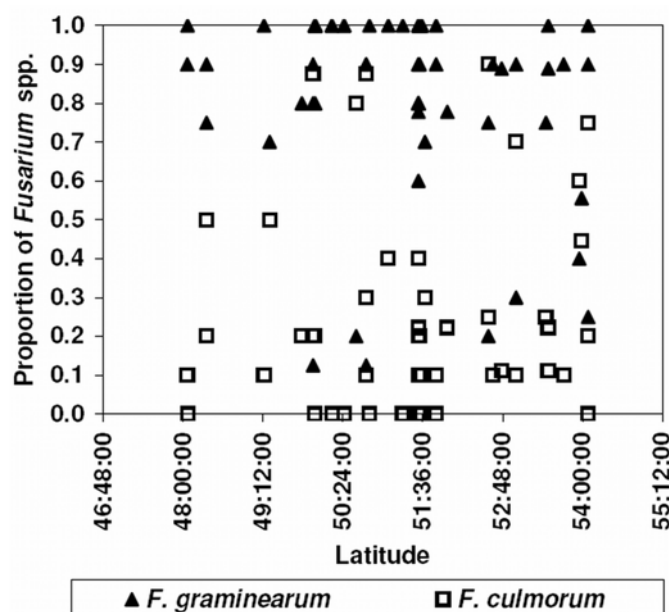
Figure 1: South-North distribution of *Fusarium* spp. The proportion of the occurrence of *F. graminearum* and *F. culmorum* in wheat rachides is drawn against the latitude of the origin of the samples. The rachides were prepared from wheat ears with symptoms of FHB collected throughout Germany in 2003 at the milk development stage (10 rachides per site).

Relationship between rachis colonization and the contamination of grain with DON

The results of DNA analysis were sorted according to the DON content of grain harvested from the corresponding fields in the fall of the same year. Fig. 2 summarizes the results. At 23 sites with wheat grain samples containing more than 500 µg per kg DON, 91.2% of the rachides contained *F. graminearum* and 4.4% contained *F. culmorum*, while both pathogens were detectable in remaining 4.4% of the samples. Grain from 31 sites contained less of DON than 500 µg per kg. A portion of 74.4% of rachides collected in these fields contained *F. graminearum* alone, 23.9% rachides contained *F. culmorum* and mixed infection by both pathogens was detected in 1.7% of the samples.

In addition to the determination of DON content, DNA of *F. graminearum* and *F. culmorum* was quantified in these grains by real-time PCR as described in Materials and Methods. The samples were grouped according to their DON content using a threshold of 500 µg kg^{-1} DON. Grain samples contaminated with more than 500 µg kg^{-1} DON (23 fields) contained 83 µg kg^{-1} *F. graminearum* DNA and 3 µg kg^{-1} *F. culmorum* DNA on the average, whereas grain contaminated with less DON than 500 µg kg^{-1}

contained 15 µg kg^{-1} *F. graminearum* DNA and 3 µg kg^{-1} *F. culmorum* DNA (Fig. 3). The difference in *F. graminearum* DNA content was significant (Mann test, $p < 0.0001$).
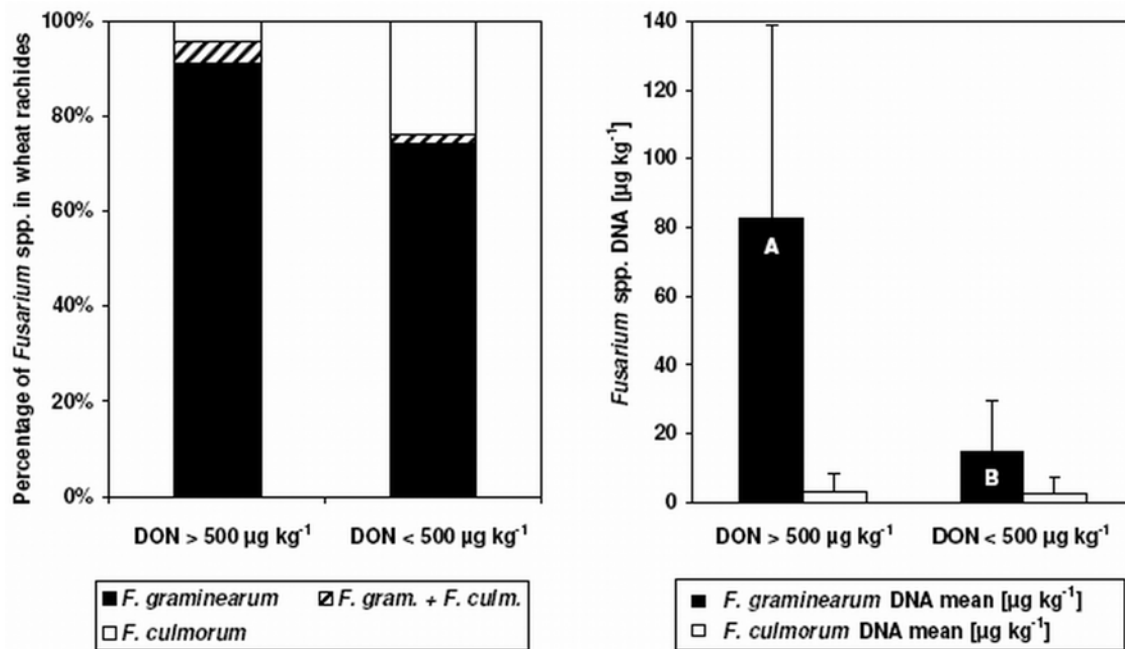
Figure 2: *F. graminearum* and *F. culmorum* DNA in wheat rachides in 2003. Wheat ears with symptoms of FHB were collected throughout Germany in 2003 at the milk development stage (10 ears per site). *Fusarium* DNA was analyzed by PCR and the results were sorted according to the DON content of grain harvested from the same field.

Figure 3: *Fusarium* DNA content of wheat grain with respect to the DON content (2003). Grain was harvested from the same fields used for collecting rachides. The *Fusarium* spp. DNA content was determined by real-time PCR and DON content was measured by ELISA as described in Materials and Methods section. The samples were grouped according to their DON content as indicated in the figure and the means and standard deviations of *F. graminearum* and *F. culmorum* DNA amounts were calculated for each group. The *F. graminearum* DNA contents differed significantly (Mann-Test, $p < 0.0001$).

Relationship between DON contamination and DNA content of grain

The analysis of rachides indicated that the major cause of DON contamination of grain was *F. graminearum.* However, the DON producer *F. culmorum* was found in a considerable number of rachides and also in grain samples. Therefore, we analyzed 388 wheat grain samples collected from 2003 to 2005 (Table 1) for their DON and *Fusarium* spp. DNA content. The 54 grain samples of 2003 evaluated in the context of the rachides analysis described above were a subset of 120 samples analyzed here from the 2003 harvest.

Looking at the whole time span from 2003 to 2005, the DON concentration correlated well with the *F. graminearum* DNA content (squared correlation coefficient $R^2 = 0.73$, p <0.001 for Pearson's test of correlation). The *F. culmorum* DNA content did not correlate with DON content. In line with the results of the rachides analysis, this finding indicates that the contamination of wheat grain with DON was caused mainly by *F. graminearum.* The contribution of *F. culmorum* to DON accumulation appears insignificant.

A comparison of the results for each year revealed clear differences. The slope of the regression line (Fig. 4) was highest in 2003 (24.0) and lowest in 2005 (5.3). The R^2 value as a measure of the quality of the correlation varied too, showing a large increase from 2003 to 2004 and 2005. The means of the DNA and DON contents in wheat of the analyzed years are given in Fig. 5.
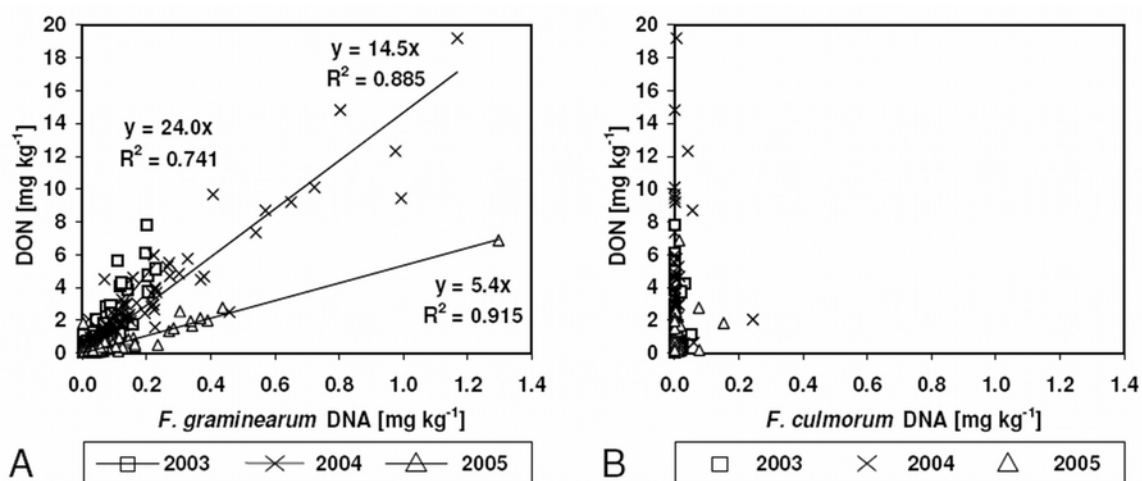
Figure 4: Correlation of DON amount in wheat grain with *Fusarium* spp. DNA content (2003-2005). The DON content of the grain is plotted against A) *F. graminearum* DNA and B) *F. culmorum* DNA for each year separately. Linear regression was calculated for each pair of variables. The regression equation and R^2 (square of the correlation coefficient) are shown in the graph.
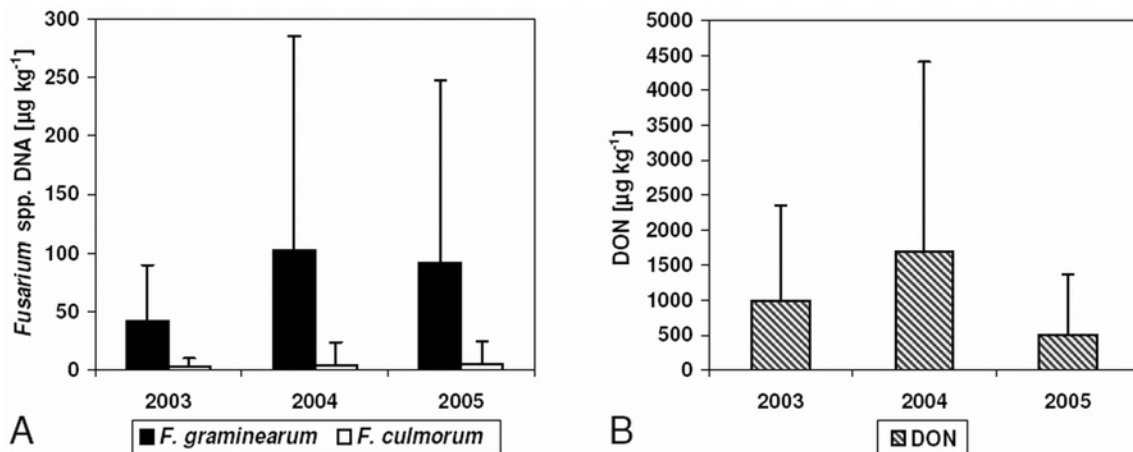
Figure 5: *Fusarium* spp. DNA and DON content in wheat grain collected throughout Germany 2003-2005. Means and standard deviations of all analyzed grain samples are shown.

