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Dedicated

To

My wife, My children and My mother

Abstract

Microbiological and molecular assessment of interactions among the major *Fusarium* head blight pathogens on wheat ears

Investigations on putative interactions among *Fusarium* species colonizing wheat ears were carried out to understand the high frequency of species with low virulence in the presence of more virulent ones. To determine inter-species interactions among the major *Fusarium* head blight pathogens, wheat ears were inoculated at mid flowering either with *F. graminearum*, *F. culmorum*, *F. avenaceum* and *F. poae* alone or in combinations of two, three or four isolates under field and greenhouse conditions. The isolates and the composition of inoculum significantly affected FHB severity, kernel weight, number of *Fusarium*-infected kernels, fungal biomass and mycotoxin production. In single or mixed inoculations, *F. graminearum* resulted in the highest disease ratings, frequency and intensity of kernel colonization and mycotoxin production, followed by *F. culmorum*, *F. avenaceum* and *F. poae*, respectively. In mixtures, the frequency of *F. culmorum*, *F. avenaceum* and *F. poae* infected kernels and fungal biomass in most cases was lower than in single inoculations, while the mycotoxin productivity significantly increased. The study demonstrated that significant interactions exist between or among *Fusarium* isolates during kernel colonization, such as competition and amensalism. These interactions were disadvantageous to less-virulent isolates, although to different degrees, no additive effects were detected. Competitiveness and virulence of isolates varied as quantified by the different parameters; they decreased in the order *Fusarium graminearum*, *F. culmorum*, *F. avenaceum* and *F. poae*, respectively. The frequency of infected spikelets increased with sampling time either in inoculation alone or in mixtures. For *F. graminearum*, the increase in the percentage of infected spikelets was unaffected by the presence of other isolates, while the other isolates were inhibited by its presence in mixed inoculations. The frequency of infection of wheat flower parts by *Fusarium* isolates decreased in the order lemma, palea, glume and developing kernel. One to three weeks after mixed inoculations, a high number of spikelets (10-20%) were infected by more than 1 isolate. In contrast, there was relatively low percentage of kernels bearing two *Fusarium* isolates at harvest (2% ≥). The highest levels of infection with two *Fusarium* isolates were observed in mixtures including *F. avenaceum* and *F. culmorum*. In contrast, infection frequencies were lowest in co-inoculations with *F. poae*. The comparison of frequency and intensity of kernel colonization proved differences in virulence and development of the isolates in the kernels. Only for the most virulent isolate, application of microbiological and real-time PCR assays gave similar results. For the other species, the intensity of kernel colonization was lower than expected from the frequency of infection. The high frequency of low-virulent *Fusarium* isolates in a FHB complex has to be attributed to others factors than direct interactions with highly virulent isolates during establishment on the wheat ears. The investigations indicated the biological complexity of multiple *Fusarium*-infections, head-Scab development and mycotoxin contamination of cereals.

Zusammenfassung

Untersuchung von Wechselwirkungen zwischen *Fusarium* spp., den Erregern der Partiellen Taubährigkeit im Weizen, unter Verwendung mikrobiologischer und molekularbiologischer Methoden

Die durchgeführten Untersuchungen bezüglich mutmaßlicher Wechselwirkungen von Fusarium-Arten in infizierten Weizen-Ähren sollen dazu beitragen, das häufige Auftreten weniger aggressiver Arten bei gleichzeitigem Vorhandensein von aggressiven Fusarium-Arten zu verstehen. Zur Bestimmung inter-spezifischer Wechselwirkungen zwischen bedeutenden Erregern der Partiellen Taubährigkeit im Weizen wurden Ähren zum Zeitpunkt der Blüte entweder einzeln mit *F. graminearum*, *F. culmorum*, *F. avenaceum* und *F. poae* oder durch die Kombination von zwei, drei oder vier Arten im Gewächshaus und in Feldversuchen inokuliert. Die einzelnen Isolate als auch deren Kombination im Inokulum beeinflussten signifikant das symptomatische Auftreten der Partiellen Taubährigkeit, die Tausendkornmasse, die Häufigkeit infizierter Körner, die pilzliche Biomasse und die Mykotoxin-Bildung. Bei Einzeinokulation oder Kombinationsinokulation mit mehreren Isolate zeigte *F. graminearum* die höchste Pathogenität, die größte Häufigkeit und Intensität infizierter Körner sowie die stärkste Mykotoxin-Bildung, gefolgt von *F. culmorum*, *F. avenaceum* und *F. poae*. Im Vergleich zur Einzelinolukation von *F. culmorum*, *F. avenaceum* und *F. poae* führte Inokulation in Kombinationen einerseits zu einer Reduktion der Häufigkeit infizierter Körner sowie der pilzlichen Biomasse, andererseits jedoch zu einer signifikant höheren Mykotoxinproduktion. Die Ergebnisse aus den Untersuchungen verdeutlichen, dass während der Infektion und Ausbreitung von *Fusarium* spp. im Korn zwischen den beteiligten *Fusarium*-Isolaten signifikante Wechselwirkungen wie Konkurrenz und Amensalismus vorkommen. Diese Wechselwirkungen benachteiligten die weniger aggressiven Isolate, wenn auch zu einem unterschiedlichen Grad. Es wurden hier keine additiven Effekte nachgewiesen. Die Häufigkeit infizierter Ährchen stieg mit dem Zeitpunkt der Probennahme, sowohl bei Einzel- als auch Kombinationsinokulationen. Bei *F. graminearum* wurde der Anstieg der Häufigkeit infizierter Ährchen durch das Vorkommen anderer Isolate nicht beeinflusst, hingegen wurden die weiteren Isolate durch ihre Präsenz in Kombinations-Inokulationen gehemmt. Die Infektionshäufigkeit der Blütensegmente durch *Fusarium* spp. verringerte sich absteigend von Deckspelze, Vorspelze und Hüllspelze zu dem sich entwickelnden Korn. Ein bis drei Wochen nach Kombinationsinokulationen waren eine hohe Anzahl von Ährchen (10-20%) von mehr als einem Isolat besiedelt. Es wurde aber nur von einem geringen Prozentsatz der geernteten Körner zwei oder mehr verschiedene *Fusarium*-Isolate isoliert (2% ≥). Der Vergleich von Häufigkeit und Intensität des *Fusarium*-Befalls infizierter Körner zeigt, dass es Unterschiede hinsichtlich Virulenz und Entwicklung der *Fusarium*-Isolate in den Körnern gibt. Nur bei dem Isolat mit der höchsten Virulenz kam es zu einem übereinstimmenden Ergebnissen bei der mikrobiologischen und der Real-Time PCR-Untersuchung. Bei den anderen Arten war die war die Intensität der Besiedlung der Körner geringer als die Infektionshäufigkeit vermuten lies. Die große Häufigkeit von *Fusarium*-Isolaten mit geringer Virulenz muss auf andere Faktoren als die direkte Wechselwirkung mit hoch virulenten Isolaten währen der Entwicklung auf den Weizenähren zurückgeführt werden. Die Untersuchungen zeigten die biologische Komplexität von multiplen *Fusarium*-Infektionen, Krankheitsentwicklung und Mykotoxin-Belastung von Weizen.

Table of contents

1. Introduction

The infection of crops by plant pathogenic fungi impairs both quality and quantity causing huge economic losses to farmers as well as immense effects on human and animal health. This implies that fungal colonization may affect seed size, weight, seed germination rate, protein and carbohydrate contents, baking quality and other quality parameters. In addition to these impairments, the most serious consequence of fungal colonization is contamination of agricultural products with mycotoxins.

Several fungi are able to produce mycotoxins, as secondary metabolites, particularly species of *Aspergillus*, *Fusarium*, *Penicillium*, *Claviceps* and *Alternaria*. Mycotoxins comprise a group of several hundreds of chemically different toxic compounds. Aflatoxins, ochratoxins, trichothecenes, zearalenone, moniliformin, enniatins and fumonisins are the most common mycotoxins produced in agricultural products (William, 1989; Moss, 1996; Rotter *et al*., 1996; Sweeney and Dobson, 1998). *Fusarium* species are able to produce a wide range of mycotoxins namely trichothecenes, zearalenone and fumonisins even though aflatoxins produced by *Aspergillus* spp. are the most dangerous group.

In nature, all fungi often interact directly or indirectly in order to survive in their habitat. Interactions among fungal species have been reported to influence the predominant species, disease-producing ability and – as a consequence - mycotoxin production.

Fusarium head blight (FHB), also known as scab, has reemerged worldwide as a disease of economic importance in recent years reducing yield quantity and quality, especially associated mycotoxin accumulation in infected grains (McMullen *et al*., 1997). The disease is caused by a complex of several *Fusarium* species in most areas, differing in ecological and biological features. Recent epidemics throughout the world have made researchers to focus on FHB pathogens and their epidemiology (Bai and Shaner, 1994; Parry *et al*., 1995; McMullen *et al*., 1997; Miedaner, 1997; Rossi *et al*., 2001; Logrieco *et al*., 2002; Rossi *et al*., 2002; Akinsanmi *et al*., 2004; Desjardins, 2006; Köhl *et al*., 2007).

The occurrence of FHB pathogens among and within fields is highly varied and independent of each other from year to year. Therefore, most of the models developed for predicting FHB pathogens outbreak may produce error under field conditions. This is because more than one species co-exist in one site, different requirements for infection and development and besides there are usually significant interactions among them. This makes the task of forecasting and understanding epidemiology of FHB pathogens and mycotoxin formation under field conditions difficult. The presence of toxin-producing and non-producing species within the disease complex, along with isolates of differing chemotypes

greatly complicate attempts to understand the factors that influence disease development and toxin accumulation (Nicholson *et al*., 2003). The infection of cereal grains with *Fusarium* species and contamination with their mycotoxins is a threat to food and feed supply throughout the world (McMullen *et al*., 1997). Accumulation of mycotoxins in grains can be harmful to humans and animals (Placinta *et al*., 1999). Concerns about human health arise when grains or other field crops are found to contain mycotoxins. Animals have a higher risk of mycotoxin contamination since they may get their feed mainly from a single or the same source unlike humans who may get their supplies from many sources, which are varied, in the content of mycotoxins. On the other hand, agricultural products with low quality are mostly used in feedstuff production rather than in human foods. To protect consumers, many countries have set up regulations to limit exposure to mycotoxins (D'Mello *et al*., 1999; Dohlman, 2003).

The existence of high variability in fungal community at ecological niches indicates interactions for nutrients and space. These interactions can broadly be classified in 9 categories based on the effects or mechanisms of the interaction between the two species and range from neutural through beneficial to harmful for one or the two species. FHB pathogens may interact in different ways on growing plant parts. Therefore, interactions can occur during the first stages of infection such as early interactions (spore germination and penetration) or late interactions (tissue colonization) using various mechanisms to occupy the ecological niches. Such interactions resulted in predominant of one species while the others occur in low levels. Therefore, it is difficult to explain how similar species can co-exist and how to measure the competition. Little information is available on effects of interactions among FHB pathogens on disease severity, the predominant species, disease development and mycotoxin production by *Fusarium* complexes under different environmental conditions.

Quantification of fungal DNA in different plant matrices is becoming more common in the epidemiology of plant pathogens (Henson and French, 1993; Martin and Le´vesque, 2000; Schaad *et al*., 2004; Schena *et al*., 2004; Waalwijk *et al.*, 2004; Mulè *et al*., 2005). Different quantitative approaches have been developed for quantification of fungal biomass in infected plant samples.

Real-time PCR is a technique of collecting data throughout the PCR process as it occurs, thus combining amplification and detection into a single step (Wong *et al*., 2005). Recently real-time PCR using non-specific double-stranded DNA intercalator dyes like SYBR Green I and internal labelled probes have been developed for the major FHB pathogens in different tissue and plant matrices. The main objective of quantification of FHB pathogens is

monitoring the individual populations to understand the nature of ecology, biology, pathogenicity and aggressiveness towards host plant.

Fusarium head blight (FHB)

In most areas, FHB is caused by a complex of various *Fusarium* species differing in important biological and ecological characteristics, e.g. virulence on cereals, host range, mycotoxin production, optimum growth conditions, survival on crop debris and in the soil (Parry *et al*., 1995; Hörberg, 2002; Logrieco *et al*., 2002; Rossi *et al*., 2002; Akinsanmi *et al*., 2004; Desjardins, 2006; Köhl *et al*., 2007). When cereal plants are infected with these fungi, there is a risk that grain may become contaminated with *Fusarium* mycotoxins, which may subsequently be transferred to compound feeds (Placinta *et al*., 1999). The distribution and predominance of FHB pathogens differ significantly among climatic conditions, geographical zones, countries, and years (Doohan *et al*., 2003; Kosiak *et al*., 2003; Waalwijk *et al*., 2003; Xu, 2003; Xu *et al*., 2005).

In Western Germany, five *Fusarium* species have been reported to predominate in the FHB complex (Schütze *et al*., 1997; Birzele *et al*., 2002; Müllenborn *et al*., 2007): *Fusarium graminearum* Schwabe (teleomorph = *Gibberella zeae* (Schwein.) Petch), *F. culmorum* (Smith) Sacc., *F. avenaceum* (Corda ex Fr.,) (teleomorph = *G. avenaceum* Cook), *F. poae* (Peck) Wollenw., and *F. tricinctum* (Corda) Sacc) (teleomorph = *G. tricincta* El-Gholl, McRitchie, Schoult. & Ridings).

F. graminearum is the predominant species in China, the USA, Canada, some European countries and Southern Germany (Parry *et al*., 1995; McMullen and Gallenberg, 1997; Bottalico and Perrone, 2002; Logrieco *et al*., 2003; Xu *et al*., 2005). *F. culmorum* is a major component of FHB in wheat growing areas with cool weather conditions. It has been suggested to be one of the main causative agents of FHB in several European countries, for example in Denmark, Romania, Bulgaria, Belgium and some parts of Germany (Mills, 1989; Birzele *et al*., 2002; Bottalico and Perrone, 2002). *F. avenaceum* appears to be the most abundant species associated with FHB in northern European countries and has been reported from a wide range of different climatic zones (Parry *et al*., 1995; Kosiak *et al*., 2003; Loiveke *et al*., 2003; Henriksen and Elen, 2005; Xu, *et al*., 2005). *F. poae* is described to be less virulent than other FHB pathogens. This species, however, is able to produce mycotoxins, e.g. nivalenol, zearalenone, diacetoxyscirpenol, fusarenone, enniatins, HT-2 toxin and T-2 toxin (Desjardins, 2006). It has been frequently reported to occur in wheat kernels in Estonia,

culmorum, allowing increased numbers of *Michrodocium nivale* (referred to by former name Fusarium nivale, Schaffnit, 1912) isolates; however this caused the possibility of underestimating *M. nivale* populations which are sensitive to benomyl.

Morphological detection of *Fusarium* species showing similarities with other species makes the task difficult when identifying closely related species, which are morphologically similar but genetically divergent, making it necessary to use DNA based methods. These are widely used in almost all disciplines in plant pathology. For example, *F. avenaceum* and *F. arthrosporioides* are very difficult to separate by their morphological characteristics and are often confused with each other (Yli-Mattila *et al*., 2004). The close relationship between these species is supported by the IGS, ITS, and β-tubulin sequence and metabolite profile results (Yli-Mattila *et al*., 2002). Using species-specific primers based on RAPD-PCR, UP-PCR and positive/negative classical PCR, it is possible to separate most of the *F. arthrosporioides* isolates from the closely related *F. avenaceum* isolates (Yli-Mattila *et al*., 2004).

The development and use of these assays is complicated by the difficulty of correctly identifying isolates in axenic culture and still limited understanding of phylogeny within the *Fusarium* genus. For example, relationship between *F. avenaceum* and *F. tricinctum* revealed a close relationship between them in spite of different morphological taxonomic sections (*Roseum* and *Sporotrichiella*, respectively). However, RAPD-based phylogenetic profiling revealed interspecies differences large enough to distinguish clearly between isolates of the two species (Turner *et al*., 1998). Generally, it would appear that the nuclear rDNA and ITS sequences may not always contain sufficient polymorphism to allow the design of completely species-specific primers (Turner *et al*., 1998).

Recent advances in molecular systematic of fungi provide extensive DNA sequence information that is of great benefit in molecular detection and diagnostics, especially for large genera whose species have overlapping morphological characteristics (e.g. *Fusarium* and *Pythium*) (Martin *et al*., 2000). Recently, a number of molecular techniques are being utilized in order to understand the nature and diversity of the FHB pathogens, interactions between pathogens and their host and pathogens themselves alone with environmental factors (Nicholson *et al*., 2003; Schena *et al*., 2004). PCR assays based on species-specific primers offer accurate, rapid identification and quantification for the major FHB pathogens in different tissues and plant matrixes in singleplex or multiplex PCR, simultaneously (Nicholson *et al*., 2003; Schena *et al*., 2004).

Current methods such as visual assessment of disease severity, infected ears or spikelets and damaged or infected kernels estimate pathogen populations, indirectly (Jones

and Mirocha**,** 1999; Paul *et al*., 2005). Direct incidence of FHB pathogens can be examined microbiologically by isolating and identifying the fungi morphologically. The biomass of various fungi may be estimated microscopically using labelled species-specific probes or using molecular techniques for the quantification of fungal DNA in infected plant samples. Detection using real-time PCR can be easily done, in less than 1 day compared to the 2-3 weeks required for the microbiological detection. Molecular tools make facility to study interactions among different strains within one species that is not possible using of the micro and macro-morphological features. Quantification of trichothecene-producing *Fusarium* spp. within harvested grain has been used to determine the efficacy of fungicides on spectrum of trichothecene-producing *Fusarium* spp. within the FHB complex disease (Edwards *et al*., 2001; Simpson *et al*., 2001).