Weather data

Weather data of the meteorological station of the DWD (German Meteorological Service) in Göttingen were used, because it is settled approximately in the centre of Germany and many samples came from the area around Göttingen each year. Averages of two time spans were calculated for each year (Table 2). The first time span includes the period of wheat flowering, extended by seven days prior the bloom according to Hooker et al. (2002) and De Wolf et al. (2003). This period represents the time when the infection with *Fusarium* spp. occurs. A second set of weather data was collected from the end of bloom up to the harvesting season. This phase represents the fungal development during grain ripening.

The infection phase of the year 2003 is characterized by the most precipitation and highest temperature in comparison to the other years. Concerning the fungal development phase, the most humid growing season was the year 2004, followed by 2005 and finally the comparable dry fungal development phase 2003.

Table 2: Weather condition in central Germany 2003-2005. Weather data of the meteorological station of the DWD in Göttingen were used. Averaged daily readings of temperature [°C] and relative humidity (Humidity in %), the sum of precipitation [mm] and the number of days with more than 3 mm precipitation are shown. The data are summarized for the periods "Head emergence and flowering" and "Ripening", which represent year-specific averaged growth stages of wheat in Göttingen. The ripening phase of 2004 is longer because a long rainy period delayed the harvest until September.

	Year	**2003**	**2004**	**2005**
Head emergence and flowering	Time span	30.05-15.06.	04.06-20.06	08.06-26.06
	Temperature [°C]	19.1	15.1	15.8
	Humidity [%]	76.3	78.8	75.0
	Precipitation [mm]	55.2	35.0	13.7
	# Precip > 3 mm	4	4	2
	Ranking infection	1	2	3
Ripening	Time span	16.06.-15.08.	21.06.-31.08	27.06-15.08
	Temperature [°C]	19.5	16.9	16.8
	Humidity [%]	67.8	76.9	76.9
	Precipitation [mm]	61.3	206.1	123.2
	# Precip > 3 mm	8	19	14
	Ranking fungal growth	3	1	2

Discussion

The aim of the study was firstly to find out whether *F. graminearum* and *F. culmorum* infection alone can explain FHB incidence in our sample set. Secondly, we investigated the relationship between DON contamination and the biomass of *F. graminearum and F. culmorum* with the goal of assessing the relative contribution of these species to DON accumulation in grain.

Causal agent of FHB

It is often stated in the literature that FHB is caused by a complex consisting of up to 17 fungal species, including mycotoxin producers *F. graminearum*, *F. culmorum*, *F. poae, F. avenaceum* and *F. sporotrichioides* (e.g., Edwards, 2004). An apparent support for this view is provided by publications reporting the isolation of numerous fungal species from surface-sterilized heads of small grain cereal plants (e.g., Birzele et al., 2002; Parry et al., 1995). However, these surveys do not distinguish between (i) fungi growing

saprophytically within dead surface tissues, which protect them from sodium hypochlorite used for the sterilization, (ii) fungal species, which can infect living tissue but remain confined to a single spikelet, and (iii) pathogens, which are able to penetrate the base of the spikelet, enter the rachis, grow within the rachis downwards and systemically colonize the ear. Only fungi of the last kind cause typical FHB symptoms, which are bleaching of the ear upwards the infection site, browning of the rachis downward the infection site carrying green spikelets at milk development stage, and colonization of the lower part of the ear in the later stages of the infection, which leads to the accumulation of high amounts of DON. We prepared rachides from wheat heads exhibiting FHB symptoms, collected from fields representing all climatic zones and major wheat growing areas in Germany. The rachides were investigated for *Fusarium* colonization by PCR and by the isolation of fungi from inside the surface sterilized rachides.

Our PCR analysis targeted *F. graminearum* and *F. culmorum*, which are the only producers of DON (Bottalico and Perrone, 2002), with the intention to subject fungi isolated from rachides not colonized by *F. graminearum* or *F. culmorum* to a detailed mycological analysis. However, it turned out that essentially all (96%) rachides from heads afflicted with FHB contained either *F. graminearum* or *F. culmorum*. The PCR result was confirmed by the analysis of fungal isolates originating from surface-sterilized rachides: apart from *F. graminearum* and *F. culmorum*, only *F. poae* has been recovered a couple of times from a total of over 500 rachides analyzed. We concluded that *F. graminearum* and *F. culmorum* are the only *Fusarium* species responsible for the systemic colonization of wheat heads afflicted with FHB. Other *Fusarium* species frequently isolated from wheat ears and kernels, notably *F. avenaceum*, *F. poae* and *F. sporotrichioides*, do not seem to be relevant for FHB.

Recently, a predominant presence of *F. graminearum* and *F. poae* has been reported from four European countries (Xu et al., 2005). In this work wheat heads were sampled randomly without taking into account the severity of FHB. The ability of the species to colonize ears systemically was not addressed. The result of Xu et al. therefore does not contradict to our conclusion. The abundance of *F. poae* in the collection by Xu et al. indicates that European populations of *F. poae* colonize dead tissues in the heads of cereal plants and infect single spikelets. *F. poae* has not been reported to colonize the axis and therefore does not cause typical FHB symptoms.

Authors claiming that FHB is caused by a complex of fungal species also regularly state that *F. culmorum* predominates at the cooler, maritime regions, whereas *F. graminearum* tend to predominate at the hotter central Europe (Parry et al., 1995). Germany extends from the northern coast up to the southern area settled in continental central Europe. Thus, a gradient of the proportion of *F. graminearum* and *F. culmorum* is to be expected. However, no such gradient was detectable in our sample set. This might be due to an extensive replacement of *F. culmorum* by *F. graminearum* in the whole area in recent years, as also discussed for the Netherlands (Waalwijk et al., 2003).

Relative contribution of *F. graminearum* and *F. culmorum* to DON accumulation

Three lines of evidence support the view that *F. graminearum* alone is responsible for the economically relevant contamination of wheat grain with DON. Firstly, the relative occurrence of *F. graminearum* as compared to *F. culmorum* in wheat rachides is higher in samples from fields yielding grain with a high DON content (Fig. 2). Secondly, the amount of *F. graminearum* DNA in wheat grain contaminated with DON is an order of magnitude higher than the amount of *F. culmorum* DNA (Fig. 3 and 4). The final and most important argument for an exclusive role of *F. graminearum* in DON contamination is a tight correlation between fungal DNA and DON content for *F. graminearum* and the lack of such correlation for *F. culmorum* (Fig. 4). The predominance of *F. graminearum* was also reported for wheat kernels from ears with FHB symptoms in the Netherlands (Waalwijk et al., 2003). It has repeatedly been reported that DON amounts in grain and the total content of *F. graminearum* and *F. culmorum* DNA in naturally infected material correlate well (Waalwijk et al., 2004; Nicholson et al., 2003). These results can be explained by the fact that the amount of *F. culmorum* DNA in our samples was one to two orders of magnitude lower than the amount of *F. graminearum* DNA. Providing that the relative amounts of *F. culmorum* and *F. graminearum* biomass in field material investigated in the work cited above were similar, the correlation between total *Fusarium* DNA and the DON content was largely determined by *F. graminearum*.

DON accumulation, FHB rating and virulence: a common lapse in the interpretation of their relationship

A tight correlation between DON content and FHB severity has been found in numerous field studies and construed to support the hypothesis that DON acts as virulence factor. Although the role of DON in virulence of *F. graminearum* has been unequivocally proven with the help of genetically engineered strains defective in the DON production

(Bai et al., 2002; Proctor et al., 2002; Desjardins et al., 2000; Proctor et al., 1997; Desjardins et al., 1996; Proctor et al., 1995), most of the reasoning to this end, based on the correlation of FHB rating with DON content, is principally flawed. For example, Wanyoike et al. (2002) found a significant correlation between the rating of head blight caused by *F. graminearum* isolates and the DON contents in infected grain (correlation coefficient 0.68-0.75) and concluded that the DON-producing capacity could, therefore, be a decisive factor of virulence of *F. graminearum*. The catch is that even if DON production had no affect on plant colonization and FHB rating, a close correlation between DON content and FHB rating would very likely occur, because both phenomena are controlled by the amount of fungal biomass in the ear. Rather than representing a cause-effect relationship, DON accumulation and FHB rating are two effects of a common cause. The logical lapse in the interpretation of DON accumulation in FHB-afflicted ears became so common that it has been perpetuated in numerous reviews (for example - Mesterhazy et al., 2005; Snijders, 2004; Wong et al., 1995).