Effective, reliable and rapid species identification and detection of FHB pathogens are essential keys and a milestone work for basic and practical research in different disciplines. Many species-specific primers for detection *Fusarium* species have been designed (Parry and Nicholson, 1996; Turner *et al*., 1998; Chelkowski *et al*., 1999; Nicholson *et al*., 2003; Waalwijk *et al*., 2003; Demeke *et al*., 2005; Waalwijk *et al*., 2004; Jurado *et al*., 2005; Leisova *et al*., 2006). Using additional sets of primers in a multiplex PCR, enables the simultaneous detection more than one species in every PCR reaction.

Quantification of *Fusarium* species

Quantification of the amount of fungal DNA in different plant matrix samples is becoming more common in the epidemiology of plant pathogens (Mulè *et al*., 2005; Henson and French, 1993; Martin and Le´vesque, 2000; Schaad *et al*., 2003; Schena *et al*., 2004; Waalwijk *et al.*, 2004).

The PCR offers a sensitive and potentially specific means to detect, identify and quantify the fungal species present within plant tissues. A number of assays have been produced to permit detection of many of the major pathogens associated with FHB (Parry and Nicholson *et al*., 1996; Schilling *et al*., 1996; Nicholson *et al*., 1998; Turner *et al*., 1998; Yoder and Christianson, 1998; Chelkowski *et al*., 1999; Nicholson *et al*., 2003; Waalwijk *et al.*, 2003; Bluhm *et al*., 2004; Nicholson *et al*., 2004; Schena *et al*., 2004; Waalwijk *et al.*, 2004; Yli-Mattila1 *et al*., 2004).

Theoretically, there is a quantitative relationship between the amount of starting material and the PCR product at any cycle of the PCR (Rasmussen *et al*., 1998). This

indicates that PCR may allow the amount of fungal DNA to be measured. In traditional PCR, results were expressed in terms of presence/absence. The major reasons why traditional PCR has not been adopted, are poor precision, Low sensitivity, short dynamic range, low resolution, non-automated, size based discrimination only, results are not expressed as numbers, ethidium bromide for staining is not quantitative, post PCR processing, detection in the plateau phase (the data would not truly represent the initial amount of starting materials).

To overcome difficulties of quantification in traditional PCR, quantitative PCR methods like competitive or non-competitive PCR has been developed (Freeman *et al*., 1999; Edwards *et al*., 2001). Developments of more recent techniques like real-time quantitative PCR (Higuchi *et al*., 1992) have been proposed for DNA quantification. These new approaches can eliminate the variability associated with traditional quantitative PCR, thus allowing routine and reliable quantification of PCR products.

Real-time PCR technologies open increasing opportunity to detect and study phytopathogenic and antagonistic fungi (Schena *et al*., 2004; Waalwijk *et al*., 2004; Leisova *et al*., 2006). They combine the sensitivity of conventional PCR with the generation of a specific fluorescent signal providing real-time analysis of the reaction kinetics and allowing the quantification of specific DNA targets (Schena *et al*., 2004). Real-time PCR is the technique of collecting data throughout the PCR process as it occurs, thus combining amplification and detection into a single step (Wong *et al*., 2005).

The quantitative information in a PCR reaction comes from those few cycles where the amount of DNA grows logarithmically from barely above background to the plateau. Often only 4 or 5 cycles out of 40 will fall in this “log-linear” portion of the curve. The position of these precious few cycles contains most of the quantitative information (Holland *et al*., 1991; Higuchi *et al.*, 1992 and Rasmussen *et al*., 1998). The advantage of this technique are (i) data collection in the exponential phase of PCR, (ii) no post PCR processing, (iii) more precise and fast to perform, (iv) simultaneous detection and quantification, (v) less laborious, (vi) can be used in multiplex PCR, and (vii) eliminating potential source of carryover contamination, gene expression, genotyping, single-nucleotide polymorphisms (SNP) and allele discrimination analysis.

One of the first things to run real-time PCR for the samples is which QPCR chemistry has to be used. Different Real-time detection systems can be divided into two approaches both of which are applicable for quantification (Ishiguro *et al*., 1995; Wittwer *et al*., 1997; Morrison *et al*., 1998; Whitcombe *et al*., 1999).

The first approach utilizes standard enzymology in the presence of two sequence-specific primers and a non-specific double-stranded DNA (dsDNA) intercalator dye such as SYBR Green I (Morrison *et al*., 1998) or YO-PRO-1 (Ishiguro *et al*., 1995). Ethidium bromide was the first double stranded DNA specific fluorescent binding dye used for simultaneously DNA amplification and detection (Higuchi *et al*., 1992; Wetmur, 1995). Later SYBR Green I as a substituted dye was used, because it has a stronger fluorescent signal (Wittwer *et al*., 1997). In solution, the unbound SYBR Green I exhibit relatively low fluorescence, but when bound to dsDNA its fluorescence enhanced upon DNA-binding. The fluorescence increases proportionately to DNA concentration. So with this property of the dye, it is possible to track the accumulation of PCR product. As the target is amplified, the increasing concentration of dsDNA in the solution can be directly measured by the increase in fluorescence signal. Since, SYBR Green I, intercalate to non-specific PCR amplification products as well target products, requires a meticulous attention to primer design and condition optimization in order to eliminate the formation of non-specific amplicons, as well as of primer-dimers. A non-specific signal can be easily detected at end of the reaction by heating the PCR products from 30–40 to 95 °C whilst continuously monitoring the fluorescence (melting curve analysis) (Ririe *et al*., 1997; Freeman *et al*., 1999). The products with different length and/or sequence can be observed as distinct fluorescent peaks. SYBR Green I fluorescent dye is extremely versatile, inexpensive, easy to use, detect any PCR products and the results are accurate enough comparison to probe based techniques (Rasmussen *et al*., 1998 and Lipsky *et al*., 2001).

The second approach utilizes internal probes labeled with different reporter dyes. This approach will provide a higher level of detection and specificity, as well as detection more than one target product in a single reaction (multiplex PCR) comparison to a non-specific dsDNA intercalator dye such as SYBR Green I. There are several amplicon sequence specific detection methods based on the use of oligonucleotide probes labeled with a donor fluorophore and an acceptor dye (quencher), (Didenko, 2001 and Schena *et al*., 2004**)**. For example, linear and Structural probes (Holland *et al*., 1991; Mergeny *et al*., 1994), scorpion primers (Whitcombe *et al*., 1999) and Molecular Beacons (Tyagi and Kramer, 1996).

Formation of mycotoxins by *Fusarium* species

Several *Fusarium* species reported throughout world are responsible for the formation of mycotoxins in infected plants and in plant products (Placinta *et al.,* 1999; Bottalico *et al.,*

2002; Lorieco *et al.,* 2003). When cereal plants are infected with these fungi, there is a risk that grain may become contaminated with *Fusarium* mycotoxins, which may subsequently be transferred to compound food and feed (Placinta *et al.,* 1999). Trichothecenes, zearalenones, fumonisins, enniatins and moniliformin may be considered as important *Fusarium* mycotoxins in animal and human health.

Trichothecenes are a family of over 150 structurally related compounds mainly produced by *Fusarium* species. Trichothecenes are relatively simple alcohols and short chain esters, share a tricycle nucleus named trichothecene, and usually contain an epoxide at c-12 and c-13, which is essential for toxicity (Desjardins *et al*., 1993). Biosynthesis of these mycotoxins proceeds from trichodiene and leads through several oxygenation, isomerization, cyclization and esterification steps to many of the more complex trichothecene toxins, such as diacetoxyscirpenol, T-2 toxin and 3-acetyldeoxynivalenol (Desjardins *et al*., 1993). According to chemical properties, trichothecenes can be subdivided into four basic groups, with type A and type B representing the most important members with regard to negative effects. The difference between of type A and B is the absence or presence of a keto group at C-8 of the trichothecene skeleton, respectively. The type a trichothecenes include T-2 toxin, HT-2 toxin, neosolaniol (NEO) and diacetoxyscirpenol (DAS), type B trichothecenes include deoxynivalenol (DON) and its 3- acetyl and 15-acetyl derivatives (3-ADON and 15-ADON, respectively), nivalenol (NIV) and fusarenon-X. Trichothecenes have been well documented to be host nonspecific in their toxicity and to inhibit protein synthesis in a wide range of eukaryotic organisms, including animals, fungi, and higher plants (Cutler, 1988; Desjardins *et al*., 1993).

According to the spectrum of mycotoxin production in *Fusarium* species, different chemotypes can be identified. Based on trichothecene production, different isolates of *F. graminearum* have been divided into nivalenol and deoxynivalenol producing chemotypes. The nivalenol chemotype include nivalenol and fusarenone X, while deoxynivalenol chemotype include deoxynivalenol and acetyldeoxynivalenol (Ichinoe *et al*., 1983). Functioning of Tri13 and Tri7 genes is required for the production of NIV and 4-acetylnivalenol, respectively. The results indicated that isolates with a non-functional tri7 gene would be capable of synthesizing NIV, but not 4-NIV, whereas isolates with a non-functional tri13 or tri 13 and tri 7 genes could make neither NIV nor 4-NIV and instead synthesized DON (Lee *et al*., 2001 and 2002 and Chandler *et al*., 2003).

It has been documented that, in some *Fusarium* species and with the same conditions, toxin production by *Fusarium* strains may vary, sharply. Some strains are able to produce

large amounts of trichothecenes, while others produce small or undetectable amounts of trichothecenes (Muthomi *et al*., 2000; Muthomi, 2001; Walker *et al*., 2001; Bakan *et al*., 2002). According to the tri5-tri6 sequence data, specific PCR primers were designed to allow differentiation of high-producing from low-producing *F. culmorum* strains using a duplex PCR (Bakan *et al*., 2002).

Several *Fusarium* species are able to produce zearalenone (ZEA) as a secondary metabolite in a wide variety of crop plants throughout world (Caldwell *et al*., 1970; Tanaka *et al*., 1988; Vesonder *et al*., 1991; Hestbjerg *et al*., 2002; Logrieco, *et al*., 2002; Binder *et al*., 2007; Glenn, 2007; Krska *et al*., 2007). However, cereal grains are the most important source of ZEA in food and feed diets. Zearalenone has been frequently reported as *Fusarium* mycotoxin in FHB from European countries (Bottalico and Perrone, 2002; Logrieco *et al*., 2002). Zearalenones differ in presence and reduction state of hydroxyl groups and their acetylation, however ZEA is the major homologue produced by *Fusarium* species, but other metabolites, such as ß-zearalenol and 4-acetylzearalenol, can occur at low levels in naturally contaminated grains (desjardins, 2006). The concentration of ZEA can vary from 0.001 and 175 mg/kg in cereal grains intended for human consumption, depending on plant variety, geographic region and climatic conditions (Vrabcheva *et al*., 1996; Binder *et al*., 2007; Fink-Gremmels and Malekinejad, 2007). This indicated when the environmental conditions favor the fungal infection and later on development; the concentration of ZEA can dramatically increase either in field or storage conditions. Zearalenone is not degraded in common food and feed processing procedures, as it has been shown its presence in grain products like bread, locally brewed beers and processed feeds (Scott, 1996; Ryu *et al*., 2003; Fink-Gremmels and Malekinejad, 2007; Jouany, 2007).

Fumonisins are mainly produced by *F. verticillioides* (syn. *F. moniliforme*), and *F. proliferatum* and some other *Fusarium* species in food and feed commodities (Gelderblom *et al*., 1988; Bolger *et al*., 2001; Seefelder *et al*., 2002; Kritzinger *et al*., 2003; Logrieco *et al*., 2003; Binder *et al*., 2007; Glenn, 2007). Fumonisins were carcinogenic in experimental rodents, and consumption of grain contaminated with fumonisins has been associated epidemiologically with human disease (Desjardins, 2006). However, causality between fumonisins and human disease is unproven; this is not the case for animals (Voss *et al*., 2007).

Several reports have been published showing that feed contaminated with *F. verticillioides*, and by inference containing fumonisins, are the cause of poultry disease (Morgavi and Riley, 2007). Fumonisins are a family of over 28 homologues and more are likely to be found (Rheeder *et al*., 2002; Humpf and Voss, 2004; Voss *et al*., 2007). Amongst

the characterized compounds, fumonisins B1 (FB1) and fumonisin B2 (FB2) present the greatest mycotoxicological concern (Logrieco *et al*., 2003). Fumonisins differ structurally in the number and placement of hydroxyl groups on the molecule's hydrocarbon backbone (Voss *et al*., 2007). Fumonisins have not any discernable role in the frequency and intensity of colonization of plant hosts (Proctor *et al*., 2002).

Enniatins are non-ribosomal, cyclic depsipeptides with general antibiotic and phytotoxic activity (Desjardins, 2006). Several *Fusarium* species are able to produce enniatins, however little is known about the occurrence of these mycotoxins and their role in human and animal disease. The results on virulence of enniatins indicated that it is not essential for the successful infection of potato tuber tissue by *Fusarium* strains. However, it could still play a role in the pathogenicity of the strains that produce it (Herrmann *et al*., 1996). The study on natural occurrence of these mycotoxins in conventional and organic grain-based products from Italian and Finnish markets showed that Enniatins as well as deoxynivalenol are the most predominant mycotoxins and were present in 97% of the samples analyzed (Jestoi *et al*., 2004). Enniatins are a group of cyclic hexadepsipeptides with low-molecular-weight produced by *Fusarium* species (Gaeumann *et al*., 1950). It has been suggested that enniatins can occur at significant levels in wheat affected by *F. avenaceum* and *F. poae* in some European countries (Logrieco *et al*., 2003) and further study concerning their occurrence and toxicity are needed. It also will be important to determine if there are synergistic effects with other toxins, e.g., moniliformin and trichothecenes, to better evaluate the toxicological risk to humans and animals (Logrieco *et al*., 2002).

Interaction between *Fusarium* species

In nature, all fungi most often interact in order to survive in their habitat. This interaction can be achieved by the development of different mechanisms, which could be direct or indirect, including: neutral, commensalisms, mutualism, competition, parasitism and synergism. The organisms and the physico-chemical conditions present in an ecological niche will delimit the type of interactions that can be observed.

Competition is a detrimental interaction in ecological niches (habitats) since fungi present need to survive. It is also known that when fungi in an ecological niche utilize the same type of substrates, they must compete in different mechanisms. Competitive exploitation is the ability of one organism or population to consume the resources (without reducing the

access of the other organisms to the same resource pool), whereas interference competition involves chemical or behavioral mechanisms that the access to resource is influenced by the presence of competitor (Carroll and Wicklow, 1992). Amensalism interaction has been used to describe interactions with a negative outcome for only one species (Tuininga, 2005).

Fusarium head blight is often caused by more than one *Fusarium* species. It can be assumed that a determined kernel colonized by one *Fusarium* species or more than one species. So interaction between or among the pathogens may happen during infection process in different parts of wheat ears. These interactions can occur during spore germination (early interactions, outside of plant tissue), penetration or tissue colonization (late interactions, inside of the plant tissue) with different mechanisms. Interactions between fungal species have been reported to influence the predominance of species and – as a consequence - mycotoxin production (Reid *et al*., 1999; Velluti *et al*., 2000; Simpson *et al*., 2004). Environmental factors such as water activity, temperature, relative humidity, irradiation and pH value may influence the spectrum of different *Fusarium* species on wheat ears and in different areas and also their interactions (Rossi *et al*., 2002; Doohan *et al*., 2003; Llorens *et al*., 2004).

It has been suggested that interaction may exist among *Fusarium* species on the ears and will influence the severity of diseases and species profile. Very little information is available on inter- species interactions among mycotoxigenic FHB pathogens on wheat ears. However, this subject has been studied on other crops, especially interaction between toxin producing and non-toxin producing fungi.

Information from studies of interactions among pathogens may be useful in the forecasting of head blight on cereals and assessing the risk of mycotoxins accumulating in grain. Interactions between fungal species have been reported to influence the predominance of species and – as a consequence - mycotoxin production (Reid *et al*., 1999; Velluti *et al*., 2000; Simpson *et al*., 2004).

Populations of *Fusarium moniliforme* and *F. proliferatum* on irradiated maize grains were reduced to a greater or lesser extent by the presence of *F. graminearum* under different conditions (different water activity and temperature) tested. While, the presence of *F. proliferatum* or *F. moniliforme* had a limited inhibitory effect on fungal population of *F. graminearum* on maize (Marin *et al*., 1998; Velluti *et al*., 2000). In contrast, Marin *et al*., (1998) reported that all the three species appear to coexist in the same niche at 25 ± 30 °C. While *F. graminearum* may be at a competitive advantage over *F. moniliforme* and *F. proliferatum* at 15 °C, *F. proliferatum* was more competitive than *F. moniliforme* over a wide

Germany, Ireland, Hungary, and UK, while other authors detected this species in low frequency (Bai and Shaner, 1994; Parry *et al*., 1995; Yli-Mattila *et al*., 2002; Rohacik and Hudec, 2005; Xu, *et al.,* 2005).

A survey in four European countries showed that prevalent pathogens differed significantly among countries. Four major FHB pathogens such as *F. graminearum*, *F. culmorum*, *F. avenaceum* and *F. poae* were identified during study. All pathogens were commonly detected in Ireland, *F. graminearum* and *F. poae*, detected in Italy and Hungary, *Fusarium culmorum* was rarely detected except in Ireland. The frequency of FHB pathogens increased significantly from anthesis to the milky-ripe stage and to harvest (Xu *et al*., 2005).