DON productivity

Because the amount of *F. graminearum* DNA and DON content correlate well, it is generally assumed that the amount of DON produced by a unit of fungal biomass in a unit of time (DON productivity) is constant. A few studies, which addressed this question directly, support this view. For example, Cumagun et al. (2004) investigated the ability to colonize a susceptible wheat cultivar and DON accumulation for a segregating popu-lation of 50 progenies of a *G. zeae* cross in three environments. They found that the ratio of DON amount and fungal biomass did not change significantly among progenies and environments. DON production by *F. graminearum* in vitro increased with relative humidity (Beyer et al., 2005) and with temperature (Hope et al., 2005; Martins and Martins, 2002).

The capacity of *F. graminearum* to synthesize DON in grain (DON productivity) is represented by the slope of the regression line in the graph of DON concentration versus fungal DNA content. Surprisingly, we found large differences in DON productivity among the years 2003, 2004 and 2005 (Fig. 4). We can exclude the influence of agronomical factors because the data were derived from a large set comprising different planting areas, pre-crops and other agronomical conditions. A significant effect of DON production by *F. culmorum* is unlikely because the biomass of *F. culmorum* was much smaller than *F. graminearum* and it has not been correlated with DON content.

Field populations of *F. graminearum* consist of strains with different DON productivity (Gilbert et al., 2002; Mesterhazy, 2002). The differences in DON productivity among the years can therefore be explained by a shifting composition of *F. graminearum* populations in time, leading to the enrichment of strains with a lower capacity to produce DON in years 2004 and 2005 as compared to 2003. An alternative explanation is the effect of weather conditions, which varied largely from 2003 to 2005.

The effect of weather on FHB and DON production

Weather data collected in Göttingen are summarized in Table 2. The so called infection phase was established according to published prediction models (De Wolf et al., 2003; Hooker et al., 2002), the fungal development phase was defined as the time span from the end of the infection phase till the harvest.

Most *Fusarium* species produce inoculum, grow best, and are most pathogenic to cereal heads at warm temperatures and under humid conditions. Conditions favorable for *in vitro* growth are also generally the most favorable for mycotoxin production on cereal grains (Doohan et al., 2003). For example, Brennan et al. (2005) found that eight wheat cultivars inoculated with single isolates of *F. graminearum* contained higher amounts of fungal DNA at 20°C compared to 16°C.

The warm and humid weather in the infection phase of the year 2003 was most favorable for the infection, but during the following ripening phase the weather became hot and dry, hardly allowing fungal growth. This was the growing season least favorable for fungal growth, resulting in the lowest averaged DNA content (Fig. 5). In contrast, the DON production per fungal biomass was highest in 2003. Hope et al. investigated the effect of water activity ($a_{(w)}$ 0.99-0.85) and temperature (15 and 25°C) on the growth and DON production by *F. graminearum* on wheat grains. They found that the production of DON occurred over a much narrower range than the growth. The maximum DON production by *F. graminearum* occurred at high water activities at both temperature levels (Hope et al., 2005). The year 2003 allowed for fungal growth mainly in the short infection period, which was favorable for both DON production and *F. graminearum* development. We speculate that fungal growth and DON production was interrupted by the hot and dry period which followed. This may explain the highest DON production efficiency in combination with the lowest biomass in 2003 (Fig. 4).

The years 2004 and 2005 were both moderately warm and wet (Tab. 2), with 2004 having more precipitation in both infection and ripening phase implicating better

conditions for fungal development in both phases. As a result, the *F. graminearum* contamination as well as the total DON production was higher in 2004 than in 2005.

In both years, the biomass of *F. graminearum* could develop continuously, including periods of low DON production. The higher DON productivity in 2004 as compared to 2005 might be explained by a longer period of conditions favorable for DON production in 2004, as 2004 had more rain at comparable temperatures.

As a result, as successful infection in Germany does not need necessarily lead to a FHB epidemic, as might be interpreted from the risk assessment model of De Wolf et al. (2003). Another approach to identify important weather variables and their timing for predicting concentrations of DON in mature grain at wheat heading (Hooker et al., 2002) is in better accordance with our results.

Conclusion

Applying a rachides analysis of wheat ears afflicted with Fusarium head blight and grain analysis for DON and *Fusarium* spp. DNA, we showed that *F. graminearum* is the main causing agent of FHB and DON contamination of wheat in Germany. This is in accordance with Waalwijk et al. (2003), who experienced an increased prevalence of *F. graminearum* in the Netherlands.

Acknowledgements

Literature Cited

Aldred, D., and N. Magan. 2004. Prevention strategies for trichothecenes. Toxicology Letters 153: 165-171.

Anonymous. 1999. Verordnung über Höchstmengen an Mykotoxinen in Lebensmitteln (Mykotoxin-Höchstmengenverordnung – MHmV), p. 1248-1251 Bundesgesetz-blatt, Vol. 1. Bundesanzeiger Verlagsges.m.b.H., Bonn.

Anonymous. 2004. Verordnung zur Änderung der Mykotoxin-Höchstmengenverordnung und der Diätverordnung, p. 151-152 Bundesgesetzblatt, Vol. 1. Bundesanzeiger Verlagsges.m.b.H., Bonn.

Bai, G.H., and G. Shaner. 2004. Management and resistance in wheat and barley to Fusarium head blight. Annual Review of Phytopathology 42: 135-161.

Bai, G.H., A.E. Desjardins, and R.D. Plattner. 2002. Deoxynivalenol-nonproducing *Fusarium graminearum* causes initial infection, but does not cause disease spread in wheat spikes. Mycopathologia 153: 91-98.

Beyer, M., J.A. Verreet, and W.S.M. Ragab. 2005. Effect of relative humidity on germination of ascospores and macroconidia of *Gibberella zeae* and deoxynivalenol production. International Journal of Food Microbiology 98: 233-240.

Birzele, B., A. Meier, H. Hindorf, J. Kramer, and H.W. Dehne. 2002. Epidemiology of *Fusarium* infection and deoxynivalenol content in winter wheat in the Rhineland, Germany. European Journal of Plant Pathology 108: 667-673.

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

Brandfass, C., and P. Karlovsky. 2006a. Simultaneous detection of *Fusarium culmorum* and *F. graminearum* in plant material by duplex PCR with melting curve analysis. BMC Microbiology 6: 4.

Brandfass, C., and P. Karlovsky. 2006b. Cost-effective quantification of *Fusarium culmorum* and *F. graminearum* in plant material by real-time PCR. submitted.

Brennan, J.M., D. Egan, B.M. Cooke, and F.M. Doohan. 2005. Effect of temperature on head blight of wheat caused by *Fusarium culmorum* and *F. graminearum*. Plant Pathology 54: 156-160.

Champeil, A., T. Dore, and J.F. Fourbet. 2004. Fusarium head blight: epidemiological origin of the effects of cultural practices on head blight attacks and the production of mycotoxins by *Fusarium* in wheat grains. Plant Science 166: 1389-1415.

Cumagun, C.J.R., F. Rabenstein, and T. Miedaner. 2004. Genetic variation and covariation for aggressiveness, deoxynivalenol production and fungal colonization among progeny of *Gibberella zeae* in wheat. Plant Pathology 53: 446-453.

De Wolf, E.D., L.V. Madden, and P.E. Lipps. 2003. Risk assessment models for wheat Fusarium head blight epidemics based on within-season weather data. Phytopathology 93: 428-435.

Desjardins, A.E., G.H. Bai, R.D. Plattner, and R.H. Proctor. 2000. Analysis of aberrant virulence of *Gibberella zeae* following transformation-mediated complementation of a trichothecene-deficient (Tri5) mutant. Microbiology-Uk 146: 2059-2068.

Desjardins, A.E., R.H. Proctor, G.H. Bai, S.P. McCormick, G. Shaner, G. Buechley, and T.M. Hohn. 1996. Reduced virulence of trichothecene-nonproducing mutants of *Gibberella zeae* in wheat field tests. Molecular Plant-Microbe Interactions 9: 775-781.

D'Mello, J.P.F., C.M. Placinta, and A.M.C. Macdonald. 1999. *Fusarium* mycotoxins: a review of global implications for animal health, welfare and productivity. Animal Feed Science & Technology 80: 183-205.

Doohan, F.M., J. Brennan, and B.M. Cooke. 2003. Influence of climatic factors on *Fusarium* species pathogenic to cereals. European Journal of Plant Pathology 109: 755-768.

Edwards, S.G. 2004. Influence of agricultural practices on fusarium infection of cereals and subsequent contamination of grain by trichothecene mycotoxins. Toxicology Letters 153: 29-35.

Gilbert, J., D. Abramson, B. McCallum, and R. Clear. 2002. Comparison of Canadian *Fusarium graminearum* isolates for aggressiveness, vegetative compatibility, and production of ergosterol and mycotoxins. Mycopathologia 153: 209-215.

Haidukowski, M., M. Pascale, G. Perrone, D. Pancaldi, C. Campagna, and A. Visconti. 2005. Effect of fungicides on the development of Fusarium head blight, yield and deoxynivalenol accumulation in wheat inoculated under field conditions with *Fusarium graminearum* and *Fusarium culmorum*. Journal of the Science of Food and Agriculture 85: 191-198.

Hooker, D.C., A.W. Schaafsma, and L. Tamburic-Ilincic. 2002. Using weather variables pre- and post-heading to predict deoxynivalenol content in winter wheat. Plant Disease 86: 611-619.

Hope, R., D. Aldred, and N. Magan. 2005. Comparison of environmental profiles for growth and deoxynivalenol production by *Fusarium culmorum* and *F. graminearum* on wheat grain. Letters in Applied Microbiology 40: 295-300.

Langevin, F., F. Eudes, and A. Comeau. 2004. Effect of trichothecenes produced by *Fusarium graminearum* during Fusarium head blight development in six cereal species. European Journal of Plant Pathology 110: 735-746.

Leonard, K.J., and W.R. Bushnell, (eds.) 2003. Fusarium Head Blight of Wheat and Barley, pp. 1-512. The American Phytopathological Society, St. Paul, Minnesota, USA.

Martins, M.L., and H.M. Martins. 2002. Influence of water activity, temperature and incubation time on the simultaneous production of deoxynivalenol and zearalenone in corn (*Zea mays*) by *Fusarium graminearum*. Food Chemistry 79: 315-318.

Mesterhazy, A. 2002. Role of deoxynivalenol in aggressiveness of *Fusarium graminearum* and *F. culmorum* and in resistance to Fusarium head blight. European Journal of Plant Pathology 108: 675-684.

Mesterhazy, A., T. Bartok, G. Kaszonyi, M. Varga, B. Toth, and J. Varga. 2005. Common resistance to different *Fusarium* spp. causing Fusarium head blight in wheat. European Journal of Plant Pathology 112: 267-281.

Nicholson, P., D.R. Simpson, G. Weston, H.N. Rezanoor, A.K. Lees, D.W. Parry, and D. Joyce. 1998. Detection and quantification of *Fusarium culmorum* and *Fusarium graminearum* in cereals using PCR assays. Physiological and Molecular Plant Pathology 53: 17-37.

Nicholson, P., E. Chandler, R.C. Draeger, N.E. Gosman, D.R. Simpson, M. Thomsett, and A.H. Wilson. 2003. Molecular tools to study epidemiology and toxicology of Fusarium head blight of cereals. European Journal of Plant Pathology 109: 691-703.

Parry, D.W., P. Jenkinson, and L. McLeod. 1995. Fusarium ear blight (scab) in small grain cereals -- A review. Plant Pathology 44: 207-238.

Peraica, M., B. Radic, A. Lucic, and M. Pavlovic. 1999. Toxic effects of mycotoxins in humans. Bulletin of the World Health Organization 77: 754-766.

Pestka, J.J., and A.T. Smolinski. 2005. Deoxynivalenol: Toxicology and potential effects on humans. Journal of Toxicology and Environmental Health-Part B-Critical Reviews 8: 39-69.

Pieters, M.N., J. Freijer, B.J. Baars, D.C.M. Fiolet, J. van Klaveren, and W. Slob. 2002. Risk assessment of deoxynivalenol in food: Concentration limits, exposure and

effects, p. 235-248 Mycotoxins and Food Safety, Vol. 504. KLUWER ACADEMIC/PLENUM PUBL, New York.

Pirgozliev, S.R., S.G. Edwards, M.C. Hare, and P. Jenkinson. 2003. Strategies for the control of Fusarium head blight in cereals. European Journal of Plant Pathology 109: 731-742.

Proctor, R.H., T.M. Hohn, and S.P. McCormick. 1995. Reduced Virulence of *Gibberella zeae* Caused by Disruption of a Trichothecene Toxin Biosynthetic Gene. Molecular Plant-Microbe Interactions 8: 593-601.

Proctor, R.H., T.M. Hohn, and S.P. McCormick. 1997. Restoration of wild-type virulence to Tri5 disruption mutants of *Gibberella zeae* via gene reversion and mutant complementation. Microbiology-Uk 143: 2583-2591.

Proctor, R.H., A.E. Desjardins, S.P. McCormick, R.D. Plattner, N.J. Alexander, and D.W. Brown. 2002. Genetic analysis of the role of trichothecene and fumonisin mycotoxins in the virulence of *Fusarium*. European Journal of Plant Pathology 108: 691-698.

Rocha, O., K. Ansari, and F.M. Doohan. 2005. Effects of trichothecene mycotoxins on eukaryotic cells: A review. Food Additives and Contaminants 22: 369-378.

Schilling, A.G., E.M. Moller, and H.H. Geiger. 1996. Polymerase chain reaction-based assays for species-specific detection of *Fusarium culmorum*, *F. graminearum*, and *F. avenaceum*. Phytopathology 86: 515-522.

Schnerr, H., R.F. Vogel, and L. Niessen. 2002. Correlation between DNA of trichothecene-producing *Fusarium* species and deoxynivalenol concentrations in wheat-samples. Letters in Applied Microbiology 35: 121-125.

Snijders, C.H.A. 2004. Resistance in wheat to *Fusarium* infection and trichothecene formation. Toxicology Letters 153: 37-46.

Waalwijk, C., P. Kastelein, I. de Vries, Z. Kerenyi, T. van der Lee, T. Hesselink, J. Kohl, and G. Kema. 2003. Major changes in *Fusarium* spp. in wheat in the Netherlands. European Journal of Plant Pathology 109: 743-754.

Waalwijk, C., R. van der Heide, I. de Vries, T. van der Lee, C. Schoen, G. Costrel-de Corainville, I. Hauser-Hahn, P. Kastelein, J. Kohl, P. Lonnet, T. Demarquet, and G.H.J. Kema. 2004. Quantitative detection of *Fusarium* species in wheat using TaqMan. European Journal of Plant Pathology 110: 481-494.

Wanyoike, M.W., F. Walker, and H. Buchenauer. 2002. Relationship between virulence, fungal biomass and mycotoxin production by *Fusarium graminearum* in winter

wheat head blight. Zeitschrift Fur Pflanzenkrankheiten Und Pflanzenschutz-Journal of Plant Diseases and Protection 109: 589-600.

Wong, L.S.L., D. Abramson, A. Tekauz, D. Leisle, and R.I.H. Mckenzie. 1995. Pathogenicity and Mycotoxin Production of *Fusarium* Species Causing Head Blight in Wheat Cultivars Varying in Resistance. Canadian Journal of Plant Science 75: 261-267.

Xu, X.M. 2003. Effects of environmental conditions on the development of Fusarium ear blight. European Journal of Plant Pathology 109: 683-689.

Xu, X.M., D.W. Parry, P. Nicholson, M.A. Thomsett, D. Simpson, S.G. Edwards, B.M. Cooke, F.M. Doohan, J.M. Brennan, A. Moretti, G. Tocco, G. Mule, L. Hornok, G. Giczey, and J. Tatnell. 2005. Predominance and association of pathogenic fungi causing Fusarium ear blight in wheat in four European countries. European Journal of Plant Pathology 112: 143-154.

Chapter 5: General discussion

Methods developed

The relative contribution of *F. graminearum* and *F. culmorum* to FHB depends upon a range of variables, which might also affect the amount of mycotoxins produced. Real-time PCR was the method of choice to determine the identity and the amount of these fungi within host tissues in a particular situation.

As we dealt with complex plant material like wheat flour, plant debris and wheat rachides, suitable DNA extraction methods had to be developed first. The starting point was a CTAB-based protocol invented by Murray and Thompson (1980) and developed and simplified by others (e.g., Stewart and Via, 1993). Looking at the wheat rachides, the small sample size and firm texture of the material put high demands on the homogenization of the samples. After unsuccessful attempts to disintegrate rachides in standard 2-ml tubes in a reciprocal mill, a large stainless steel ball (32 g) in a stainless steel container eventually enabled us to pulverize of rachides reproducibly. The DNA extraction for quantitative PCR faced another problem, because the colonization of plant material by *Fusarium* spp. is inhomogeneous, causing a large variation in DNA content among replicas when standard-size samples (100 mg) are analyzed. We aimed at constant extraction efficiencies and a possibly large sample volume at low costs to improve the representation of inhomogeneous material in the sample. This should result in a reduction of the sampling errors (Mulfinger, 2000). Sonification (Knoll et al., 2002) and some modifications in the upscaled extraction protocol enabled us to achieve this goal. Beside the difficulties mentioned so far, the inhibition of the PCR by DNA extracts, particularly in case of wheat flour and plant residues, had to be circumvented (Wilson, 1997). This was achieved primarily by diluting the DNA extract before analysis. Other cleaning procedures like a second precipitation of DNA or a column-based purification were also investigated, but the extra workload and costs of these procedures eventually led us to use a simple dilution instead, in spite of the potential loss of sensitivity it may cause.

To establish PCR specific for *F. graminearum*, we looked for published primers suitable for our real-time PCR conditions. The most promising primers were those published by Schilling et al. (1996) due to their thoroughly tested specificity. However, we failed to

adapt the *F. graminearum* specific primers to real-time PCR conditions. The gaoA targeted primers published by Niessen and Vogel (1997) were not preferred due to the size of the amplified product and published problems with the specificity (Kingsley, 2001). Therefore, we focused on the optimization of primers Fg16N (Nicholson et al., 1998), as these primers produce a product of constant size in contrast to primers Fg16 described in the same publication. The constant product size was important because melting temperature as well as signal intensity, which is crucial for both duplex and quantitative PCR, changes with the product size.

O'Donnell et al. (2004) proposed the existence of nine phylogenetically distinct species within the *F. graminearum* clade. Only lineage 7 has so far been reported in Europe and America (Ward et al., 2002; O'Donnell et al., 2000). According to Waalwijk et al., *F. graminearum* lineages 1, 2, 6 and 7 were amplified using the Fg16 primer set, whereas no PCR products were detected for lineages 3, 4 and 5 (Waalwijk et al., 2003). As the primers Fg16N used in our study amplify a section of the sequence amplified with the Fg16 primer set (Nicholson et al., 1998), it is not sure that all the lineages amplified using the Fg16 primer set can be detected with the help of Fg16N primers. However, all *F. graminearum* isolates used to test the specificity of Fg16N tested positive suggesting that these primers ensure a reliable detection of *F. graminearum* isolates originated from Germany.

With respect to *F. culmorum*-specific primers, we succeeded to adapt conditions for Opt18 primers (Schilling et al., 1996). The specificity for *F. graminearum* and *F. culmorum* could be confirmed for the particular assays both in duplex and in quantitative PCR.