Research findings in some European countries during the last two decades ago indicated a shift of the population from *F. culmorum* to a higher percentage of *F. graminearum* (Birzele *et al*., 2002; Obst *et al.,* 2002; Waalwijk *et al*., 2003; Xu *et al*., 2005). Additionally, these species are able to produce important mycotoxins such as trichothecenes in food and feed that are hazardous to human and animal health.

Studies on the virulence of major FHB pathogens indicated that *F. graminearum* and *F. culmorum* are the most virulent species; *F. avenaceum* is mildly to moderately virulent and *F. poae* is the least virulent species (Mihuta-Grimm and Forster, 1989; Wong *et al*., 1995; Miedaner and Schilling, 1996; Miedaner *et al*., 1996; Akinsanmi *et al.* 2004; Fernandez and Chen, 2005). In addition to inter-species variability in virulence, isolates within a species have been reported to differ significantly in disease-causing ability. Nevertheless, in experiments determining the frequency of *Fusarium*-infected wheat kernels, the less virulent species *F. avenaceum* and *F. poae* predominated in the FHB complex in the Rhineland area, Germany.

Recently, a number of nucleic acid-based techniques have been utilized to understand the nature and diversity of FHB pathogens, the size and dynamics of populations of *Fusarium* species, interactions between pathogens and their host and among pathogens (Nicholson *et al*., 2003; Schena *et al*., 2004). Molecular tools allow the detection, identification and quantification of closely related fungal species in a one-step procedure.

Identification of *Fusarium* species

Attempts to evaluate the frequency of fungal species in a particular context, in the absence of selective media is confound, because the relative amount of each pathogen may not be accurately determined (Nicholson, 2003). For example, Pettitt *et al*., (1993) reported that benomyl in agar media effectively reduced competition from fast growing *Fusarium*

range of temperature and water activity conditions. Reid *et al.* (1999) reported that dry and warm conditions during growth of maize strongly affect the infection of ears by *F. moniliforme* and *F. graminearum*. *Fusarium moniliforme* has at least one competitive advantage over *F. graminearum*, because of a broader response to temperature that confers direct and indirect benefits. Marin *et al*. (1998) showed that *F. moniliforme* and *F. proliferatum* are able to dominate several other common maize-contaminating fungi over a wide range of temperature and water availability conditions – e.g. *Aspergillus* spp. and *Penicillium* spp.

Marin *et al*. (2001) showed complex interactions between aflatoxin and fumonisin producing fungi in maize grain and influence of environmental conditions on the outcome of dominance fungi and mycotoxin production. Generally, *Aspergillus parasiticus* reduced populations of *Fusarium* spp., but did not affect fumonisin B1 production by them. While, *Fusarium* spp. were not able to decrease populations of *A. parasiticus*, they significantly reduced the amount of aflatoxin B1 accumulation.

Data on effects of different fungicides to control FHB indicated that fungicides are able to produce differential controls depending on spectrum of *Fusarium* spp., efficacy of fungicides, and concentrations of fungicides, time of applications, perhaps method of application and interactions among them and also with cultivar. Greenhouse and field trials carried out to assess the efficacy of fungicides against FHB pathogens have yielded inconsistent results.

During 2 years of field studies, application of prochloraz and tebuconazole at mid flowering caused a significant reduction in FHB severity and incidence as well as DON content. The use of tebuconazole-azoxystrobin mixture in the same study significantly decreased FHB severity and incidence as well as the DON contents in 2003. However, in 2002 the DON content of kernels was higher than that for the untreated control, with particularly high values in the cooler and wetter sites (Blandino *et al*., 2006). In contrast, Haidukowski *et al*. (2005) showed that application of tebuconazole-azoxystrobin mixture significantly reduced the FHB severity and DON content in the grain as compared to the inoculated control (a mixture of *F. culmorum* and *F. graminearum*). A report by Simpson *et al*. (2001) showed that fungicides could change pathogen populations on wheat ears. For example, following the application of azoxystrobin *Microdochium nivale* was selectively controlled while *Fusarium* spp., were largely unaffected or markedly increased. In contrast, tebuconazole generally reduced *Fusarium* more than *Microdochium.*

The level of efficacy of different fungicides against certain *Fusarium* species can be different. For example, the efficacy of some fungicides tested against the major FHB pathogens decreased towards the species with higher pathogenicity (Kamil, 2006). For example, a mixture of triazoles and strobilurins significantly reduced FHB severity, while they have different effects on DON content. Applications of some fungicides may give certain combinations or alter the proportion of *Fusarium* spp. within the FHB complex and the rate of mycotoxins synthesis (Jennings and Turner, 2000; Edwards *et al*., 2001). Cromey *et al*. (2001) showed that application of azoxystrobin, tebuconazole and carbendazim at end of heading or mid flowering on winter wheat plots naturally infected with *Fusarium* spp. reduced the FHB severity compared to the control treatment. Tebuconazole and carbendazim significantly decreased the amount of DON and NIV in grain, while azoxystrobin did not have any effect on these mycotoxins. *Fusarium graminearum* reacted in various ways when incubated in the presence of sub-lethal concentrations of some fungicides such as prochloraz, tebuconazole, benomyl, carbendazim and thiabendazole (Matthies *et al*., 1999).

Effective chemical control of FHB is further confounded by the fact that FHB is a disease caused by a complex of pathogens where interaction may exist among them and also with common saprophytic fungi such as *Alternaria* spp. and *Cladosporium* spp. One of the best ways to reduce the risk of FHB is applying fungicide in a timely manner. For example, an early fungicide application may suppress the saprophytic microflora or reduce inhibitory effects of other micro-organisms on the grains, while the *Fusarium* species are less affected. This could have given an advantage to the *Fusarium* species and increase level of FHB after fungicide application (Henriksen and Elen, 2005). Liggitt *et al*. (1997) stated that common saprophytic flora of wheat ears and *F. culmorum* could be competing for the same niche and therefore applications of some fungicides may preferentially inhibit the saprophytes, allowing the niche to become occupied by the pathogen and lead to greater colonization of wheat ears by the pathogen, due to the removal of antagonistic saprophytes. These workers demonstrated that inoculation saprophytic fungi prior to *F. culmorum* at mid flowering were able to reduce FHB severity by 46 % to 78 % compared to plants inoculated only with *F. culmorum*. This information indicated that fungicides may influence the spectrum of fungal species and change the ratio between toxigenic and non-toxigenic or common saprophytic fungi.

Applying fungicides or herbicides need knowledge on inter-species interactions among FHB complex disease on wheat ears. Some researchers used a mixture of inoculum to favour the approach. However, it is questionable, because of the possibility of multiple ecological interactions among these pathogens in negative or positive ways in natural

infections, the pathogens may differ in prevalence and potential damage and some fungicides may be effective for the suppression of one pathogen but not for the other.

There is evidence that glyphosate based herbicides may or may not increase FHB pathogens under controlled conditions and field (Hanson and Fernandez, 2003; Henriksen and Elen, 2005). In Canada, the observation has indicated that the effect of glyphosate is incremental or detrimental on growth of *F. avenaceum* and *F. graminearum*, respectively (Hanson and Fernandez, 2003).

Aims of study

Investigations on putative interactions among *Fusarium* species colonizing wheat ears were carried out to explain the preference of infection of wheat kernels by *F. avenaceum* and *F. poae* in the presence of inoculum of more virulent *Fusarium* species, which affect the spectrum of mycotoxins. To achieve this aim, molecular and microbiological tools were used to determine frequency and intensity of colonization of wheat ears. This was quantified by determining the amount of *Fusarium* DNA and infection for individual species in single or mixed inoculations and establishing the species effect on the plants (symptoms, yield formation).

The specificity of available species-specific primers for *F. graminearum, F. culmorum, F. avenaceum* and *F. poae* were evaluated using conventional PCR and then a SYBR green real-time PCR was optimized to determine the content of fungal biomass in a matrix including fungal and plant DNA. Different DNA extraction methods were tested in order to quantify the amount of *Fusarium* DNA in wheat using real-time PCR. There were some problems to produce inoculum in *F. graminearum* in high quantities. Thus, a rapid and simple method for spore production was developed for the tested *Fusarium* species.

Objectives

Different experiments were conducted to understand the nature of virulence (to the host plant), interactions among the *Fusarium* species as well as with host plant and the effects on the plants (yield formation) during host physiological maturation under greenhouse and field conditions. The most important aspect of the study was to get knowledge on mycotoxin productivity under interactions of different *Fusarium* isolates.

This was done with the following investigations:

1- Establish the preference of *Fusarium* isolates in two consecutive inoculations with one-day interval.

2- Study the effects of single and mixed inoculations of the isolates on the virulence of FHB pathogens to wheat ears under greenhouse and field conditions.

3- Investigate competitive abilities among the isolates on wheat ears. In these experiments, all possible combinations of four *Fusarium* isolates with 2, 3 and 4 isolates in mixture which result in 6, 4 and 1 combinations, respectively. The total inoculum concentration varied or not in order to compare the frequency of infected kernels, intensity of kernel colonization, disease severity and the content of mycotoxins in kernels by the isolates in the presence of one, two, or three other isolates.

4- Investigate susceptibility of glume, lemma, palea and kernel to *Fusarium* isolates under field conditions.

5- Study development of *Fusarium* isolates on spikelets and wheat flower parts in single and mixed inoculations.

6- Study the frequency of spikelets and kernels infected by more than one *Fusarium* isolates.

Various parameters were assessed to characterize the interactions among the isolates in different experiments including ratings of disease severity, 1000-kernel weight, frequency of infected kernels, species–specific DNA amount of fungal isolates and the content of mycotoxins in kernels.

2. Experimental parts

2.1 Materials and methods

2.1.1 Organisms

2.1.1.1 Plant

The following wheat cultivars (*Triticum aestivum* L.) were used in this study:

- Cultivar 'Munk' for greenhouse experiments
- Cultivars 'Drifter' and 'Taifun' for field experiments in 2005 and 2006, respectively.

2.1.1.2 Fungi

The fungal isolates listed in the Table 1 were used in this study. The source of all *Fusarium* isolates were wheat kernels, except *F. poae* DSM 62376 that its origin was *Avena sativa*. Other fungal isolates were isolates from wheat plant, which had been grown under greenhouse conditions.

2.1.2 Chemicals for molecular and microbiological assays

Biomol (Hamburg): Phenol, Phenol/Chlorophorm/Isoamyl alcohol (25:24:1), Lambda DNA Eco91I (BstE II) and Lambda DNA Hind*III*

Biozym Dianostic: SequiTherm EXCELTMII, Sequagel XR sequencing gel (National Diagnostics)

Qiagen (Hilden): RNeasy, DNAse plant Mini kit

Roth (Karlsruhe): Acetic acid, Agar-Agar, Ampicillin, Ammonium peroxide sulphate (APS), Ethylenediaminetetraacetic acid (EDTA), Ethanol, Ethidium bromide, Hydrochloric acid, Isopropyl β-D-thiogalactoside (IPTG), Kohrsolin. FF, Nitric acid, Pepton, Potassium dihydrogen phosphate, 2- Propanol, Silver nitrate, Sodium acetate, Sodium carbonate, Sodium chloride, Sodium hydroxide, Trichloromethane/chlorophorm, Tris, X-Gal (5-bromo-4- chloro-3-indolylbeta-Dgalactopyranoside), Yeast extract.

Sigma-Aldrich Chemie GmbH (Munich): Agarose, Ammonium acetate, Calcium chloride, Calcium chloride dihydrate, Calcium lactate, Dulbecco's phosphate buffered saline (D-PBS), Isopropanol, Magnesium chloride, Magnesium chloride hexahydrate, 2-Mercaptoethanol, Oligonucleotide primers, Penicillin, 10 X PCR reaction buffer, Potassium chloride, Sodium hydrogen carbonate, Sodium hydrogen phosphate, Sodium hydrogen

sulfate, Streptomycin sulfate, Taq DNA polymerase, GenEluteTM plasmid mini kit

STARLAB GmbH (Ahrensburg): Rigid thin wall 96 X 0.2 mL skirted microplates

Promega: PGEM®-T and PGEM®-T Easy Vector Systems Promega

Table 1: Fungal species used in this study (all isolates were from the Institute of Crop Science and Resource Conservation, University of Bonn, Germany; besides of DSM 62376, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany)

Fungal species	Isolate	Fungal species	Strain
Fusarium graminearum	5.1	*Fusarium poae*	7.3
Fusarium graminearum	5.10	*Fusarium poae*	7.5
Fusarium graminearum	5.15	*Fusarium poae*	7.6
Fusarium graminearum	5.17	*Fusarium poae*	7.8
Fusarium graminearum	5.19	*Fusarium poae*	7.9
Fusarium culmorum	3.6	*Fusarium poae*	7.10
Fusarium culmorum	3.2	*Fusarium poae*	7.11
Fusarium culmorum	3.6	*Fusarium poae*	7.12
Fusarium culmorum	3.9	*Fusarium poae*	7.14
Fusarium culmorum	3.11	*Fusarium poae*	7.15
Fusarium culmorum	3.12	*Fusarium poae*	7.16
Fusarium culmorum	3.14	*Fusarium poae*	DSM 62376
Fusarium culmorum	3.16	*Fusarium langsethae*	18.1
Fusarium culmorum	3.17	*Fusarium tricinctum*	10.10
Fusarium culmorum	3.18	*Fusarium sporotrichioides*	9.9
Fusarium culmorum	3.22	*Fusarium sporotrichioides*	9.10
Fusarium culmorum	3.33	*Fusarium cerealis*[*]	
Fusarium culmorum	3.35	*Blumeria graminis*[*]	
Fusarium culmorum	3.36	*Puccinia triticina*[*]	
Fusarium culmorum	3.37	*Alternaria* sp.[*]	
Fusarium avenaceum	1.7	*Trichoderma* sp.[*]	
Fusarium avenaceum	1.8	*Aspergillus* sp.[*]	
Fusarium avenaceum	1.12	*Penicillium* sp.[*]	
Fusarium avenaceum	1.12		
Fusarium avenaceum	1.16		

Table 2: Sequences of species-specific primers for detecting *Fusarium* species, PCR chemotypes and large and small amount of DON

Fragment name	Sequence of primer	Target	Size (bp)	Annealing temperature
[A] *F. graminearum*	GGCGCTTCTCGTGAACACA TGGCTAAACAGCACGAATGC	*F. graminearum*	94	55 ºC
[A] *F. culmorum*	TCACCCAAGACGGGAATGA GAACGCTGCCCTCAAGCTT	*F. culmorum*	60	55 ºC
[A] *F. avenaceum*	CAAGCCCACAGACACGTTGT CCATCGCCGTGGCTTTC	*F. avenaceum*	58	57 ºC
[B] *F. poae*	CAAGCAAACAGGCTCTTCACC TGTTCCACCTCAGTGACAGGTT	*F. poae*	220	60 ºC
[C] GzTri7F GzTri7R	GGCTTTACGACTCCTCAACAATGG AGAGCCCTGCGAAAG(C\T)ACTGGTGC	NIV & DON	162+11 × n	60 ºC
[D] Tri13F Tri13R	TACGTGAAACATTGTTGGC GGTGTCCCAGGATCTGCG	NIV & DON	234 or 415	60 ºC 30 s
[E] 4056 3551	ATCCCTCAAAAACTGCCGCT ACTTTCCCACCGAGTATTTC	DON, Low [I]	600	55 ºC
[E] N1-2 N1-2R	CTTGTTAAGCTAAGCGTTTT AACCCCTTTCCTATGTGTTA	DON, High [I]	200	50/40 ºC
[F] Tri303F Tri303R	GATGGCCGCAAGTGGA GCCGGACTGCCCTATTG	3-AcDON	586	51/46 ºC
[F] Tri315F Tri315R	CTCGCTGAAGTTGGACGTAA GTCTATGCTCTCAACGGACAAC	15-AcDON	549	60 ºC 30
[G] ITS-FUF ITS-FUF	CAACTCCCAAACCCCTGTGA GCGACGATTACCAGTAACGA	*Fusarium* genus	398	55 ºC 30
[H] ITS-1F ITS-4R	CTTGGTCATTTAGAGGAAGTAA TCCTCCGCTTATTGATATGC	ITS	500-600	54 ºC 30

A, Waalwijk *et al*., (2004); B, Parry and Nicholson, (1996); C, Lee *et al*., (2001); D, Waalwijk *et al*., (2003); E, Bakan, (2002); F, Jennings *et al* ., (2004); G, Abd-Elsalam *et al*., (2003); H, White *et al*., (1990); I, detection low and high producer of deoxynivalenol NIV, nivalenol; DON, deoxynivalenol; 3-AcDON,3-acetyldeoxynivalenol;15-AcDON,15-acetyl-deoxynivalenol; ITS, internal transcribed spacer

2.1.3 The following buffers were used for molecular assays

TAE (50x) buffer, PH 8	Hydroxymethyl (Tris)	242.0 mg
	Acetic acid	57.1 mL
	EDTA (186.1 mg/mL)	100.0 mL
	Water	1000.0
TE buffer, PH = 8, for 250 mL	Tris-HCl, 10 (mM)	2.5 mL M
	EDTA (1 mM)	0.5 mL M
	Water	250 mL
	Filter sterilized	
Ethanol (70 % v/v)	Ethanol (100 %)	70 mL
	Water	30 mL
Proteinase K (10mg/mL)	Proteinase K	50 mg
	Water	5 mL
RNase (10 mg/mL)	RNase	50 mg
	Water (Boiled for 5 min)	5 mL
CTAB buffer		
	Tris	1.21 g
	EDTA	7.44 g
	Cetyltrimethylammonium bromide (CTAB)	7.28 g
	NaCl	46.4 g
	N-lauryl sarcosine	8.80g
	Sorbitol	23.68 g
	Polyvinylpolypyrrolidone (PVPP)	10.0 g
	Water	1000.0
X-gal	X – gal	50.0 mg
	N, N'-dimethylformamide	1.0 mL
LB-broth:	Sodium chloride	8.0 g
	Peptone	8.0 g
	Yeast extract	4.0 g
	Sodium hydroxide (40.0 mg/mL)	480.0 µL
	Water up to	800.0 mL

2.1.4 Equipments for nucleic acid and mycotoxin quantification

ABI PRISM 7000 SDS	Applied Biosystems, Foster city, USA
CEQ 8000 genetic analysis	apparatus Beckman Coulter, Inc, USA
Electrophoresis (for agarose gels)	Bio Rad München, Germany
Icycler	Bio Rad Laboratories, München, Germany
Memmert CO2 incubator	Fisher Scientific Leicestershire, UK
Millipore apparatus	Millipore Corporation, USA
Millipore Corporation (PTC 100)	Millipore Corporation
Spectrophotometer (Ultrospec™ 2100)	Amersham Bioscience Freiburg, Germany
QTrap 4000 LC/MS/MS system	Applied Biosystems, Foster City, USA
HPLC system (1100 Series)	Agilent, Waldbronn, Germany
C18 HPLC column	Phenomenex, Torrance, USA
security guard cartridge	Phenomenex, Torrance, USA

2.1.5 Culture media for cultivation of *Fusarium* species

All the media are recipes per litre of distilled water. The culture media were autoclaved at 121 ºC for 20 min at one bar pressure.