The first real-time PCR assay to be developed was the qualitative detection of *F. culmorum* and *F. graminearum* to enable the identification of the two pathogens within rachides to determine the causing agent of FHB symptoms. A duplex assay was targeted in order to reduce the workload and costs occurring in the analysis of a high number of samples. As the fluorescent dye SYBR Green I intercalates unspecifically to all double-stranded DNA products, the main problem was the adjustment of the PCR signal intensity of the two products during the melting curve analysis. This problem was increased by the fact that the longer *F. culmorum* product binds the dye more efficiently than the *F. graminearum* product, leading to a suppression of the *F. graminearum* signal. This effect was partially compensated for by increasing the dye concentration. The same

phenomenon was reported by Giglio et al. (2003) for different PCR products. These problems were eliminated by reducing the total *Fusarium* spp. DNA amount used in the assay, as the competition for dye binding does not play a significant role as long as the amount of *F. culmorum* DNA is low. Although the quantity of *F. culmorum* DNA in a particular sample is not known before the analysis, limitation of the total rachis DNA amount used in the assay secured reliable detection of both fungi.

The second obstacle to be overcome was the analysis of iCycler data, as the form in which the results of the melting curve analysis are provided has not been designed for evaluation in a high-throughput environment. This may be a reason why none of the published duplex PCR assays with SYBR Green uses the iCycler (Fukushima et al., 2003; Hernandez et al., 2003; Jothikumar et al., 2003). Therefore, we designed a spreadsheet, which transformed the output from the thermocycler into a user-friendly, color-enhanced tabular report. It is interesting to remark that the areas under the peaks in the first derivative of the melting curves, which can be used to estimate the quantity of a PCR product, are not available within the iCycler interface, but appear during the transfer of melting curve data when copied to a spreadsheet. These data are used to evaluate different signals in the melting curve to exclude false positives by setting thresholds in relation to the positive standards representing the detection limit. This was necessary as the peak analysis of the first derivative of the melting curves is very sensitive, responding to small changes in the slopes of the melting curve. These extremely small peaks are by far not visible in an electrophoretic gel, but rather originate from system-specific processing of optical data.

One reason for the difficulties we met might be the relatively large size of PCR products used in the duplex reaction (280 and 472 bp). Other published duplex or multiplex assays used shorter PCR products, which might be more easy to separate by melting curve analysis and also less prone to competition in SYBR Green binding. The product sizes used in other multiplex assays with SYBR Green were 85 and 98 bp for a duplex assay (Jothikumar et al., 2003), and 75 to 280 bp (Hernandez et al., 2003) or 95 to 383 bp (Fukushima et al., 2003) for multiplex assays using SYBR Green.

To compare our method with a classical approach, morphological taxonomy requires cultivation for at least 12 days, followed by time-consuming microscopic examination. The method developed in this study allows the simultaneous identification of *F. graminearum* and *F. culmorum* in one PCR without the use of doubly labeled hybridization probes or electrophoresis. Species-specific PCR products are differentiated

by melting curve analysis, allowing a fast and cost-effective analysis of a large number of samples.

The second main task of this study was the quantitative detection of *F. graminearum* and *F. culmorum* in complex plant material like wheat flour and plant debris via real-time PCR. When we started the optimization of the primer Fg16N using a native Taq polymerase, the sensitivity of detection was insufficient to detect *F. graminearum* in many wheat flour samples of 2003 contaminated with DON. As we found out later, the cause was in part a high DON productivity in 2003 in combination with a low disease severity of FHB, leading to relatively low *Fusarium* spp. DNA contents accompanied by detectable DON amounts. After comparing different commercial kits and polymerases, we found that a particular PCR premix containing a hot-start polymerase (SYBR Premix Ex Taq, produced by Takara Bio, Otsu, Japan) secured the best performance for the *F. graminearum* assay, lowering the detection limit tenfold. No other premix or hot-start polymerase tested that time performed as well with this assay. We suppose that the higher specificity of amplification achieved with the help of hot-start polymerase might partly explain the superiority of this premix, as the primers tend to generate unspecific PCR products at low DNA concentrations.

For quantification purpose, a suitable standard curve had to be established to relate the PCR results to the amount of fungal DNA in the sample. As in any indirect analytical method, the quality of the standard curve is crucial for quantitative real-time PCR. Coamplification of specific plant DNA sequences (Winton et al., 2002) could not serve as an internal standard because plant DNA in plant debris is degraded by saprophytes to a variable degree. Mixing defined amounts of fungal mycelium with uncontaminated plant material to prepare standard curves was also hardly possible as fungal biomass in our plant samples was very low, making the preparation of defined mycelium/plant mixtures difficult. Therefore, the calibration curve was constructed with pure *Fusarium* spp. DNA mixed with DNA extracted from healthy maize or wheat flour. This procedure ensured that DNA standards and DNA from unknown samples were amplified under identical conditions. However, we found that standard curves analyzed with or without the addition of plant DNA did not differ significantly.

We are aware of the fact that our quantitative real-time PCR detection system is not the only one available. Nevertheless it has several advantages, which we believe to make this system preferable in comparison with other PCR-based methods. As we wanted to quantify *Fusarium* spp., qualitative PCR assays to detect a *Fusarium* species (Demeke et al., 2005; Jurado et al., 2005; Nicolaisen et al., 2005) were per se not suitable for our purposes. In addition, the assay of Nicolaisen et al. does not allow the differentiation of *F. graminearum* and *F. culmorum*. Real-time PCR assays available so far for the quantification of toxigenic *Fusarium* spp. in infected plants are costly because they utilize doubly-labeled probes (Reischer et al., 2004a; Reischer et al., 2004b; Waalwijk et al., 2004). Thereby the assay of Reischer et al. detects only *F. graminearum*, whereas the TaqMan based detection system of Waalwijk et al. quantifies five species in separate reactions. Here the costs will play a dominant role, as for some primer and probe combinations further modifications (Minor Groove Binder) were necessary to ensure specificity. A recently published competitive microsatellite-based *F. graminearum* detection (Naef et al., 2006) is basically unsuitable for naturally infected material because it requires prior knowledge of microsatellite alleles present in the target strain. Therefore, this assay can only be used in experiments with artificial inoculation. Finally, the real-time PCR systems discussed were not available when we started our work.

The method developed in this study is suitable for cost-effective, high- throughput quantification of *F. graminearum* and *F. culmorum* in plant material by real-time PCR to be applied in epidemiological studies.

Causal agent of FHB

The collected data should serve for a further elucidation of the epidemiology of FHB by identifying the causing agent of this disease in Germany. This was accomplished by the qualitative detection of *F. graminearum* and *F. culmorum* in 10 symptomatic wheat rachides per field on 54 fields in 2003 and the determination of the relative importance of these *Fusarium* spp. in relation to the DON content of the corresponding harvested grain. We only analyzed the DON-producing *Fusarium* spp. *F. graminearum* and *F. culmorum,* as deoxynivalenol is the most frequently encountered *Fusarium* mycotoxins in FHB (Bottalico and Perrone, 2002), and DON is a virulence factor responsible for the fungal spread via the rachis causing the typical head blight symptoms (Jansen et al., 2005; Langevin et al., 2004). In addition, we wanted to know to which extent the often stated fact that *F. graminearum* is the dominant species in hotter central Europe and that *F.*

culmorum tends to predominate in cooler maritime regions of northwest Europe (Bottalico and Perrone, 2002; Parry et al., 1995), is true for Germany. This statement cannot be supported by our data, as *F. graminearum* was found to be the dominant species all over Germany including the northern area near the coast. *F. culmorum* played only a minor role in rachides of 2003 derived from fields with a DON content of the corresponding harvested grain less than the legal limit for processed products (500 µg kg^{-1}). This may be due to a higher aggressiveness of this fungus when only few infections occur or due to lacking ascospore inoculum of *G. zeae* on the corresponding field. The major prevalence of *F. graminearum* was also reported from the Netherlands in wheat kernel samples of symptomatic ears (Waalwijk et al., 2003). The same holds true for the United States, where FHB is exclusively accredited to *F. graminearum* (Leonard and Bushnell, 2003).

Recently, a predominance presence of *F. graminearum* and *F. poae* has been reported in four European countries (Xu et al., 2005). Thereby, it has to be considered that the sampling was done randomly, neither associated with the severity of FHB nor with the toxin contamination of harvested grain. Hence, their results cannot be directly compared with our results, as *F. graminearum* is discussed being more aggressive and pathogenic than *F. poae*. The frequently occurring *F. poae* might therefore have a high potential of dispersal, with limited or varying effects on toxin content of the harvested grain. For example, Bottalico and Perrone report that the occurrence of T-2 toxin derivatives produced by *F. poae* have only been recorded in conjunction with sporadic epidemics of *F. sporotrichioides* and *F. poae* (Bottalico and Perrone, 2002).

The data attained in 2003 by the in-depth analysis of the rachides were confirmed by the results of the wheat flour samples of 2003 to 2005. In all three years *F. graminearum* was the by far dominating contaminating agent of wheat grain. *F. culmorum* was the only other noteworthy species, but it was still found only rarely in samples of 2003.

Plant residues on the soil surface are reported to be a primarily inoculum source for FHB (Xu, 2003; Parry et al., 1995). Spores produced on plant debris are ascospores of *G. zeae* as well as conidia of *F. graminearum* and *F. culmorum*. As both types of spores have been shown to be important for causing FHB (Xu, 2003), and both have mainly field-specific effects (Dill-Macky and Jones, 2000; Fernando et al., 1997), the content of *F. graminearum* and *F. culmorum* in plant debris was quantified from 2002 to 2005 to gain knowledge about the composition of the potential inoculum on a field. In 310 maize

debris samples collected throughout Germany in autumn and spring, *F. culmorum* was found more frequently than in wheat kernel samples, probably due to a higher saprophytic fitness of this fungus. However, *F. graminearum* was still the dominant species, especially in samples collected in autumn after maize harvest. In spring of the following year, samples were collected from the same locations cropped with wheat in this year. Despite the comparable amounts of *F. culmorum* and *F. graminearum* at a lower total level in the spring samples serving as sources of inoculum for FHB, only *F. graminearum* was detectable in the corresponding wheat samples. This emphasizes the role of the formation of ascospores on maize residues for the risk of a FHB epidemic. In plant residues of other crops collected, namely wheat and rapeseed straw, no *Fusarium* spp. DNA was detectable.