Czapek-Dox-Iprodione-Dicloran Agar (CZID) (Abildgren *et al*., 1987)

Czapek Dox agar	35.0 g
$CuSo_4 \cdot 5H_2O$	5.0 mg
$ZnSo_4 \cdot 7H_2O$	10.0 mg
Chloramphenicol	50.0 mg
Dicloran	2.0 mg
Agar	10.0 g
When the media had cooled to about 55 ºC, the following were added.	
Penicillin	50.0 mg
Streptomycin	50.0 mg
Rovral (Iprodione)	6.0 mg

Synthetic Nutrient Agar (SNA) (Nirenberg, 1981)

KH_2PO_4	1.0 g
KNO_3	1.0 g
$MgSO_4.7H_2O$	0.5 g
KCl	0.5 g
Glucose	0.2 g
Sucrose	0.2 g
Agar	20.0 g

Potato Dextrose Agar (PDA) (Merck, Darmstadt Germany)

Potato dextrose agar	39.0 g
Penicillin	50.0 mg
Streptomycin	50.0 mg
Ampicillin	50.0 mg

Low Strength Potato Dextrose Agar (LSPDA, Merck, Darmstadt Germany)

Potato dextrose agar	12.5 g
Penicillin	50.0 mg
Streptomycin	50.0 mg
Ampicillin	50.0 mg
Agar	19.0 g

Banana Leaf Agar (BLA)

Fresh banana leaves were cut into 1 - 2 cm^2 pieces and dried in an oven at 50 - 55 ºC between 2 and 3 h. The banana leaf pieces were sterilized in 1.3 % sodium hypochlorite (NaOCl) for 5 min and then in ethanol for 30 s. The pieces were dried under a laminar flow cabinet for 30 min. Five leaf pieces were placed on Petri-dishes including cooled water agar or SNA. To check for possible contaminations, the plates were left at room temperature for three days before use.

2.1.6 Plant cultivation

2.1.6.1 Greenhouse

Seeds of spring wheat (cv. Munk) were planted in 10 L-pots, (22 cm diameter) with 15 seeds per pot using a 12:7:1 mixture of organic potting substrate, field soil (soil horizon C) and sand, respectively. After emergence, plants were thinned to 12 and were grown under greenhouse conditions with a 16-h light photoperiod at 23.5 ± 4 ºC and 24.6 ± 2 ºC in 2005 and 2006, respectively. The intensity of light was 300-µmol m^{-2} s^{-1} in plant height. Plants were fertilized at GS 29 using NPK (4-2-3 g/l).

2.1.6.2 Field

Field trials were conducted at the Poppelsdorf experimental station, University of Bonn, Germany. In 2005, winter wheat cv. Drifter was planted in October and sprayed with Fandango ® (prothioconazole + Fluoxastrobin) to control powdery mildew. In 2006, spring wheat cv. Taifun was sown in April and sprayed with Tristar ® (Acetamiprid) to control aphids. The plots were separately harvested using a combine harvester on August 03 and July 24 in 2005 and 2006, respectively. The kernels were stored at −20°C for further analysis.

2.1.7 Design of experiments

2.1.7.1 Greenhouse

Wheat ears cv. Munk were inoculated at mid flowering - growth stage (GS) 65 (Meier, 1997) with one species and combinations of two, three or four *Fusarium* spp. (Table 3 and 4). In 2005, wheat ears were inoculated with variable inoculum concentrations, while variable and constant inoculum concentrations were used in the investigations in 2006.

In the variable concentration approach, mixtures of two, three and four species were produced resulting in 120 mL inoculum with a final concentration of 1×10^5, 1.5×10^5 and 2×10^5 conidia mL^{-1}, respectively, while the concentration of individual species in the mixture was maintained at 5×10^4 conidia mL^{-1} (Table 5).

In the constant concentration approach, the final inoculum concentration was 5×10^4 conidia mL^{-1} for all treatments; in mixtures of two, three and four *Fusarium* spp. the conidia concentration of each species was half, one third and one quarter the concentration in single inoculations. Control plants were sprayed with sterile distilled water in the same way. Experiments were conducted in a completely randomized design with 5 replicates.

Table 3: Experimental design using a variable concentration of conidia approach

Inoculation	Concentration of conidia mL^{-1} [x 1000]				
	FG	FC	FA	FP	Total
F. graminearum (FG)	50				50
F. culmorum (FC)		50			50
F. avenaceum (FA)			50		50
F. poae (FP)				50	50
FG + FC	50	50			100
FG + FA	50		50		100
FG + FP	50			50	100
FC + FA		50	50		100
FC + FP		50		50	100
FA + FP			50	50	100
FG + FC + FA	50	50	50		150
FG + FC + FP	50	50		50	150
FG + FA + FP	50		50	50	150
FC + FA + FP		50	50	50	150
FG + FC + FA + FP	50	50	50	50	200

Table 4: Experimental design using a constant concentration of conidia approach

Inoculation	Concentration of conidia mL^{-1} [x 1000]				
	FG	FC	FA	FP	Total
F. graminearum (FG)	50				50
F. culmorum (FC)		50			50
F. avenaceum (FA)			50		50
F. poae (FP)				50	50
FG + FC	25	25			50
FG + FA	25		25		50
FG + FP	25			25	50
FC + FA		25	25		50
FC + FP		25		25	50
FA + FP			25	25	50
FG + FC + FA	16.7	16.7	16.7		50
FG + FC + FP	16.7	16.7		16.7	50
FG + FA + FP	16.7		16.7	16.7	50
FC + FA + FP		16.7	16.7	16.7	50
FG + FC + FA + FP	12.5	12.5	12.5	12.5	50

Table 5: Experimental designs using a variable (2005, 2006) and a constant (2006) conidia concentration approach

Number of *Fusarium* species in inoculum	Concentration of conidia mL^{-1} [x 1000]			
	Variable conc. approach		Constant conc. approach	
	Per species	Total	Per species	Total
1	50	50	50	50
2	50	100	25	50
3	50	150	16.7	50
4	50	200	12.5	50

2.1.7.2 Field

Wheat ears cv. Drifter were inoculated at mid flowering (GS 65) (Meier, 1997) in two consecutive days with 1-day interval. The first day (01.06.2005), *F. graminearum*, *F. culmorum*, *F. avenaceum* and *F. poae* were inoculated in four plots comprising of four sub-plots (0.5 m^2), separately (Table 6). The second day (02.06.2005), single inoculations of the four *Fusarium* spp. was carried out in every sub-plot resulting in either a two species combination or a double-single species inoculation. For the single inoculations, the first day, every sub-plot was inoculated singly with *Fusarium* spp. The sub-plots were sprayed with sterile distilled water in the second day. A concentration of 9.1 x 10^4 conidia mL^{-1} was used in all of the inoculations. Experiments were conducted in a completely randomized design.

In 2006, wheat ears cv. Taifun were inoculated alone or in two, three and four species combination of *Fusarium* species in constant and variable approaches, as described for greenhouse experiments (Table 3 and 4). Each treatment was inoculated separately in 4 replicate plots (1.5 m^2). The inoculated plots were separated at least 1 m^2 from each other to minimize plot-by-plot interferences. Experiments were conducted in a completely randomized block design with 4 replications.

Table 6: Experimental design of consecutive inoculations approach in 2005

Inoculation	Inoculation	
	First day (01.06.2005)	Second day(02.06.2005)
FG + FG	FG	FG
FC + FG	FC	FG
FA + FG	FA	FG
FP + FG	FP	FG
FG + FC	FG	FC
FC + FC	FC	FC
FA + FC	FA	FC
FP + FC	FP	FC
FG + FA	FG	FA
FC + FA	FC	FA
FA + FA	FA	FA
FP + FA	FP	FA
FG + FP	FG	FP
FC + FP	FC	FP
FA + FP	FA	FP
FP + FP	FP	FP
FG + W	FG	W
FC + W	FC	W
FA + W	FA	W
FP + W	FP	W

FG, *F. graminearum*; FC, *F. culmorum*; FA, *F. avenaceum*; FP, *F. poae*; A concentration of 9.1 x 10^4 conidia mL^{-1} was used in all of the inoculations

2.1.8 Pathogens

The *Fusarium* isolates FA 1.7 (*F. avenaceum*), FC 3.11 (*F. culmorum*), FG 5.1 (*F. graminearum*), and FP 7.8 (*F. poae*) had been isolated from winter wheat fields in North Rhine-Westphalia, Germany; FA 1.7 (Dormagen, 1995), FC 3.11 and FP 7.8 (Lage-Ohrsen, 2002), FG 5.1 (Blankenheim, 1996) were used. Single-hypha isolates were used for the all experiments.

The following *Fusarium* isolates were used for rapid spore production method; *F. avenaceum* (1.7, 1.15, 1.16, 1.17), *F. culmorum* (3.2, 3.11, 3.35, 3.38, AG 6a), *F. graminearum* (5.1, 5.15, 5. 17, 5.18, AG 23d), *F. poae* (7.8, 7.9, 7. 15, R8 a), *F. tricinctum* (10.2, 10.12, 10.11), *F. verticillioides* (AG 11i) and *F. proliferatum* (AG 31g).

2.1.9 Inoculation

2.1.9.1 Inoculum production

Conventional method

In 2005, *F. graminearum*, *F. culmorum*, *F. avenaceum* and *F. poae* were grown LSPDA for 21 days under near ultra violet light. Conidia were harvested by adding sterile distilled water including some droplets of Tween 20 and slightly scraping with a spatula. The suspension was passed through double-layered cheesecloth.

Rapid spore production method

In 2006, a new method of spore production was developed. In the new method, *Fusarium* spp. were cultured in potato-dextrose broth (24 gl^{-1}) in 1000 mL Erlenmeyer flasks for five days in darkness on a shaker (200 rpm) at 22 ºC. After 1, 2, 3, 4 and 5 days of incubation, 500-1000 microliter suspension were spread over the surface of petri-dishes containing LSPDA, water agar (agar-agar 20 g/l^{-1}) and synthetic nutrient-poor mineral agar (SNA, Nierenberg, 1981) using a sterile bent-glass rod in five replications. To remove excess water, the cultures were dried under a laminar flow cabinet for 20 to 30 min.

The plates were incubated under near ultra violet light at 22 ºC for 2 to 14 days. The cultures were examined daily during this period for spore production. Conidia were harvested by adding sterile distilled water including some droplets of Tween 20 and slightly scraping with a spatula. The suspension was passed through double layered cheesecloth. The number of conidia per mL was determined using a haemocytometer. The produced conidia on PDA were adjusted to 5×10^4 conidia mL^{-1} and used in experiments aimed at studying the quality of conidia. Other media such as SNA and WA were included in the experiments to assess the ability of conidia production on different media. The concentration of conidia was determined using a haemocytometer. Inoculum concentrations of 5×10^4 conidia mL^{-1} were prepared for individual isolates of *Fusarium* species.

At mid flowering (GS 65, Meier *et al*., 1997) sixty wheat ears (*Triticum aestivum*, cv. Munk) were inoculated with 120 mL spore suspension (5×10^5 conidia mL^{-1}) of the *Fusarium* isolates 1.7 (*F. avenaceum*). 3.11 (*F. culmorum*), 5.1 (*F. graminearum*), and 7.8 (*F. poae*), respectively. Control plants were sprayed with sterile distilled water in the same way. After inoculation, the plants were covered with plastic bags for 48 hours to ensure high relative humidity for optimum infection conditions. The experiments were conducted in a completely randomized design with 5 replicates (5 pots with 12 ears).

2.1.9.2 Inoculation techniques

The concentration of conidia was adjusted as summarized in the Tables 3 to 6 and used for the inoculations. The plants were inoculated using a hand sprayer under greenhouse experiments, while a manual sprayer used for field inoculations. After inoculation, plants were covered with plastic film for 48 hours to ensure high relative humidity for infection.

2.1.10 Assessment of disease

2.1.10.1 Visual disease ratings and yield assessment

Assessment of disease severity was done in two trial experiments under greenhouse conditions, while in field experiments the symptoms were visible to assess only in 2006. Disease severity was assessed as the percentages of bleached spikelets 14, 21 and 28 days after inoculation using a nine-class rating scale (Miedaner *et al.*, 1996) with 1 = no infection, 2 = <5; 3 = 5-15; 4 = 16 to 25%; 5 = 26 - 45%; 6 = 46 - 65%; 7 = 66 - 85%; 8 = 86 - 95%, and 9 = 96 - 100% of bleached spikelets. The mean value of the three disease severity ratings was determined.

2.1.10.2 Microbiological detection of *Fusarium*-infected kernels

Czapek-Dox-Iprodione-Dicloran agar (Abildgren *et al*., 1987) was used for re-isolation of *Fusarium* spp. Synthetic nutrient-poor mineral agar (Nierenberg, 1981) and Banana leaf agar (Seifert, 1996) were used to grow the isolates for morphological identification. *Fusarium* species were identified according to Nelson *et al.* (1983).

2.1.10.3 Morphological identification of *Fusarium* species

Fungal isolates were cultured on PDA for 21 days under near ultra violet light. Conidia were harvested by adding sterile distilled water including some drops of Tween 20 to wash the mycelium and lightly scraping with a spatula to dislodge the conidia. The suspension was passed through double layered cheesecloth. The suspension was diluted and spread on WA (water agar 2 %) using a sterile bent-glass rod. After 10 to 24 h the plates were examined using a stereo-microscope and single germinated conidia were transferred on PDA.

For morphological identification, *Fusarium* species were sub-cultured on PDA, SNA and BLA. The cultures were incubated under near ultra violet light for 2-3 weeks. *Fusarium*

species were identified according to Nelson *et al.* (1983). PDA was used for morphological features such as growth rates, aerial mycelium, and colour of back side of the colony, cultural appearances and pigmentation. Microscopic observations were carried out on SNA and BLA.

The microscopic characters used were the macroconidia morphology (size, abundance, number of septa, shape, length of macroconidia, basal and apical cells, production in aerial mycelia or sporodochia), microconidia (present or absent, abundance, shape, number of septate, produced in false heads or in chains), types of conidiophores and chlamydospores (present or absent, abundance, whether produce intermediately or terminally and appearances).

2.1.11 Sampling

2.1.11.1 Greenhouse

For the frequency of kernel colonization and the amount of fungal DNA, 50 kernels per replicate were cut into two pieces perpendicular to kernel length axis (one part for microbiological detection, the other for real-time PCR). To extract DNA and later on real-time PCR assays the pieces of the kernels were ground to fine powder using a blender machine and 250 mg of kernels weight were included in the experiments. The mean value per replicates was recorded and used for statistical analysis.

2.1.11.2 Consecutive field inoculations (2005)

Wheat flower parts

To determine the frequency of infection of individual parts of the wheat flower parts sampling was carried out 7, 21 and 35 days after inoculation. Wheat flower parts were manually separated to glume, palea, lemma and kernel. The mean value of infection for each part per ear was used for statistical analysis.

At harvest

Firstly, 5 wheat ears with FHB symptoms from each sub-plot were randomly harvested. Wheat ears were separately cut and threshed using small combine harvester (Kurt pelz, Waldendurg, Germany) to obtain the grains. The kernels from every ear were cut into two pieces perpendicular to the length axis (one piece for microbiological assays, the other

one for the content of *Fusarium*-kernel DNA using real-time PCR). To extract DNA and later on real-time PCR assays the pieces of the kernels were ground to fine powder using a blender machine and 250 mg of kernels weight were included in the experiments. The mean value of frequency of infected kernels of every wheat ear and the amount of fungal DNA per milligram dry weight were determined.

Secondly, the Wheat ears in every sub-plot were separately cut and threshed using combine harvester to obtain the grains. Thirty kernels in five replicates from every sub-plot were randomly used to determine the frequency of *Fusarium*-infected kernels using microbiological assays. 10-grams of kernels per sub-plots were ground to fine powder using a blender machine. To assess the content of species-specific content of *Fusarium*-kernel DNA 250-mg of the fine powder in three replicates were used for molecular assays.

2.1.11.3 Simultaneous field inoculations (2006)

Spikelet

Ten wheat ears per plot were randomly sampled seven (GS 73), 14 (GS 83) and 21 (GS 89) days after inoculation. The frequency of *Fusarium*-infected spikelets was determined for each wheat ear. The mean values of infected spikelets were determined for each plot and used for statistical analysis.

Wheat flowers parts

To determine the frequency of infection of wheat flower parts sampling was carried out seven (GS 73), 14 (GS 83) and 21 (GS 89) days after inoculation from plots, which had been inoculated with a mixture of four isolates (constant inoculum concentration, 5×10^4). Wheat flower parts were manually separated to glume, palea, lemma and kernel. The mean value of infection for each part of the florescence was used for statistical analysis.

At harvest

The Wheat ears in each plot were separately cut and threshed using combine harvester to obtain the grains. Thirty kernels from every plot with five replicates were randomly used to determine the frequency of *Fusarium*-infected kernels and the content of fungal DNA using microbiological and molecular assays. The kernels were cut into two pieces perpendicular to

the length (one piece for microbiological assays, the other one for the content of *Fusarium*-kernel DNA using real-time PCR). The mean value of frequency of infected kernels and amount of fungal DNA for every plot was determined.

2.1.12 Yield assessment

In greenhouse experiments kernel weight was recorded for every replicate. Data are shown based on 1000-kernel weight which was calculated from kernel weight and number of kernels per replicate. In field experiment, data were determined based on 3 or 4 times calculating kernel weight for 1000-kernels per replicate.

2.1.13 Molecular assay

2.1.13.1 DNA extraction methods

DNA purification for quantitative PCR

DNA was extracted from a 5 day cultures of *Fusarium* spp. in potato-dextrose broth (Merk, Darmstadt, Germany) (24g/L) in 1000 mL Erlenmeyer flasks (25 °C, 200 rpm). The contents of flasks were filtered and washed three times with sterile distilled water using a vacuum pump. The mycelia were collected in 2 milliliter tubes and frozen at -80 °C. Mycelia (100 - 250 mg) were ground under liquid nitrogen to a fine powder using mortar and pestle and then transferred to 50 milliliter tubes. DNA was extracted as described below for grain. Further chloroform-isoamyl alcohol (24:1) and ammonium acetate precipitation steps were done to obtain high quality DNA. The quality and quantity of isolated DNA were checked on agarose gel and with a spectrophotometer. A 10-fold dilution series (from 0.9 to 9000 pg/µL) of Purified DNA were used as standard curve in every real-time PCR assays.

DNA extraction from grain samples

The efficacy and suitability of CTAB method (Stewart and Via 1993; Brandfass and Karlovsky, 2005) with some modifications, Plant Mini kit Method (QIAGEN Germany) and combination of two methods were compared on wheat grain samples that had been colonized by *Fusarium* spp. using quantitative PCR, agarose gel and spectrophotometer assays. Based on quality and quantity a modified CTAB method was used in all further experiments.

CTAB method

Twenty mL of CTAB-extraction 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, pH set to 8.0 with NaOH), 40 µL mercaptoethanol and 50 µL proteinase K (from a stock solution 10 mg/mL), were added to 250 mg ground grains in 50–mL centrifugation tube and mixed vigorously. The mixture was incubated at 65 °C for 60 min and mixed after every 10 min. Eight hundred µL of the upper phase was transferred to a 2 mL new tube containing 5 µL of RNAase (50 mg/mL) and incubated for 10 min at 65 °C, Eight hundreds µL of chloroform-isoamyl alcohol (24:1) was added into each tube. The samples were mixed by inverting the tubes and centrifuged for 10 min at 5,000 g at room temperature. The aqueous phase was transferred into a 1.5 mL tube containing 500 µL isopropanol, mixed and 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 buffer and incubated at 4 °c over night and then in -20 °c.

Plant Mini kit Method, QIAGEN Germany

DNA was extracted according to the manufacturer's protocol.

CTAB and DNA DNeasy Plant Mini kit Method, QIAGEN Germany

Twenty mL of CTAB-extraction buffer (10 mM Tris, 20 mM EDTA, 0.02 M CTAB, 0.8 M NaCl, 0.03 M N-lauryl sarcosine, 0.13 M sorbitol, 1 % (w/v) PVPP, pH set to 8.0 with NaOH), 40 µL mercaptoethanol and 50 µL proteinase K (from a stock solution 10 mg/mL), were added to 250 mg ground grains in 50–mL centrifugation tube and mixed vigorously. The mixture was incubated at 65 °C for 60 min and mixed after every 10 min. Eight hundred µL of the upper phase was transferred to a 2 mL new tube containing 5 µL of RNAase (50 mg/mL) and incubated for 10 min at 65 °C, Eight hundreds µL of chloroform-isoamyl alcohol (24:1) was added into each tube. The samples were mixed by inverting the tubes and centrifuged for 10 min at 5,000 g at room temperature. The aqueous phase was transferred into a 1.5 mL tube containing 500 µL isopropanol, mixed and transfer into the DNeasy Mini Spin Column sitting of Plant mini kit (Qiagen, Germany). Subsequently, the samples were further processed according to the manufacturer's protocol and DNA was dissolved in 200 µL AE buffer.

2.1.13.2 Primer specificity

Sequence specific primers were used to amplify specific fragment using classical PCR (Thermocycler BIO RAD iCycler BIO RAD, München) (Table 2). All of the primers were synthesized by Carl Roth Company (Karlsruhe, Germany). DNA was extracted from 50-100 mg fresh mycelia growing on the surface of agar media using the Plant DNeasy Mini Kit (Qiagen, Hilden, Germany). The DNA was used to determine the specificity of primers (Species-specific amplification). The PCR products were loaded on 0.7 % - 1 % (w/v) agarose gel and run in 1 X TAE buffer by staining with ethidium bromide. The bands were then visualized under UV light and specific gel slices containing the bands were cut for further analysis. The fungal strains and matrix DNA utilized for the specificity and sensitivity of primers are listed in Table 1.

2.1.13.3 DNA purification and sequencing

The PCR products were loaded on the 0.8 % agarose gel and run in 1x TAE buffer containing ethidium bromide. The target band was visualized under UV light. The band were cut and placed in 1.5 mL tube and frozen at -20 ^{o}C for 30 min. The frozen gel was ground using 1 mL pipette tip. 500 µL of 1 x TE buffer was added and homogenized using syringe. An equal volume (500 µL) of phenol-chloroform (1:1) was added. The mixture was homogenised by needle and syringe and then centrifuged for 20 min at 10,000 g at 20 ^{o}C. The supernatant was transferred into a new tube including 500 µL chloroform. The mixtures was mixed and centrifuged for 15 min. The upper phase was carefully transferred into a new 2-mL tube and 1:10 volume (v/v) of 3 M sodium acetate as well as two volumes (v/v) of 100 % ethanol were added and incubated at -20 °C overnight. The samples were centrifuged for 30 min at 14,000 g at 4 ^{o}C. The DNA pellet was washed by 1mL ethanol (70 %) and centrifuged for 10 min. The DNA was dried for 5 - 10 min at room temperature and then dissolved in 7 µL Millipore water.

For further purification, 1 µL of ExoSAP-IT were added to 5 µL of PCR products and incubated at 37 ^{o}C for 45 min and then followed by ExoSAP-IT inactivation at 80 ^{o}C for 15 min. The clean DNA template was subsequently used for the sequencing PCR which contained 8 µL of milli-pore water, 2 µL of either forward and reverse primer and 4 µL of DTCS Quick Start Master Mix (Beckman Coulter). The PCR reaction was performed with 30 cycles (96 ^{o}C for 20 sec, 50 ^{o}C for 20 sec, 60 ^{o}C for 4 min). The stop solution was prepared in a volume of 2.0 µL of 3M NaOAc (pH = 5.2), 2.0 µL of 100 mM EDTA (pH = 2.0) and 1.0

µL of glycogen (20 mg/mL). The PCR product was transferred to a 1.5 mL sterile tube and mixed with 5 µL stop solution. A volume of 60 µL cold ethanol (98 %) was added and mixed by vortex and then centrifuged for 15 min at 4 ^{o}C. The supernatant was removed and the pellet washed 2 times with 200 µL cold ethanol (70 %) and centrifuged for 5 min at 4 ^{o}C. The pellet was dried by the speed vacuum machine at 35 ^{o}C and re-suspended in 40 µL SLS (Sample loading solution). The sample was loaded into plates and sequenced using the CEQ8000 Genetic Analysis System. The sequence of the fragments was utilized to confirm the specificity of primers using available information in data bank (http://www.ncbi.nlm.nih.gov/BLAST/).

2.1.13.4 Real-time PCR reactions

Real-time PCR reactions were performed in an ABI Prism®7000 SDS instrument. Prior to quantification, primer optimisation was carried out for forward and reverse primers. Different combinations of forward and reverse (0.1, 0.2 and 0.4) were used in presence of template (DNA) and non-template as control to avoid primer dimmer formation (Table 7). At the end of the run, the dissociation curves were generated to check the absence of the non-specific amplification and subsequent confirmation by analysis of the PCR products on agarose gel electrophoresis. The combination with the lowest threshold cycle and without primer dimmer formation was used to perform subsequent PCRs.

Table 7: Concentration (pg/µL) of forward and reverse primers used in real-time PCR for primer optimization

Forward primer	Reverse primer		
	10	20	40
10	10/10	10/20	10/40
20	20/10	20/20	20/40
40	40/10	40/20	40/40

Standard curves were generated using a serial dilution (0.9, 9, 90, 900 and 9000 pg) of purified genomic DNA of *Fusarium* spp. Polymerase chain reactions (PCRS) were carried out in 20 µL reaction volume containing 10 µL Sybr® Green Jump startTM Taq Ready MixTM, 0.2 µl Rox as internal reference dye, 0.4 µM of each forward and reverse primers, 2 µL

genomic DNA. PCR reactions were performed in duplicates for standard curves and samples to control the reproducibility of quantitative results. A universal thermal cycling parameter (10 s at 50 ^{o}C, 10 min at 95 ^{o}C, 40 cycles of 15 s at 95 ^{o}C and 60 s at 60 ^{o}C) was used for the quantification. The specificity of amplification was confirmed by generating melting curve at the end of PCR reactions. The curves were used as control for the specificity of real-time PCR during the quantification. Final quantification of fungal DNA analysis was performed using the standard curve method (User bulletin of ABI PRISM 7700 SDS, Http://docs.appliedbiosystems.com). The results were reported as the absolute amount of individual specific-species DNA for the *Fusarium* species using the standard curves for the each species.

2.1.13.5 Validation experiments

Reproducibility of the experiments were checked on 250 mg aliquots of a positive sample for *F. graminearum*, *F. culmorum*, *F. avenaceum* and *F. poae* using real-time PCR assays as described by Waalwijk *et al*. (2004).

2.1.14 Analysis of mycotoxins

In field experiments, the mycotoxin content of kernel samples were measured as described by Sulyok *et al*. (2006 and 2007). Methanol and acetonitrile (both LC gradient grade) were purchased from J.T. Baker (Deventer, The Netherlands) and ammonium acetate (MS grade) and glacial acetic acid (p.a.) were obtained from Sigma-Aldrich (Vienna, Austria). Water was purified successively by reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France). Mycotoxin standards were purchased from different sources and were dissolved in acetonitrile (ACN) if not stated otherwise. Stock solutions of nivalenol (NIV), deoxynivalenol (DON), fusarenon X (FUSX), 3-acetyldeoxynivalenol (3ADON), Diacetoxyscirpenol (DAS), HT-2 toxin (HT-2), T-2 toxin (T-2), zearalenone (ZON), alpha-zearalenol (a-ZOL), fumonisins B1 and B2 (FB1, FB2, in ACN/H2O 1+1) were obtained from Biopure Referenzsubstanzen GmbH (Tulln, Austria). Moniliformin (MON, dissolved in MeOH) were received from Sigma-Aldrich. A stock solution of enniatin A, A1, B and B1 (ENN A, ENN A1, ENN B, ENN B1) was provided by Dr. Marika Jestoi (EELA Helsinki, Finland). Deoxynivalenol-3-glucoside (D3G) was isolated from wheat treated with DON.

Four combined working standard solutions were prepared weekly by dilution of the stock solutions of the analytes in the related solvents, i.e. MeOH (for MON), MeOH/H2O

1 + 1 (for Z4S, ERA and ERC), ACN/H2O 1 + 1 (for FB1 and FB2) and ACN (for all other analytes), respectively. All solutions were stored at -20 ºC and were brought to room temperature before use.

Instrumental conditions

Detection and quantification was performed with a QTrap 4000 LC/MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with a TurboIonSpray ESI source and a 1100 Series HPLC system (Agilent, Waldbronn, Germany). Chromatographic separation was performed at 25 ºC on a Gemini1 C18 column, 150 × 4.6 mm i.d., 5 mm particle size, equipped with a C18 4 × 3 mm i.d. security guard cartridge (all from Phenomenex, Torrance, CA, USA). Both eluents contained 5mM ammonium acetate and were composed of methanol/water/acetic acid 10+89+1 (v/v/v; eluent A) or 97+2+1 (eluent B), respectively. After an initial time of 2 min at 100 % A, the proportion of B was increased linearly to 100 % within 12 min, followed by a hold time of 3 min at 100 % B and 4 min column re-equilibration at 100 % A. The flow rate was 1 mL/min. The column effluent was transferred via a six-port valve (VICI Valco Instruments, Houston, TX, USA) either to the mass spectrometer (between 2 and 17 min; no flow splitting was used) or to the waste. ESI-MS/MS was performed in multiple reactions monitoring (MRM) mode both in positive and negative polarity in two separate chromatographic runs per sample.

Calibration solutions

For external calibration, a multi-analyte stock solution was freshly prepared by mixing the four combined working solutions (200 mL each) and 800 mL of mobile phase A. This solution was further diluted with mobile phase A to obtain appropriate concentrations (for the method validations experiments. For the validation experiments, the concentrations were matched on each level to the expected analyte concentration in the final diluted extract.

Spiking

Ground wheat kernels (0.250 g) were spiked by consecutively adding the appropriate amounts of the four combined working solutions. The samples were subsequently stored for overnight at 40 ºC to allow evaporation of the solvent and to establish equilibration between the analytes and the matrix.

Extraction

A volume of 1 mL of extraction solvent (CH3CN/H2O/HAc 79+20+1) was added to 0.25 g of ground wheat kernels. The samples were extracted for 90 min using a GFL 3017 rotary shaker (GFL, Burgwedel, Germany) and subsequently centrifuged for 2 min at 3000 rpm (radius: 15 cm) on a GS-6 centrifuge (Beckman Coulter Inc., Fullerton, CA, USA). The extracts were transferred into glass vials using Pasteur pipettes and aliquots of 350 µL were diluted with the same amount of a mixture containing CH3CN/H2O/HAc (20+79+1). After appropriate mixing, 5 µL of the diluted extract were injected into the LC/MS/MS system without further pre-treatment. To perform the optimization of the extraction solvent, samples were spiked at one concentration level in duplicate or triplicate and extraction was performed by adding 2mL of the investigated solvent mixture. Crude extracts were diluted 1+9 with eluent A prior to analysis in this experiment to reduce signal suppression/enhancement due to matrix effects.

2.1.15 Statistical analysis

The average values of diseases severity ratings, 1000-kernel weight, frequency of re-isolation and the content of fungal DNA were separately determined for each replication, sub-plot or plots. The effects of trials, fungal species, treatment and their interactions on frequency of infected kernels, diseases severity ratings, the content of fungal DNA and 1000- kernel weight reduction were analysed using Proc GLM procedures (SAS Release Version 9.0, SAS Institute, Inc., Cary, NC). Fungal species and treatments considered as a fixed effects in these analyses. Mean comparisons were made using Duncan's new multiple range test at 5 % probability. When it was necessary data were log-transferred prior to analysis. Simple linear regressions were calculated for pairwise comparisons among content fungal DNA, frequency of infected kernels, diseases severity ratings and 1000-kernel weight. Pairwise comparisons of mean values were made using t-test (Elliott, 2006). The Chi-square goodness of fit test was used to study Shift in the ratio among *Fusarium* species during *Fusarium* head blight development.

2.2 Results

2.2.1 Specificity of primers

PCR was performed with the species-specific primer pairs resulted in single fragments for individual *Fusarium* species. The Fp82F/R, MGB (minor group binding) GRA, MGBCUL and MGBAVE primers amplified fragments with the size of 220, 100, 60 and 58 bp that were unique for all isolates of *F. poae, F. graminearum, F. culmorum* and *F. avenaceum*, respectively. No cross reactivity was observed with other fungal and matrix DNA as well as in the negative control (lacking DNA template) (Table 8, Figure 1-7), indicating the specificity of the primer sets.

In some cases, MGBGRA, MGBCUL primers amplified a PCR product with matrix plant DNA (Figures 4 and 6). Further assays showed that when the annealing temperature increased to 60 ºC, the primers showed specific amplification (Figures 3 and 5). In most cases the results of morphological identification of tested *Fusarium* species was confirmed using PCR assays with species-specific primers for individual species. However, there was no amplification in three isolates (7.2, 7.10 and 7.13) of *F. poae*, with Fp82F/R primer (Figure 1).

Table 8: Specificity of primers for the detection of *F. graminearum*, *F. culmorum*, *F. avenaceum* and *F. poae* in the presence of matrix DNA and other fungal species

Fungal and matrix DNA Primer	*F. graminearum*[1]	*F. culmorum*[2]	*F. avenaceum*[3]	*F. poae*[4]	*Fusarium* spp.[5]	Matrix DNA[6]	Fungal species[7]
MGBGRA	+	-	-	-	-	-	-
MGBCUL	-	+	-	-	-	-	-
MGBAV	-	-	+	-	-	-	-
Fp82	-	-	-	+	-	-	-

[1]*F. graminearum* isolates, 5.1, 10, 15, 17 and 5.19; [2] *F. culmorum* isolates, 3.6, 9, 12, 14, 16, 17, 18, 22, 33, 35, 36 and 3.37; [3] *F. avenaceum* isolates, 1.7, 8, 12 and 1.16; [4] *F. poae* isolates, 7.3, 5, 6, 8, 9, 11, 12, 14, 15, 16 and DSM 62376; [5] *F. langsethae* isolate, 18.1, *F. tricinctum* isolate, 10.10, *F. sporotrichioides* isolates, 9.9 and 9.10, and *F. cerealis*; [6] Matrix DNA, DNA of wheat stem, leaf and kernel; [7] fungal species, *Blumeria graminis*, *Puccinia* sp., *Trichoderma* sp., *Alternaria* sp., *Aspergillus* sp. and *Penicillium* sp. The isolates were sourced from Institute of Crop Science and Resource Conservation, University of Bonn, Germany.