Relationship of toxin contamination and *Fusarium* spp. DNA
It is known that DON and DNA content of the sum of *F. graminearum* and *F. culmorum* correlate well (Waalwijk et al., 2004; Nicholson et al., 2003). The same is valid for artificially inoculated trichothecene producing *Fusarium* spp. (Schnerr et al., 2002). This might indicate that these two species, which are the only known DON producing *Fusarium* spp., produce quite constant amounts of DON in relation to their biomass. However, these data do not answer the question, which of these two fungi is the causing agent of DON contamination.

Our results show that the DON production relevant for grain contamination is caused nearly exclusively by *F. graminearum*. Looking at the whole time span 2003 to 2005, a significant correlation of the DON concentration and the *F. graminearum* DNA content could be detected. ($R^2 = 0.73$ (square of the correlation coefficient), $p < 0.001$, Pearson's test of correlation). No correlation could be found between *F. culmorum* DNA and the DON concentration. Even the analysis of the sum of *F. graminearum* and *F. culmorum* DNA did not change the square of the correlation coefficient. This might explain the good correlations found for the sum of *F. graminearum* and *F. culmorum* in the literature, as these correlations are at least mainly caused by *F. graminearum*. In experiments with artificial inoculation, the use of visual FHB ratings may be also suitable, as the main source of inoculum is known (Haidukowski et al., 2005; Mesterhazy et al., 2005).

Despite these facts, the coherency of the DON contamination with FHB ratings is still in discussion. Thereby the term DON production or DON content needs to be separated from DON productivity, which is the DON production per unit of fungal biomass or

DNA. Wanyoike et al. found significant correlation between head blight rating (AUDPC) of different *F. graminearum* isolates and the DON contents in infected grain (r = 0.68-0.75, Wanyoike et al., 2002) indicating that the visual FHB rating may give information about the DON content of samples infected with different isolates, at least under the same environmental conditions. Contrasting results were found by Brennan et al., who investigated the effect of small temperature differentials (16 vs. 20°C) on the pathogenicity of single isolates of *F. graminearum* and on the FHB severity determined on eight wheat cultivars. In their studies *F. graminearum* caused greater overall visual disease symptoms at 16°C than at 20°C. Contrary results were obtained for the fungal DNA content. Plants contained higher amounts of *F. graminearum* DNA at 20°C than at 16°C (Brennan et al., 2005). This makes the use of visual disease assessment unsuitable as a measure of fungal colonization. Cumagun et al. investigated the relationship of *F. graminearum* biomass to DON production. They found a good correlation between DON production and *Fusarium* exoantigen content among a progeny population of *G. zeae* in wheat, even across 3 environments (Cumagun et al., 2004). In summary, the relation of FHB ratings to fungal biomass of *F. graminearum* might be questionable, whereas constant amounts of DON seem to be produced per unit of fungal biomass.

Our results show that the DON production relevant for grain contamination in relation to the fungal biomass changes among years. The factor of the regression equation to convert the *F. graminearum* DNA content to DON content (both in µg kg^{-1}), representing the DON productivity, decreases from 24.6 in 2003 to 5.3 in 2005 with an intermediate position of 14.9 in 2004. This might be due to a general shift within the *F. graminearum* population of Germany to strains with a lower DON productivity in the last two years, or due to an effect of different weather constellations.

The DON productivity is not correlated with the total DON or *F. graminearum* DNA contents of the three years. Similar results were obtained under constant environmental conditions by Wanyoike et al., who found only a low to moderate association between DON productivity of different isolates of *F. graminearum* and head blight ratings or DON content produced by these isolates (Wanyoike et al., 2002).

To describe weather parameters relevant for the development of FHB in Germany, a set of weather data recorded in Göttingen was chosen because it is settled in the centre of Germany and in all years many samples were collected in the area around Göttingen. Weather data for each year were summarized in two time spans, designated infection phase and fungal development phase to represent the meteorological differences of the

three analyzed years. The infection phase includes the period of flowering plus seven days prior the bloom according to Hooker et al. (2002) and De Wolf et al. (2003). A second set of weather data is summarized from the end of bloom up to the harvesting season. This phase represents the fungal development during the ripening.

The year 2003 was the year with most precipitation at highest temperature in the infection phase. If the model of De Wolf et al. held true for our situation, a FHB epidemic could be expected. As this was not the case, either the fungal development phase had a decisive impact on the FHB development or the time span set for perithecia formation and infection was not sufficient for our conditions.

Looking at the time span in which the fungal development takes place, the most humid growing season is the year 2004, followed by 2005 and finally the comparable hot and dry fungal development phase of 2003. The same order was found looking at the total amount of *F. graminearum* DNA present in the samples. This suggests an important role of this phase in relation to the fungal colonization. This assumption is supported by the fact that most *Fusarium* species grow best and are most pathogenic to cereal heads at warm temperatures and under humid conditions (Doohan et al., 2003).

The year 2003 displays a special constellation of weather conditions. The warm and humid weather in the infection phase was most favorable for infection with *Fusarium* spp., but in the following ripening phase the weather became comparatively hot and dry, hardly allowing fungal growth within spikes. As a result, the averaged DNA content of the grain was the lowest of all years. In contrast, the DON production per fungal biomass was the highest in 2003. Hope et al. found that the production of DON occurred over a much narrower range of temperature and water availability than that for growth of *F. graminearum* (Hope et al., 2005). The year 2003 may have allowed the fungal growth and DON production mainly in the short infection period. As the fungal development was interrupted by the hot and dry weather afterwards, comparatively large amounts of DON were produced in relation to the total fungal biomass. This may explain the highest DON production efficiency in combination with the least biomass in 2003.

Due to the comparatively cold and wet summer in 2004 and 2005, the biomass of *F. graminearum* could develop continuously including periods of low DON production. The DON productivity might have been higher in 2004 than in 2005 due to longer periods of conditions favorable for DON production, as in 2004 more precipitation occurred at comparable temperatures. However, we found an interesting phenomenon, which has to be further elucidated.

In a conclusion, a successful infection in Germany does not necessarily cause a FHB epidemic, as might be interpreted from the risk assessment model of De Wolf et al. (2003). Another approach to identify important weather variables and their timing for predicting concentrations of DON in mature grain (Hooker et al., 2002) is in better accordance with our results. However, this model becomes more inaccurate at high DON concentration and is only based on data from parts of Ontario limiting the applicability of the results. Therefore, Xu called for future research focusing on the development of more generally applicable forecasting models and on understanding the relationships between disease severity, fungal biomass and the production of associated mycotoxins (Xu, 2003). FHB is a disease, which appear in epidemics highly dependent of the weather constellation of a specific year. This becomes even clearer after this study, as not only the total DON and DNA contents, but also the averaged DON productivity of the *F. graminearum* population in Germany are subject to change between the years. In addition, the extent of an FHB epidemic depends on a given inoculum source, for example maize residues available due to a reduced tillage system.

Therefore, the obtained data will help to adapt the model FUSOPT to predict FHB and DON contamination in Germany. The model shows the effect of agronomical risk constellations and demonstrates possibilities to reduce FHB by adapting agronomical practices. Fields and regions with high risk of a FHB infection are identified and field-specific threshold for plant protection are calculated. Thus, the model shall become an essential part of good agricultural practice to reduce FHB and toxin contamination in wheat.

References

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

Brennan, J.M., D. Egan, B.M. Cooke, and F.M. Doohan. 2005. Effect of temperature on head blight of wheat caused by *Fusarium culmorum* and *F. graminearum*. Plant Pathology 54: 156-160.

Cumagun, C.J.R., F. Rabenstein, and T. Miedaner. 2004. Genetic variation and covariation for aggressiveness, deoxynivalenol production and fungal colonization among progeny of *Gibberella zeae* in wheat. Plant Pathology 53: 446-453.

De Wolf, E.D., L.V. Madden, and P.E. Lipps. 2003. Risk assessment models for wheat Fusarium head blight epidemics based on within-season weather data. Phytopathology 93: 428-435.

Demeke, T., R.M. Clear, S.K. Patrick, and D. Gaba. 2005. Species-specific PCR-based assays for the detection of *Fusarium* species and a comparison with the whole seed agar plate method and trichothecene analysis. International Journal of Food Microbiology 103: 271-284.

Dill-Macky, R., and R.K. Jones. 2000. The effect of previous crop residues and tillage on Fusarium head blight of wheat. Plant Disease 84: 71-76.

Doohan, F.M., J. Brennan, and B.M. Cooke. 2003. Influence of climatic factors on *Fusarium* species pathogenic to cereals. European Journal of Plant Pathology 109: 755-768.

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

Fukushima, H., Y. Tsunomori, and R. Seki. 2003. Duplex real-time SYBR green PCR assays for detection of 17 species of food- or waterborne pathogens in stools. Journal of Clinical Microbiology 41: 5134-5146.

Giglio, S., P.T. Monis, and C.P. Saint. 2003. Demonstration of preferential binding of SYBR Green I to specific DNA fragments in real-time multiplex PCR. Nucleic Acids Research 31.

Haidukowski, M., M. Pascale, G. Perrone, D. Pancaldi, C. Campagna, and A. Visconti. 2005. Effect of fungicides on the development of Fusarium head blight, yield and deoxynivalenol accumulation in wheat inoculated under field conditions with *Fusarium graminearum* and *Fusarium culmorum*. Journal of the Science of Food and Agriculture 85: 191-198.

Hernandez, M., D. Rodriguez-Lazaro, T. Esteve, S. Prat, and M. Pla. 2003. Development of melting temperature-based SYBR Green I polymerase chain reaction methods for multiplex genetically modified organism detection. Analytical Biochemistry 323: 164-170.

Hooker, D.C., A.W. Schaafsma, and L. Tamburic-Ilincic. 2002. Using weather variables pre- and post-heading to predict deoxynivalenol content in winter wheat. Plant Disease 86: 611-619.