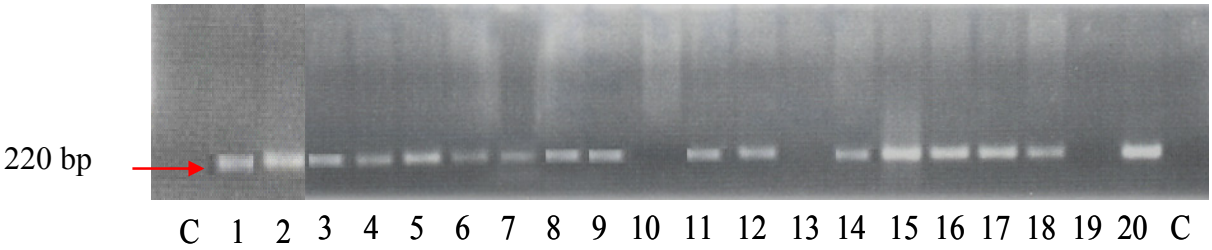

Figure 1: Agarose gel electrophoresis of DNA amplification from different isolates of *F. poae* using primer pair Fp82F/R. Lanes 1-6, *F. poae* 5.1, 2, 3, 4, 5 and 5.6; Lanes 7-20, *F. poae* 7.16, 15, 14, 13, 12, 11, 10, 9, 8, 6, 5, 3, 2 and 62376, respectively.

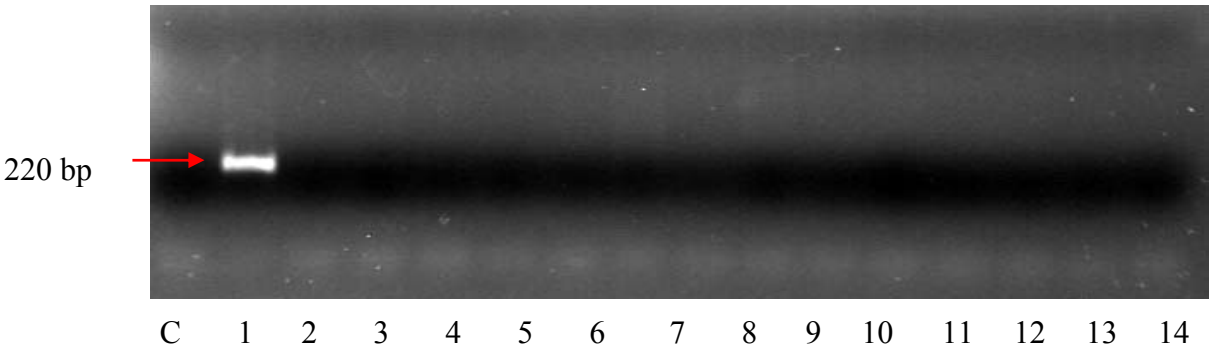

Figure 2: Agarose gel electrophoresis of DNA amplification of fungal species commonly found with head blight and matrix DNA using primer pair Fp82F/R. C, non-template control (water); Lanes 1-4, *F. poae,* wheat stem, kernel and leaf; Lanes 5-9, *Blumeria graminis*, *Puccinia* sp.*, Trichoderma* sp., two isolates of *Alternaria* sp.; Lanes 10-13, *F. tricinctum*, two isolates of *F. sporotrichioides, F. cerealis*; Lanes 14-15, *Aspergillus* sp. and *Penicillium* sp., respectively. Wheat stem and leaf are from healthy plant (greenhouse) and wheat kernel from field infected by *F. graminearum* and *F. culmorum*.

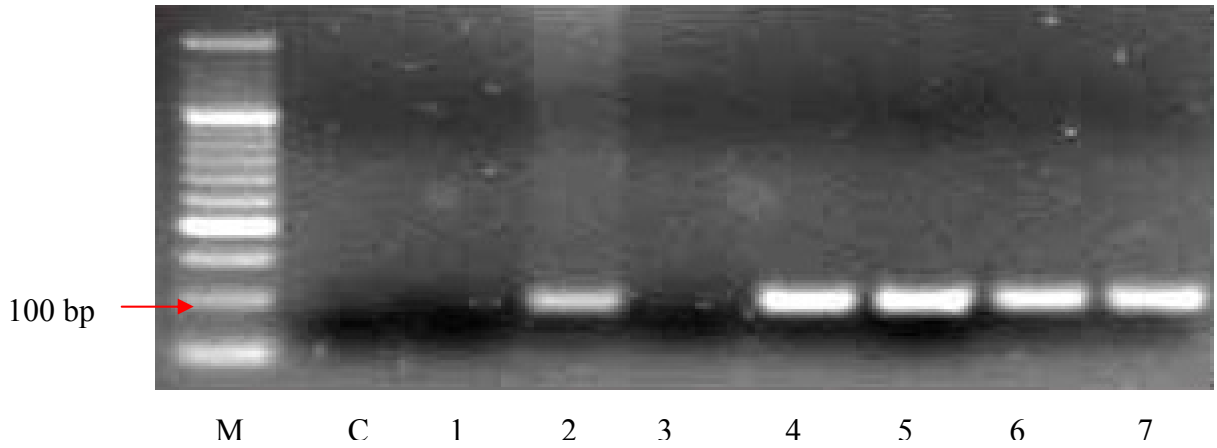

Figure 3: Agarose gel electrophoresis of DNA amplification from different isolates of *F. graminearum* using primer pair MGBGRA F/R. M, DNA ladder; C, non-template control (water); Lanes, 1-3 wheat stem, kernel and leaf; Lanes 4-7, *F. graminearum* 5.10, 15, 17 and 5.19 isolates, respectively.

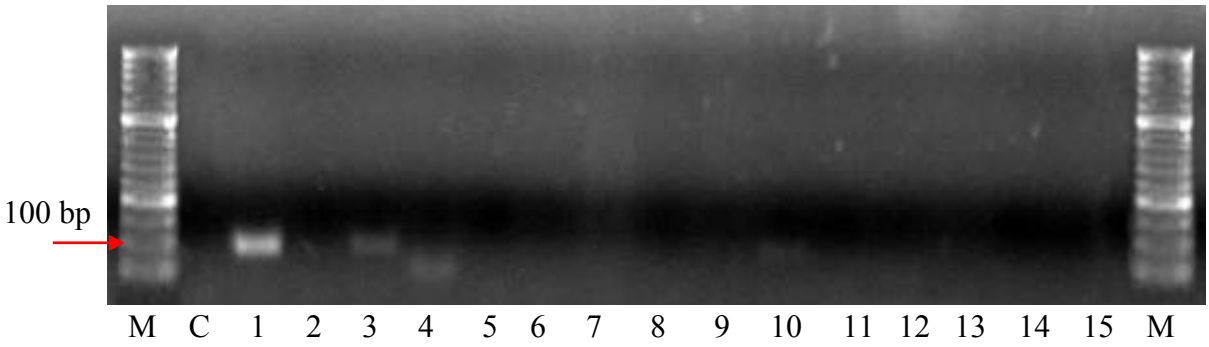

Figure 4: Agarose gel electrophoresis of DNA amplification of fungal species commonly found with Fusarium head blight and matrix DNA using primer pair MGBGRA F/R. M, DNA ladder; C, non-template control (water); Lanes 1-4, *F. graminearum,* wheat stem, kernel and leaf; Lanes 5-9, *Blumeria graminis*, *Puccinia* sp.*, Trichoderma* sp. and two isolates of *Alternaria* sp.; Lanes 10-13, *F. tricinctum* and two isolates of *F. sporotrichioides, F. cerealis*; Lanes 14-15, *Aspergillus* sp. and *Penicillium* sp., respectively.

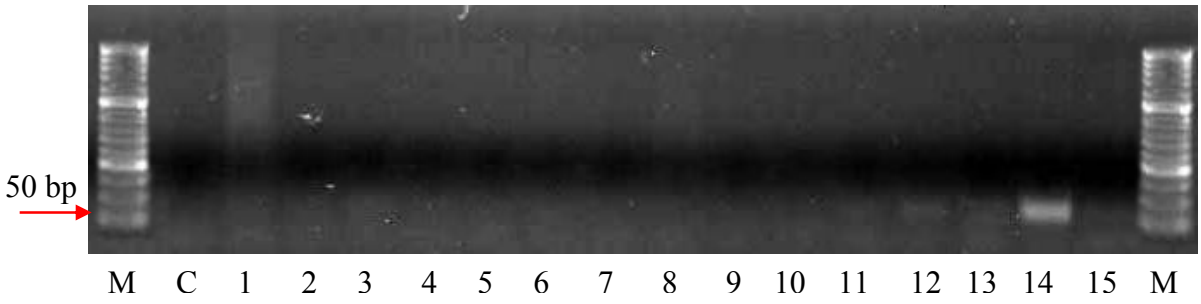

Figure 5: Agarose gel electrophoresis of DNA amplification of fungal species commonly found with *Fusarium* head blight and matrix DNA using primer pair MGBFCUL F/R. M, DNA ladder; C, non-template DNA (water); Lanes 1-2, *Aspergillus* sp. and *Penicillium* sp.; Lanes 3-7, *Blumeria graminis*, *Puccinia* sp.*, Trichoderma* sp. and two isolates of *Alternaria* sp.; Lanes 8-11, *F. tricinctum*, two isolates of *F. sporotrichioides* and *F. cerealis*; Lanes 12-15, wheat leaf, kernel, and *F. culmorum* tester isolate and wheat stem, respectively.

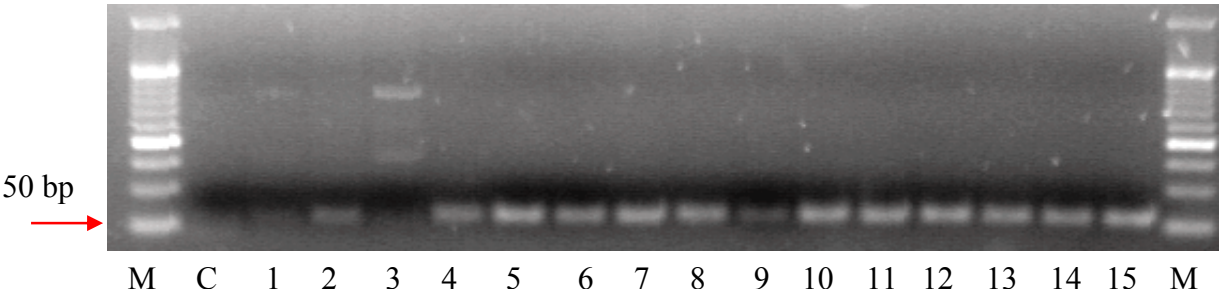

Figure 6: Agarose gel electrophoresis of DNA amplification from different isolates of *F. culmorum* using primer pair MGBCULF/R. M, DNA ladder; C, non-template control (water); Lanes 1-3, wheat stem, kernel and leaf; Lanes 4-15 *F. culmorum* 3.6, 9, 12, 14, 16, 17, 18, 22, 33, 35, 36 and 3.37 isolates, respectively.

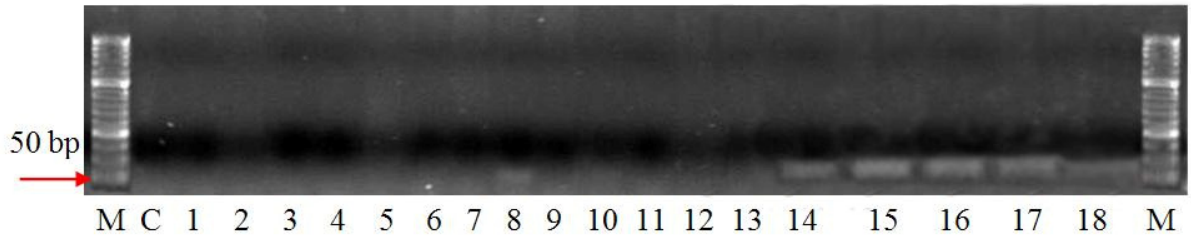

Figure 7: Agarose gel electrophoresis of DNA amplification of fungal species commonly found with *Fusarium* head blight, matrix DNA and different isolates of *F. avenaceum* using primer pair MGBAVEF/R. M, DNA ladder; C, non-template control (water); Lanes 1-3, wheat stem, kernel and leaf; Lanes 5-7, *Blumeria graminis*, *Puccinia* sp., *Trichoderma* sp. and *Alternaria* sp.; Lanes 8-11, *F. tricinctum*, two isolates of *F. sporotrichioides* and *F. cerealis*; Lanes 12-13 *Aspergillus* sp. and *Penicillium* sp.; Lanes, 13-18 *F. avenaceum* 1-7, 8, 9, 12, and 1.16, respectively.

2.2.2 Development of real-time PCR for the quantification interactions among *Fusarium* species on wheat ears

Melting curve analysis

The results of real-time PCR using SYBR Green I for *Fusarium graminearum*, *F. culmorum*, *F. avenaceum* and *F.* poae confirmed the results of the primer specificity obtained with conventional PCR. Using the species-specific primers for *F. graminearum*, *F. culmorum*, *F. avenaceum* and *F. poae* no PCR amplification or melting curve was observed neither for the negative control nor for other *Fusarium* species or other fungal species commonly found on wheat (Figure 8), which indicates the specificity of primers and accuracy of the real-time PCR. Melting curve analysis revealed the presence of a single peak for individual *Fusarium* species. Melting temperatures (MT) for amplicons were 81.2 °C, 77.5 °C, 79.0 °C and 81.4 °C for *F. graminearum, F. culmorum, F. avenaceum* and *F. poae*, respectively. The concentration of fungal DNA did not affect the melting temperature of the amplicons. A direct correlation existed between the amounts of PCR product and the height of the melting curve.

Standard curve

For the quantification of *Fusarium* species via real-time PCR, threshold cycles correlated with a known amount of *Fusarium* DNA. For all *Fusarium* species, standard curves were prepared based on ten threshold cycles from ten-fold serially diluted DNA in two replications of real-time PCR. Standard curves are presented in Figure 9 for *F. graminearum, F. culmorum*, *F. avenaceum* and *F. poae*.

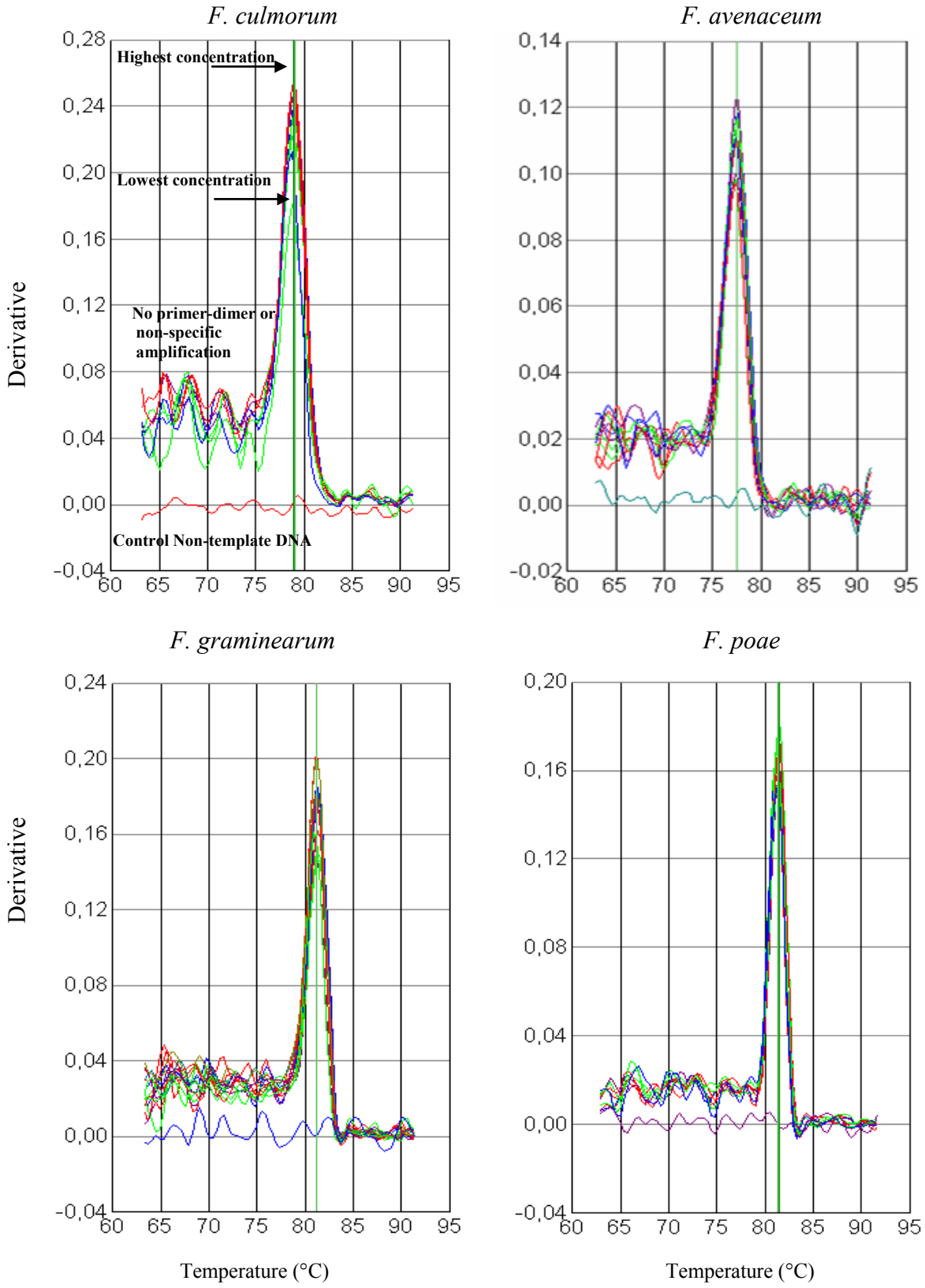

Figure 8: Melting curves (fluorescence derivative dF/dT versus temperature °C) of specific amplicons for *F. avenaceum*, *F. culmorum, F. graminearum* and *F. poae* in a matrix of fungal and plant DNA.
* Peaks or curves indicated species-specific amplification in real-time PCR with a mixture of plant and fungal DNA in different samples

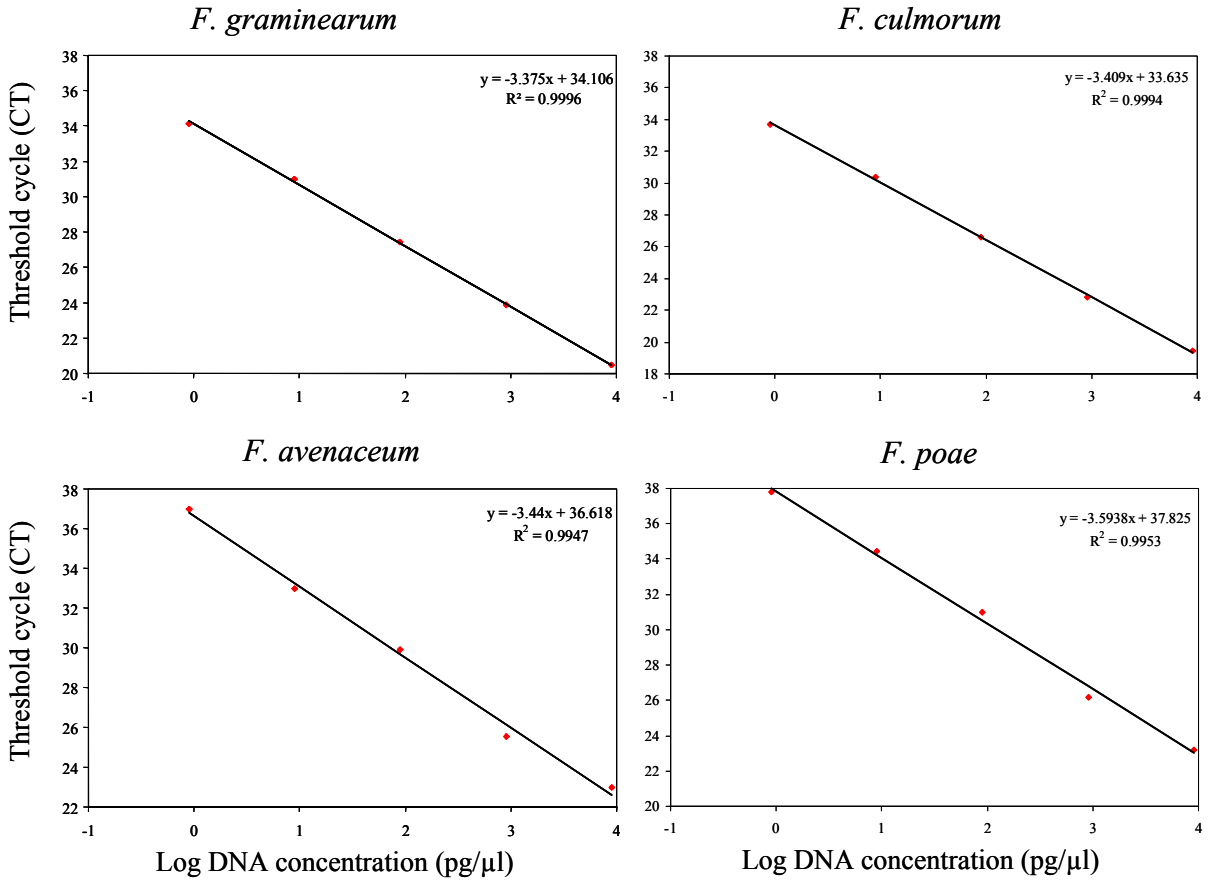

Figure 9: Standard curve for the quantification of *Fusarium* spp. using Sybr green real-time PCR

Overall, there were highly negative correlations ($R^2 \geq 0.99$) between threshold cycle values and the amounts of fungal DNA for all species. The negative correlations ranged from $R^2 = 0.99$ to 0.999 for *F. culmorum* and *F. graminearum*, $R^2 = 0.989$ to 0.997 for *F. avenaceum* and $R^2 = 0.985$ to 0.995 for *F. poae*. The standard deviation of the negative correlations between the threshold cycle values and amount of fungal DNA in different real-time PCR runs was less than 0.007 for the *Fusarium* species tested. These results indicated that real-time PCR technique was able to quantify the amount of *Fusarium* DNA in unknown samples with high reproducibility. The slope ranged from –3.32 to –3.75 and the amplification efficiency varied from 1.88 to 2.0.

Validation assays

Results of reproducibility of DNA extraction procedure, intra and inter assays has been summarized in Table 9. The standard deviation of threshold cycles value of nine different DNA preparations, intra- and inter-assays of *F. graminearum* ranged from 0.10 to

0.44, *F. culmorum* 0.10 to 0.15, *F. avenaceum* 0.2 to 0.33 and *F. poae* 0.11 to 0.66. The standard deviation of threshold cycle values among DNA preparations was higher than that for the other assays and reached 0.66 for *F. poae*, while intra-assays had the lowest standard deviations. *Fusarium culmorum* had the lowest standard deviation of threshold cycle values in all assays.

Table 9: The threshold cycle values and standard deviation among DNA preparations using CTAB method (n = 9), intra assays and inter assays using real-time PCR assays

Fusarium species	Reproducibility		
	DNA preparations	Intra-assays	Inter-assays
F. graminearum	20.84 ± 0.24	20.95 ± 0.10	20.30 ± 0.44
F. culmorum	20.37 ± 0.15	20.39 ± 0.10	20.30 ± 0.14
F. avenaceum	23.98 ± 0.31	24.00 ± 0.20	24.49 ± 0.33
F. poae	24.80 ± 0.66	24.80 ± 0.11	24.70 ± 0.18

2.2.3 DNA extraction methods

DNA was extracted from 250 mg ground wheat kernels inoculated with *F. graminearum*, *F. culmorum*, *F. avenaceum* and *F. poae* using three different DNA extraction methods. The mean value of optical density in 260/280 and 260/230 ratios was higher than 1.5 for the CTAB and CTAB + DNeasy Plant Mini kit, while it was less than 1.0 for Plant Mini kit method (Table 10). For the DNeasy Plant Mini kit the absorbance of purified DNA had two decimal zeros compared to the other methods, which had one decimal zero. The CTAB method gave the highest amount of DNA, followed by CTAB + DNeasy Plant Mini kit and Plant Mini kit with the average of 90 % and 16 % of the CTAB DNA, respectively.

Table 10: Comparison of absorbance of the purified DNA using three different DNA extraction methods in 230, 260 and 280 nm and 260/280 and 230/260 ratios

	Method	Starting material (mg)	230	260	280	260/230	260/280	Amount (ng/µl)
1	CTAB	250	0.013	0.030	0.017	2.21	1.77	59
2	CTAB	250	0.019	0.035	0.019	1.88	1.81	70
3	CTAB	250	0.013	0.027	0.015	2.1	1.75	53
4	CTAB+KIT	250	0.016	0.030	0.017	1.87	1.72	60
5	CTAB+KIT	250	0.003	0.022	0.013	4.05	1.66	44
6	CTAB+KIT	250	0.010	0.031	0.017	3.11	1.79	61
7	KIT	250	0.005	0.009	0.007	1.8	1.21	17
8	KIT	250	0.007	0.004	0.003	0.59	1.40	9
9	KIT	250	0.013	0.003	0.003	0.2	0.87	5

The CTAB method had the lowest threshold cycles (19.2-23.35) from 250 mg of kernel material for detecting different *Fusarium* species as well as little variation among the replicates (0.09 - 0.69) and the extraction process (Table 11). The threshold cycles (19.7-23.8) and standard deviations (0.21 - 0.71) in CTAB + DNeasy Plant Mini kit method were a little higher than CTAB. The DNeasy Plant Mini kit method had the highest threshold cycles (21.3 - 25.7) and standard deviation (0.56-0.85). The threshold cycles were different among *Fusarium* species and increased in the order *F. culmorum, F. graminearum*, *F. avenaceum* and *F. poae* (Table 11).

2.2.4 Inoculum production

All species produced a large amount of mycelium in PDB. Mucilaginous colonies with large quantities of conidia characterized by great reduction in mycelia formation were formed within 2 days after transferring the mycelia onto LSPDA (**L**ow **S**trength **P**otato **D**extrose **A**gar), SNA (**S**ynthetic **N**utrient **A**gar**)** or WA (Figure 10). With the method described here, a high amount of conidia was produced in a short time for *Fusarium* spp. Most isolates of *F. graminearum* did not led to produce enough spores with the most common methods, while all isolates of tested *Fusarium* species gave a high amount of conidia in a very short time. For example, two days in PDB and two days incubation on LSPDA, WA and SNA, the number of conidia produced by *F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. poae* and *F. tricinctum* was 14.0, 29.0, 15.0, 2.3, and 74.0 times higher than with the conventional method,

respectively (Table 12). This was in comparison to the conventional methods which required 1 to 3 weeks and still resulted in lower conidiation especially for *F. graminearum*.

Table 11: The mean threshold cycle values and standard deviations among three different DNA extraction methods using real-time PCR assays

Fusarium species	Threshold cycle		
	CTAB	CTAB + Plant Mini kit	Plant Mini kit
F. graminearum	19.70 ± 0.20	20.20 ± 0.60	22.30 ± 0.60
F. culmorum	19.27 ± 0.09	19.70 ± 0.44	21.30 ± 0.56
F. avenaceum	22.77 ± 0.63	23.14 ± 0.21	24.50 ± 0.85
F. poae	23.35 ± 0.69	23.85 ± 0.71	25.70 ± 0.66

The conidia-producing ability using the new method decreased in the order *F. tricinctum*, *F. culmorum, F. avenaceum*, *F. poae* and *F. graminearum*. *F. avenaceum, F. poae* and *F. tricinctum* produced all types of conidia with the new method, while with the conventional method they mostly produced microconidia and only very few number of macroconidia.

In tests on the virulence of conidia produced with the different methods, the frequency of kernel infection, FHB severity and *Fusarium* biomass of kernels as measured *Fusarium* DNA content were not significantly different for both methods (Table 13). For highly virulent species, symptoms were observed within 1 to 2 days after inoculation. For both methods, *F. graminearum* resulted in the highest rates of infection and colonization as well as FHB severity, followed by *F. culmorum*, *F. avenaceum* and *F. poae,* respectively.

Table 12: Comparison of number of conidia produced by conventional and the new spore production methods on different media (data × 10^6 are the number of conidia per plate)

Fusarium species	Conventional on	New method on		
	LSPDA[1]	LSPDA[1]	SNA[2]	WA[3]
F. graminearum	1.3	19.5	20.5	14.5
F. culmorum	2.8	145.0	60.0	35.5
F. avenaceum	4.3	28.0	120.0	42.5
F. poae	23.8	50.0	65.0	50.0
F. tricinctum	2.3	200.0	225.0	85.0

[1] Low-strength PDA; [2] Synthetic nutrient-poor agar; [3] Water agar

Table 13: Comparison conventional and new spore production methods in frequency and intensity of kernel colonization, and ratings of disease severity of wheat ears (cv. Munk) inoculated with *Fusarium* spp. at GS 65

Inoculation	Disease severity [ratings]		Freq. infected kernel [%]		Amount of fungal biomass [ng/mg kernel dry weight]	
	CONVE.	NEW	CONVE.	NEW	CONVE.	NEW
Non-inoculated	1.0	1.0				
F. graminearum	6.5	7.1	65.2	63.5	15.9	17.4
F. culmorum	4.5	5.2	49.6	47.5	9.4	10.5
F. avenaceum	3.4	3.5	35.2	28.5	1.0	0.6
F. poae	1.0	1.0	2.0	7.0*	0.05	0.06

* Significantly different from conventional method (t-test, $p \leq 0.05$)

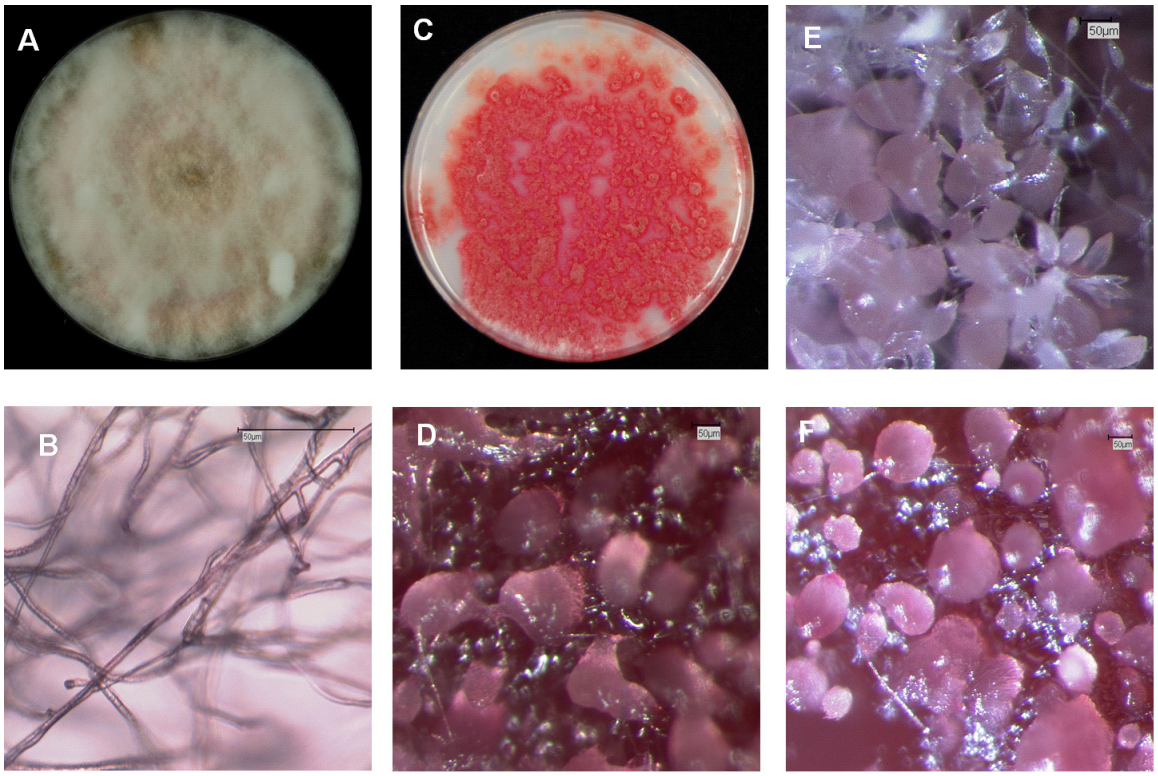

Figure 10: Comparison of mycelia and conidia produced with a conventional and the new spore production method. A and B, conventional method, *F. graminearum* 10 days old on LSPDA; C and D, new method, *F. graminearum* 2 days on LSPDA; E and F, new spore production *F. culmorum* and *F. avenaceum*, respectively.

2.2.5 Greenhouse experiments

Wheat ears inoculated at mid-flowering with one single isolate of *Fusarium* species or a mixture of two to four isolates, respectively, were investigated under controlled conditions for visible disease development during ripening, the effect on 1000-kernel weight, the frequency of infected kernels as well as the *Fusarium* species-specific amount of DNA. In both years of investigation, inoculum concentration increased with the number of *Fusarium* isolates in the mixture (5 x 10^4 for one isolate, 2 x 10^5 for the mixture of four isolates; = variable conc.). In 2006, additional treatments with a constant concentration (5 x 10^4 conidia ml^{-1}; = constant conc.) were used.

2.2.5.1 Effects of single and mixed inoculations with *Fusarium* spp. on disease severity ratings

The *Fusarium* species isolates as well as the composition of inoculum significantly affected visible FHB severity. With single isolate inoculation, *F. graminearum* (Fg) resulted in the highest disease level, followed by *F. culmorum* (Fc), *F. avenaceum* (Fa) and *F. poae* (Fp), respectively (Table 14).