Hope, R., D. Aldred, and N. Magan. 2005. Comparison of environmental profiles for growth and deoxynivalenol production by *Fusarium culmorum* and *F. graminearum* on wheat grain. Letters in Applied Microbiology 40: 295-300.

Jansen, C., D. von Wettstein, W. Schafer, K.H. Kogel, A. Felk, and F.J. Maier. 2005. Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. Proceedings of the National Academy of Sciences of the United States of America 102: 16892-16897.

Jothikumar, N., X.W. Wang, and M.W. Griffiths. 2003. Real-time multiplex SYBR green I-based PCR assay for simultaneous detection of *Salmonella serovars* and *Listeria monocytogenes*. Journal of Food Protection 66: 2141-2145.

Jurado, M., C. Vazquez, B. Patino, and M.T. Gonzalez-Jaen. 2005. PCR detection assays for the trichothecene-producing species *Fusarium graminearum*, *Fusarium culmorum*, *Fusarium poae*, *Fusarium equiseti* and *Fusarium sporotrichioides*. Systematic and Applied Microbiology 28: 562-568.

Kingsley, M.T. 2001. Nucleic Acid-Based Detection and Identification of Bacterial and Fungal Plant Pathogens PNNL-13457. Pacific Northwest National Laboratory, Richland, Washington.

Knoll, S., S. Mulfinger, L. Niessen, and R.F. Vogel. 2002. Rapid preparation of *Fusarium* DNA from cereals for diagnostic PCR using sonification and an extraction kit. Plant Pathology 51: 728-734.

Langevin, F., F. Eudes, and A. Comeau. 2004. Effect of trichothecenes produced by *Fusarium graminearum* during Fusarium head blight development in six cereal species. European Journal of Plant Pathology 110: 735-746.

Leonard, K.J., and W.R. Bushnell, (eds.) 2003. Fusarium Head Blight of Wheat and Barley, pp. 1-512. The American Phytopathological Society, St. Paul, Minnesota, USA.

Mesterhazy, A., T. Bartok, G. Kaszonyi, M. Varga, B. Toth, and J. Varga. 2005. Common resistance to different *Fusarium* spp. causing Fusarium head blight in wheat. European Journal of Plant Pathology 112: 267-281.

Mulfinger, S., Niessen, L. and Vogel, R.F. 2000. PCR based quality control of toxigenic *Fusarium* spp. in brewing malt using ultrasonication for rapid sample preparation. Advances in Food Science 22: 38-46.

Murray, M.G., and W.F. Thompson. 1980. Rapid Isolation of High Molecular-Weight Plant DNA. Nucleic Acids Research 8: 4321-4325.

Naef, A., M. Senatore, and G. Defago. 2006. A microsatellite based method for quantification of fungi in decomposing plant material elucidates the role of *Fusarium graminearum* DON production in the saprophytic competition with *Trichoderma atroviride* in maize tissue microcosms. FEMS Microbiology Ecology 55: 211-220.

Nicholson, P., D.R. Simpson, G. Weston, H.N. Rezanoor, A.K. Lees, D.W. Parry, and D. Joyce. 1998. Detection and quantification of *Fusarium culmorum* and *Fusarium graminearum* in cereals using PCR assays. Physiological and Molecular Plant Pathology 53: 17-37.

Nicholson, P., E. Chandler, R.C. Draeger, N.E. Gosman, D.R. Simpson, M. Thomsett, and A.H. Wilson. 2003. Molecular tools to study epidemiology and toxicology of Fusarium head blight of cereals. European Journal of Plant Pathology 109: 691-703.

Nicolaisen, M., A.F. Justesen, U. Thrane, P. Skouboe, and K. Holmstrom. 2005. An oligonucleotide microarray for the identification and differentiation of trichothecene producing and non-producing *Fusarium* species occurring on cereal grain. Journal of Microbiological Methods 62: 57-69.

Niessen, M.L., and R.F. Vogel. 1997. Specific identification of *Fusarium graminearum* by PCR with gaoA targeted primers. Systematic and Applied Microbiology 20: 111-123.

O'Donnell, K., H.C. Kistler, B.K. Tacke, and H.H. Casper. 2000. Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. Proceedings of the National Academy of Sciences of the United States of America 97: 7905-7910.

O'Donnell, K., T.J. Ward, D.M. Geiser, H.C. Kistler, and T. Aoki. 2004. Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. Fungal Genetics and Biology 41: 600-623.

Parry, D.W., P. Jenkinson, and L. McLeod. 1995. Fusarium ear blight (scab) in small grain cereals -- A review. Plant Pathology 44: 207-238.

Reischer, G.H., M. Lemmens, A. Farnleitner, A. Adler, and R.L. Mach. 2004a. Quantification of *Fusarium graminearum* in infected wheat by species specific real-time PCR applying a TaqMan Probe. Journal of Microbiological Methods 59: 141-146.

Reischer, G.H., M. Lemmens, A. Farnleitner, A. Adler, and R.L. Mach. 2004b. Erratum to "Quantification of *Fusarium graminearum* in infected wheat by species specific real-time PCR applying a TaqMan probe" [J. Microbiol. Methods 59 (2004) 141-146]. Journal of Microbiological Methods 59: 437-437.

Schilling, A.G., E.M. Moller, and H.H. Geiger. 1996. Polymerase chain reaction-based assays for species-specific detection of *Fusarium culmorum*, *F. graminearum*, and *F. avenaceum*. Phytopathology 86: 515-522.

Schnerr, H., R.F. Vogel, and L. Niessen. 2002. Correlation between DNA of trichothecene-producing *Fusarium* species and deoxynivalenol concentrations in wheat-samples. Letters in Applied Microbiology 35: 121-125.

Stewart, C.N., and L.E. Via. 1993. A rapid CTAB DNA isolation technique useful for rapid fingerprinting and other PCR applications. Biotechniques 14: 748-751.

Waalwijk, C., P. Kastelein, I. de Vries, Z. Kerenyi, T. van der Lee, T. Hesselink, J. Kohl, and G. Kema. 2003. Major changes in *Fusarium* spp. in wheat in the Netherlands. European Journal of Plant Pathology 109: 743-754.

Waalwijk, C., R. van der Heide, I. de Vries, T. van der Lee, C. Schoen, G. Costrel-de Corainville, I. Hauser-Hahn, P. Kastelein, J. Kohl, P. Lonnet, T. Demarquet, and G.H.J. Kema. 2004. Quantitative detection of *Fusarium* species in wheat using TaqMan. European Journal of Plant Pathology 110: 481-494.

Wanyoike, M.W., F. Walker, and H. Buchenauer. 2002. Relationship between virulence, fungal biomass and mycotoxin production by *Fusarium graminearum* in winter wheat head blight. Zeitschrift Fur Pflanzenkrankheiten Und Pflanzenschutz-Journal of Plant Diseases and Protection 109: 589-600.

Ward, T.J., J.P. Bielawski, H.C. Kistler, E. Sullivan, and K. O'Donnell. 2002. Ancestral polymorphism and adaptive evolution in the trichothecene mycotoxin gene cluster of phytopathogenic *Fusarium*. Proceedings of the National Academy of Sciences of the United States of America 99: 9278-9283.

Wilson, I.G. 1997. Inhibition and facilitation of nucleic acid amplification. Applied and Environmental Microbiology 63: 3741-3751.

Winton, L.M., J.K. Stone, L.S. Watrud, and E.M. Hansen. 2002. Simultaneous one-tube quantification of host and pathogen DNA with real-time polymerase chain reaction. Phytopathology 92: 112-116.

Xu, X.M. 2003. Effects of environmental conditions on the development of Fusarium ear blight. European Journal of Plant Pathology 109: 683-689.

Xu, X.M., D.W. Parry, P. Nicholson, M.A. Thomsett, D. Simpson, S.G. Edwards, B.M. Cooke, F.M. Doohan, J.M. Brennan, A. Moretti, G. Tocco, G. Mule, L. Hornok, G. Giczey, and J. Tatnell. 2005. Predominance and association of pathogenic fungi causing Fusarium ear blight in wheat in four European countries. European Journal of Plant Pathology 112: 143-154.

Summary

Fusarium head blight (FHB) is a disease of cereal crops, which has a severe impact on wheat production worldwide. Apart from reducing the yield and impairing grain quality, FHB leads to contamination of grain with mycotoxins. The *Fusarium* species primarily involved in FHB are *F. graminearum* and *F. culmorum,* both producing deoxynivalenol (DON), the most important mycotoxin in wheat grain. Although the relative importance of *F. graminearum* and *F. culmorum* and their infection pathways has great impact on epidemiological questions, it has not be clarified in all details so far. For the differentiation of these pathogens, two real-time PCR methods were developed and applied, which allow a qualitative and quantitative detection of fungal biomass within host tissue. Samples for this study have been collected throughout Germany from 2003 to 2005.

F. graminearum and *F. culmorum* were determined qualitatively in symptomatic wheat rachides with a duplex PCR assay enabling the identification of the causal agent of FHB in a single PCR reaction. The method is based on the analysis of melting curves of amplification products generated in a real-time PCR thermocycler. The quantitative detection of both fungi in complex plant material like wheat flour and plant debris was realized in separated real-time PCR reactions, applying SYBR Green I fluorescence detection. The specificity of both assays for *F. graminearum* and *F. culmorum* was confirmed. Suitable high-throughput DNA extraction methods were developed for all types of samples analyzed in this study, including wheat rachides, grain samples and plant debris. For quantitative PCR, an upscaled DNA extraction protocol with increased extraction efficiency was used to reduce the sampling error. In addition to the analysis with the quantitative PCR, the DON content of the grain samples was measured.

The scope of the study was both the identification of the infecting agent in the wheat rachides and the determination of the relative importance of *F. graminearum* and *F. culmorum* in relation to the DON content of wheat samples to establish a risk assessment model for FHB. The results show that *F. graminearum* is the main causing agent of FHB and DON contamination in Germany. *F. culmorum* was sometimes detected in wheat rachides of 2003, but did not play a significant role in wheat grain samples 2003 to 2005. The collected data serve to adjust quantitative parameters of a computer-based

decision support system aiming at the minimization of *Fusarium* infection and toxin contamination of grain in Germany, which is being developed by our collaborators.

Zusammenfassung

Die Getreidekrankheit „Partielle Weißährigkeit" (Ährenfusariose oder Fusarium head blight, FHB) beeinträchtigt den Weizenanbau weltweit. Neben Ertrags- und Qualitätsverlusten verursacht FHB die Kontamination des Weizens mit Mykotoxinen. Die hauptsächlich an FHB beteiligten *Fusarium*-Arten sind *F. graminearum* und *F. culmorum*, die beide das wichtigste Mycotoxin im Weizen, Deoxynivalenol (DON), bilden. Die relative Bedeutung von *F. graminearum* und *F. culmorum* und ihrer Infektionswege hat für epidemiologische Fragestellungen große Bedeutung und konnte für Deutschland bisher nicht abschließend geklärt werden. Für die Unterscheidung wurden zwei Real-time PCR-Methoden entwickelt und angewendet, die den qualitativen und quantitativen Nachweis dieser Pathogene in den verschiedenen Wirtsgeweben ermöglichen, die in Weizenanbaugebieten aus dem Bundesgebiet 2003 bis 2005 entnommen wurden.

F. graminearum und *F. culmorum* wurden qualitativ in symptomatischen Weizenspindeln mit einer Duplex PCR-Methode bestimmt, wodurch die Identifizierung des Verursachers der FHB-Symptome ermöglicht wurde. Die Methode basiert auf einer Schmelzkurvenanalyse des Amplifikates im Real-time PCR-Gerät. Der quantitative Nachweis beider Pilze im komplexen Pflanzenmaterial, wie Weizenmehl und Ernterückstände, wurde unter Verwendung der Detektion des Fluoreszenzfarbstoffes SYBR Green I in getrennten Real-time PCR-Reaktionen realisiert. Die Spezifität beider Methoden für *F. graminearum* und *F. culmorum* wurde bestätigt. Für den Hochdurchsatz geeignete DNA-Extraktionsmethoden wurden für alle Probenarten, wie Weizenspindeln, Getreideproben und Pflanzenrückstände, entwickelt. Für die quantitative PCR reduzierte eine DNA-Extraktionsmethode mit einer homogenen Extraktionseffizienz in größerem Maßstab den Fehler der Probenahme. Neben der Analyse mit der quantitativen PCR wurde auch der DON-Gehalt der Getreideproben gemessen.

Das Ziel der Arbeit war zum einen die Identifizierung des infizierenden Pathogens in Weizenspindeln und zum anderen die Bestimmung der relativen Bedeutung von *F. graminearum* und *F. culmorum* im Verhältnis zum DON-Gehalt des Weizens, um ein Modell zur Risikobewertung für FHB zu etablieren. Die Ergebnisse zeigen, dass *F. graminearum* der Hauptverursacher von FHB und DON-Kontamination in Deutschland ist. *F. culmorum* wurde in den Weizenspindeln aus 2003 vereinzelt

gefunden, spielt aber keine bedeutende Rolle in den Weizenproben aus 2003 bis 2005. Mit den gesammelten Daten sollen außerhalb dieser Arbeit die quantitativen Parameter einer computergestützten Entscheidungshilfe eingestellt werden. Diese soll dazu dienen, das Risiko einer FHB-Infektion und einer Toxinbelastung in Deutschland zu minimieren.

Danksagung

An dieser Stelle möchte ich mich ganz besonders bei Herrn Prof. Dr. Petr Karlovsky für die Bereitstellung des Themas, die Diskussionen und die Hilfsbereitschaft sowie für die gewährte Freiheit und die Unterstützung bei der Durchführung dieser Arbeit und schließlich für die Durchsicht der Manuskripte bedanken.

Bei Herrn Prof. Dr. Andreas von Tiedemann bedanke ich mich für die bereitwillige Übernahme des Korreferates.

Bei Dr. Joachim Weinert bedanke ich mich für 3 Jahre sehr gute Zusammenarbeit, für die umfassende Bereitstellung von Daten und Proben sowie für die ständige Diskussions- und Hilfsbereitschaft.

Mein Dank gilt auch Herrn Dr. Birger Koopmann für die immer gewährte Hilfsbereitschaft bei Problemen jeglicher Art.

Heike Rollwage und Petra Romanczuk-Schulz möchte ich für die gute Zusammenarbeit im Labor und die Unterstützung bei technischen Problemen danken.

Bei unseren Azubis Sarah Greco, Martin Brokop, Patricia Bartoschek und Claudia Nordmann bedanke ich mich ganz herzlich für die technische Unterstützung.

Mein herzlicher Dank gilt auch Frau Martina Bode für die Hilfe und Unterstützung bei allen administrativen Problemen.

Bei Ulrike Brandfaß, Christina Eynck, Nadine Riediger und Joachim Weinert möchte ich mich ganz herzlich für die kurzfristige Korrektur der Dissertation bedanken.

Allen Mitarbeitern des Fachgebietes Pflanzenpathologie und Pflanzenschutz danke ich für das gute Arbeitsklima und für die mir entgegengebrachte Unterstützung bei dieser Arbeit.

Publications

Brandfass, C., J. Weinert, and P. Karlovsky. 2006. Detection of *Fusarium graminearum* and *F. culmorum* in rachides from wheat ears afflicted with Fusarium head blight and in grain identifies *F. graminearum* as the major cause of FHB and DON contamination. submitted.

Brandfass, C., and P. Karlovsky. 2006. Cost-effective quantification of *Fusarium culmorum* and *F. graminearum* in plant material by real-time PCR. submitted.

Brandfaß, C., J. Weinert, B. Kleinhenz, and P. Karlovsky. 2006. Anwendung Real-time PCR basierter Nachweisverfahren für *Fusarium graminearum* und *F. culmorum* als Grundlage für die Etablierung eines Prognosemodells. Tagungsbericht des Arbeitskreises Mykologie, Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA), Berlin Dahlem Deutsche Phytomedizinische Gesellschaft, in press.

Brandfass, C., and P. Karlovsky. 2006. Simultaneous detection of *Fusarium culmorum* and *F. graminearum* in plant material by duplex PCR with melting curve analysis. BMC Microbiology 6: 4.

Brandfaß, C., J. Weinert, B. Kleinhenz, A. von Tiedemann, and P. Karlovsky. 2005. *Fusarium*-Arten in Spindeln symptomatischer FHB-Ähren - Eine Bestimmung mit Hilfe der Real-time PCR als Grundlage für die Etablierung eines Prognosemodells. Phytomedizin 35 (2): 54-55.

Brandfaß, C., J. Weinert, B. Kleinhenz, A. von Tiedemann, and P. Karlovsky. 2005. Bestimmung der wichtigsten am Komplex der Ährenfusariose in der BRD beteiligten Fusariumarten mit Hilfe der Real-time PCR als Grundlage eines Prognosemodells. Tagungsband der 18. Tagung der DPG Projektgruppe "Krankheiten im Getreide", Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA), Braunschweig Deutsche Phytomedizinische Gesellschaft, http://www.phytomedizin.org/70.html.

Weinert, J., B. Kleinhenz, C. Brandfaß, P. Karlovsky, and A. von Tiedemann. 2005. Untersuchungen zum unterschiedlichen Auftreten von FHB und DON-Belastungen im Erntegut von Weizen als Grundlage eines Prognose- und Entscheidungsmodells. Tagungsband der 18. Tagung der DPG Projektgruppe "Krankheiten im Getreide", Biologische Bundesanstalt für Land- und

Forstwirtschaft (BBA), Braunschweig Deutsche Phytomedizinische Gesellschaft, http://www.phytomedizin.org/70.html.

Brandfaß, C., I. Pöhler, J. Weinert, and P. Karlovsky. 2004. Etablierung von Real-time PCR basierten Nachweisverfahren für *Fusarium graminearum* und *F. culmorum* sowie deren Anwendung. 54. Deutsche Pflanzenschutztagung, Hamburg; Mitt. BBA 396: 221-222.

Lebenslauf

Name:	Christoph Brandfaß
Geburtstag:	06.08.1974
Geburtsort:	Northeim
Familienstand:	verheiratet

Promotion:

05.2003 - 07.2006 Promotion am Fachgebiet Pflanzenpathologie und Pflanzenschutz der Georg-August-Universität Göttingen im Rahmen des Promotionsstudienganges „International Ph.D.-Program for Agricultural Sciences in Göttingen (IPAG)" zu dem Thema: „Establishment and application of real-time PCR-based methods to study the epidemiology of Fusarium Head Blight"

Studium:

10.1997 – 09.2002 Diplomstudium der Agrarwissenschaften, Fachrichtung Pflanzenproduktion an der Justus-Liebig-Universität Gießen, zweiteilige Diplomarbeit:
„Zytologische Untersuchungen zur Infektion von Zuckerrüben (*Beta vulgaris*) durch *Cercospora beticola* und Isolierung von zwei Promotoren aus der Genfamilie Subtilisin-ähnlicher Proteasen"
am Instituts für Phytopathologie und Angewandte Zoologie (IPAZ) der Universität Gießen und bei der PLANTA - Angewandte Pflanzengenetik und Biotechnologie GmbH in Einbeck
Abschluss: Dipl.-Ing. agr.

Berufsausbildung:

08.1995 – 07.1997 Ausbildung mit Abschluss zum Landwirt

Wehrdienst:

08.1994 – 09.1995 Zivildienst beim Arbeiter Samariter Bund in Nörten-Hardenberg

Schulbildung:

08.1987 – 06.1994 Gymnasium Corvinianum Northeim, Abschluss: Abitur

08.1981 – 07.1987 Grundschule und Orientierungsstufe in Katlenburg-Lindau

Eidesstattliche Erklärung

Hiermit erkläre ich eidesstattlich, dass diese Dissertation selbständig und ohne unerlaubte Hilfe angefertigt wurde.

Göttingen, im Mai 2006