Table 14: Visual ratings of *Fusarium* head blight severity (1 to 9) after inoculation of wheat ears (cv. Munk) with different combinations of *Fusarium* species at GS 65 (data are the average of three ratings 14, 21 and 28 days after inoculation)

Inoculum [1]	2005 Variable conc. [2]	2006 Variable conc. [2]	2006 Constant conc. [3]	
F. graminearum (FG)	6.5 C [4]	7.1 D	7.1 A-C	
F. culmorum (FC)	4.5 DE	5.2 F	5.2 F	
F. avenaceum (FA)	3.4 F	3.5 G	3.5 G	
F. poae (FP)	1.0 H	1.0 H	1.0 H	
FG + FC	7.6 B	7.8 BC	7.4 AB	*
FG + FA	7.7 B	6.5 E	7.7 A	*
FG + FP	7.0 BC	7.2 CD	6.9 BC	*
FC + FA	4.9 D	6.6 E	5.0 F	*
FC + FP	3.4 F	6.9 DE	6.0 DE	*
FA + FP	2.3 G	3.2 G	3.7 G	
FG + FC + FA	7.6 B	7.8 B	7.5 AB	
FG + FC + FP	7.4 B	7.5 BC	7.7 A	
FG + FA + FP	6.3 C	7.8 B	6.9 BC	*
FC + FA + FP	3.8 EF	6.5 E	5.9 E	*
FG + FC + FA + FP	8.7 A	8.5 A	6.5 CD	*

[1] FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

[2] Inoculation with variable inoculum concentrations of (50,000 conidia ml $^{-1}$ per species)

[3] Inoculation with constant amount of inoculum (50,000 conidia ml $^{-1}$)

[4] Means with the same letters within a column are not significantly different (Duncan test, $p \leq 0.05$)

* Pairwise comparison variable and constant inoculum (2006), t-test ($p \leq 0.05$)

Fusarium head blight (FHB) severity slightly increased with increasing inoculum concentration in the mixture (variable conc.). However, no significant differences were

observed for most of the treatments including *F. graminearum* in mixture with one or two other isolates. The mixture of all isolates resulted in the highest disease ratings, with FHB indices 1.5 to 2 and 0.7 to 1.0 higher than those for ears inoculated with *F. graminearum* alone and the other mixtures, respectively. In 2005, FHB severity of ears inoculated with *F. culmorum* was similar to or a little bit higher than FHB indices for the combinations with *F. avenaceum*, *F. poae* and the three-party mixture. In 2006, these combinations gave significantly higher disease indices. Combinations of the less virulent *F. avenaceum* and *F. poae* with other more virulent isolates resulted in disease indices very similar to the more virulent ones alone.

With a constant inoculum concentration, *F. graminearum* isolate alone or in mixtures with one, two or three other isolates, gave high disease severities, which did not differ significantly from each other (Table 14). Mixtures of *Fusarium* species isolates differing in virulence gave disease indices similar to or only slightly higher (Fc with Fp, Fc with Fa and Fp, Fa with Fp) than those of the more virulent ones alone. As expected from the lower total number of conidia inoculated, the inoculation with constant number of conidia resulted in a significantly lower disease index than that with variable inoculum concentrations for 7 out of 11 combinations; there was only one case with an opposite effect (Fg with Fa). Also with the constant inoculum approach, combinations of isolates had no additive or synergistic effects on disease severity.

2.2.5.2 Effects of single and mixed inoculations with *Fusarium* spp. on reduction of 1000-kernel weight

For variable concentrations of inoculum, inoculations with *F. graminearum* isolate alone or in mixtures with other isolates reduced the kernel weight by 40 to 50 %; the effect of *F. culmorum* was significantly lower (Table 15). In mixtures, the reduction of kernel weight by *F. graminearum* was hardly affected by the other isolates, except for the mixture of all isolates and the combinations with *F. culmorum* in 2006, which increased the damage. Kernel weights of plants inoculated with *F. avenaceum* and/or *F. poae* were not significantly different from those of non-inoculated control plants. Mixtures with *F. culmorum* gave slightly higher damage, but only in 2006, the combination Fa with Fc resulted in an additive effect on 1000-kernel weight reduction. Pairwise comparisons of the 2006 data demonstrated that only five treatments with constant inoculum concentration resulted in a lower reduction of kernel weight than the variable concentration approach.

Table 15: Effect of different combinations of *Fusarium* species on the 1000-kernel weight of wheat (cv. Munk) after inoculation of ears at GS 65

Inoculum [1]	2005 Variable conc.[2]	2006 Variable conc.[2]	2006 Constant conc.[3]
Non-inoculated	25.8 A [4]	27.7 A	27.7 A
F. graminearum (FG)	12.9 E-G	16.1 DE	16.1 E-G
F. culmorum (FC)	18.4 B-D	24.5 AB	24.5 AB
F. avenaceum (FA)	21.6 A-C	26.1 A	26.1 A
F. poae (FP)	22.5 AB	27.7 A	27.7 A
FG + FC	11.3 FG	10.6 FG	13.3 G
FG + FA	10.6 FG	11.7 E-G	14.5 G
FG + FP	10.9 FG	14.3 EF	12.6 G
FC + FA	18.9 B-D	19.2 CD	25.8 A *
FC + FP	20.3 BC	21.0 BC	21.6 B-D
FA + FP	22.7 AB	23.0 A-C	23.8 A-C
FG + FC + FA	12.5 E-G	9.1 G	15.3 FG *
FG + FC + FP	14.6 D-F	10.7 FG	16.6 E-G *
FG + FA + FP	16.5 DE	12.6 E-G	19.0 D-F *
FC + FA + FP	21.0 A-C	20.1 B-D	21.7 B-D
FG + FC + FA + FP	8.4 G	10.0 FG	20.1 C-E *

[1] FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

[2] Inoculation with variable inoculum concentrations of (50,000 conidia ml $^{-1}$ per species)

[3] Inoculation with constant amount of inoculum (50,000 conidia ml $^{-1}$)

[4] Means with the same letters within a column are not significantly different (Duncan test, $p \leq 0.05$)

* Pairwise comparison variable and constant inoculum (2006), t-test ($p \leq 0.05$)

2.2.5.3 Effects of single and mixed inoculations with *Fusarium* spp. on frequency of *Fusarium*-infected kernels

In single isolate inoculations, *F. graminearum* resulted in the highest rate of infected wheat kernels followed by *F. culmorum*, *F. avenaceum* and *F. poae*, respectively (Table 16 and 17). For variable concentrations of inoculum, the frequency of kernels infected with *F. graminearum* was similar for all combinations including this isolate, except the mixture with *F. culmorum* isolate, which resulted in a significant lower frequency of *F. graminearum* infected kernels. In this combination, however, the frequency of *F. graminearum* was twice as high as that of *F. culmorum*. The frequency of kernels infected with *F. culmorum*, *F. avenaceum* and *F. poae*, respectively, was lower in mixed inoculations than when inoculated alone; in most cases this effect was significant demonstrating decreasing competitiveness of the isolates in the order *F. graminearum*, *F. culmorum*, *F. avenaceum*, and *F. poae*. Mixtures generally resulted in a higher total frequency of *Fusarium* infected kernels with the mixture of all isolates reaching almost 97% in 2005 and 81% in 2006; mixtures of two or three isolates gave lower infection rates. The total infection rates resulting from *Fusarium* mixtures were significantly lower than the sum derived from single isolate inoculations demonstrating significant interactions between isolates during kernel colonization.

With a constant inoculum concentration of 5 x 10^4 conidia ml^{-1} in all treatments, inoculation with *F. graminearum* isolate alone gave the highest infection rate (Table 17). The reduction in the number of *F. graminearum* conidia in mixtures by the factor of 2, 3, and 4 reduced the frequency of *F. graminearum*-infected kernels by 14 % (in combination with *F. poae*) to 45 % (with *F. culmorum*) demonstrating significant differences in the competitiveness of the isolates. In all mixtures, the reduction in the frequency of *Fusarium* infected kernels compared to single isolate inoculations was more pronounced than in the experiments with variable inoculum concentrations; this effect was strongest for *F. graminearum* and often resulted in a frequency of almost 0 for *F. poae*. Especially for the combinations involving *F. graminearum* and *F. culmorum* isolates, the total rate of infected kernels was significantly lower than that detected with variable inoculum concentrations. This effect increased with the number of isolates in the mixture resulting in similar total rates for mixtures of two, three or four isolates.

Table 16: Frequency of *Fusarium*-infected wheat kernels (cv. Munk) after inoculation of ears with different combinations of *Fusarium* species at GS 65 (2005, variable inoculum concentrations)

Inoculum [1]	Infected kernels [%]				
	FG	FC	FA	FP	Total
F. graminearum (FG)	65.2 A [2]				[3] 65.2 C-E [2]
F. culmorum (FC)		49.6 C			49.6 E-G
F. avenaceum (FA)			35.2 DE		35.2 GH
F. poae (FP)				2.0 H	2.0 I
FG + FC	52.0 BC	24.0 EF			76.0 BC
FG + FA	68.0 A		10.0 GH		78.0 A-C
FG + FP	71.2 A			0 H	71.2 B-D
FC + FA		37.2 D	19.6 FG		56.8 D-F
FC + FP		37.6 D		1.6 H	39.2 F-H
FA + FP			27.2 D-F	2.2 H	29.4 H
FG + FC + FA	60.0 A-C	19.4 FG	10.0 GH		89.4 AB
FG + FC + FP	60.0 A-C	18.8 FG		1.2 H	80.0 A-C
FG + FA + FP	65.2 A		18.0 FG	0 H	83.2 A-C
FC + FA + FP		35.2 DE	20.0 FG	0.4 H	55.6 D-F
FG + FC + FA + FP	62.8 AB	24.0 EF	9.2 GH	0.8 H	96.8 A
LSD	16.6	13.9	7.3	1.4	

[1] FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

[2] Means were separated by independent multivariate analyses for *Fusarium* isolates and total *Fusarium* infected kernels, respectively (Duncan test, $p \leq 0.05$)

[3] The total number of infected kernels is the sum of the individual isolates

LSD, least significant difference within columns (Duncan test, $p \leq 0.05$)

Table 17: Frequency of *Fusarium*-infected wheat kernels (cv. Munk) after inoculation of ears with variable and constant inoculum concentrations, respectively, of *Fusarium* species at GS 65 (2006)

Inoculum [1]	Variable inoculum concentration [2]					Constant inoculum concentration [3]				
	FG	FC	FA	FP	Total	FG	FC	FA	FP	Total
F. graminearum (FG)	63.5 AB [3]				[5] 63.5 CD [4]	63.5 A [3]				[5] 63.5 A [4]
F. culmorum (FC)		47.5 EF			47.5 E		47.5 C			47.5 B-D
F. avenaceum (FA)			28.5 G		28.5 F			28.5 F		28.5 E
F. poae (FP)				7.0 K-M	7.0 G				7.0 IJ	7.0 F
FG + FC	56.0 DC	12.5 JK			68.5 C	35.0 E *	13.0 GH			48.0 B-D *
FG + FA	65.0 AB		3.0 M		68.0 C	47.0 C *		2.0 JK		49.0 B-D *
FG + FP	67.0 A			0 M	67.0 C	55.0 B			0 K	55.0 B
FC + FA		37.5 G	6.0 K-M		43.5 E		27.0 F *	1.5 J *		28.5 E *
FC + FP		45.0 F		3.0 M	48.0 E		41.5 D		0.4 K *	42.0 D
FA + FP			24.0 HI	5.5 K-M	29.5 F			24.0 F	2.0 J	26.0 E
FG + FC + FA	53.0 DE	11.5 J-L	5.0 K-M		69.5 BC	35.0 E *	11.0 I	2.0 JK *		48.0 B-D *
FG + FC + FP	59.0 B-D	17.5 IJ		1.0 M	80.5 AB	38.0 DE *	16.0 G		0 K *	54.0 BC *
FG + FA + FP	61.0 A-C		7.0 K-M	2.0 M	70.0 A-C	50.0 C *		2.0 JK *	1.0 K	53.0 BC *
FC + FA + FP		47.0 EF	6.5 K-M	0 M	53.5 DE		41.5 D *	4.0 JK	0 K	45.5 CD
FG + FC + FA + FP	59.5 B-D	15.0 J	5.0 K-M	1.5 M	81.0 A	40.0 DE *	9 HI	3.0 JK	0 K *	52.0 BC *
LSD	9.6	6.6	4.0	2.6		6.6	6.6	4.2	1.0	

[1] FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

[2] Inoculum concentration 5 x 10^4 conidia ml^{-1} per species in mixture

[3] Inoculum concentration constant (5 x 10^4 conidia ml^{-1})

[4] Means were separated by independent multivariate analyses for *Fusarium* isolates and total *Fusarium* colonization within inoculation (Duncan test, $p \leq 0.05$)

[*] Significantly different from variable inoculum concentration (t-test, $p \leq 0.05$)

[5] The total number of infected kernels is the sum of the individual isolates; LSD, least significant difference within columns (Duncan test, $p \leq 0.05$)

2.2.5.4 Effects of single and mixed inoculations with *Fusarium* spp. on fungal biomass measured as species-specific DNA content

Results from experiments in two years demonstrated high reproducibility of the variability among the isolates regarding fungal biomass of wheat kernels (Table 18 and 19). For inoculations with one isolate, *F. graminearum* gave by far the highest amount of fungal DNA, followed by *F. culmorum*, *F. avenaceum* and *F. poae* with 60%, 5%, and 0.3%, respectively, of the fungal DNA content in kernels as compared to *F. graminearum*. Similar to the frequency of infected kernels, the *F. graminearum* biomass was not affected in most combinations with other isolates, except for the two-isolate mixture with *F. culmorum* when DNA content was significantly reduced. In these interactions, the content of *F. graminearum* DNA decreased - relative to single isolate inoculation – by 30% compared to almost 50% for *F. culmorum* (Tables 5 and 6).

The inherent low DNA content of *F. avenaceum* and *F. poae* was further reduced in mixtures with the other isolates even though to a non-significant level. For *F. poae* isolate, some mixtures even resulted in a non-significant increase in DNA content at a very low level. Although low in biomass, these isolates significantly reduced the biomass production by *F. culmorum* in kernels when inoculated in mixtures with this isolate. Wheat ears inoculated with a mixture of all four isolates (variable inoculum concentration) had the highest total DNA content, which was similar to those produced by the mixtures including *F. graminearum* isolate.

Mixed inoculations with a constant concentration of conidia resulted in a decrease in the total fungal DNA content of the four isolates in wheat kernels largely reflecting the dilution factors in the inoculum mixtures (Table 19). The combination of all four isolates resulted in a total DNA concentration almost 50 % lower than that of the most virulent isolate and very close to the arithmetic mean for all isolates (8.8 *vs*. 7.2 ng mg^{-1} kernel dry weight). This effect was more pronounced for the DNA content than for the frequency of infected kernels (52 *vs*. 37 %). The reciprocal inhibition in development among isolates was very pronounced in the combination *F. graminearum* with *F. culmorum* when both isolates produced only about one third of the biomass they did after single isolate inoculations.

Table 18: Biomass of *Fusarium* species measured as fungal DNA content in wheat kernels (cv. Munk) after inoculation with variable inoculum concentrations at GS 65 (2005)

Inoculum [1]	Fungal DNA content [pg/mg kernel dry weight]				
	FG	FC	FA	FP [2]	Total
F. graminearum (FG)	15921 A [3]				15921 C [3]
F. culmorum (FC)		9356 BC			9356 D
F. avenaceum (FA)			1065 FG		1065 E
F. poae (FP)				46 G	46 E
FG + FC	11241 B	5014 DE			16255 C
FG + FA	17565 A		679 G		18244 A-C
FG + FP	16387 A			95 G	16482 BC
FC + FA		3228 D-G	620 G	0 G	3848 E
FC + FP		4534 D-F		61 G	4595 DE
FA + FP			845 G	53 G	898 E
FG + FC + FA	15597 A	6596 CD	521 G		22714 A
FG + FC + FP	15007 A	2073 E-G		30 G	17110 A-C
FG + FA + FP	18111 A		864 G	145 G	19120 A-C
FC + FA + FP		2279 E-G	287 G	29 G	2595 E
FG + FC + FA + FP	17392 A	4181 D-F	529 G	0 G	22102 AB
LSD	4775	2983	754	ns	

Inoculation with variable inoculum concentrations (5 x 10^4 conidia ml^{-1} per species)

[1]FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

[2] DNA content in pg/mg kernel dry weight

[3] Means were separated by independent multivariate analysis within *Fusarium* isolates and total *Fusarium* DNA content (Duncan test, $p \leq 0.05$)

LSD, least significant difference within columns (Duncan test, $p \leq 0.05$)

NS, Not significant

Table 19: Biomass of *Fusarium* species measured as fungal DNA content of wheat (cv. Munk) kernels after inoculation with variable and and constant inoculum concentrations, respectively, of *Fusarium* species at GS 65 (2006)

Inoculum [1]	Fungal DNA content of wheat kernels [pg/mg kernel dry weight]									
	Variable inoculum concentration [2]					Constant inoculum concentration [3]				
	FG	FC	FA	FP	Total	FG	FC	FA	FP	Total
F. graminearum (FG)	17360 AB [4]				17360 AB [4]	17360 A [4]				17360 A [4]
F. culmorum (FC)		10571 DE			10571 C		1057 B			1057 B-D
F. avenaceum (FA)			620 H		620 D			620 F		620 EF
F. poae (FP)				59 H	59 D				59 F	59 F
FG + FC	11904 DC	5460 G			17364 AB	5195 CD *	3173 D-F			8368 B-D *
FG + FA	15263 AB		333 H		15596 B	10976 B		253 F		11229 B
FG + FP	17005 AB			77 H	17082 AB	10571 B			36 F	10607 B-D
FC + FA		7169 FG	286 H		7455 C		5410 C-E	188 F		5598 C-E
FC + FP		7969 E-G		187 H	8156 C		5911 CD		27 F	5938 B-D
FA + FP			334 H	170 H	504 D			122 F	92 F	214 EF
FG + FC + FA	14625 BC	4936 G	347 H		19908 AB	4814 DE *	4234 DE	174 F		9222 B-D *
FG + FC + FP	15684 AB	5579 G		142 H	21405 A	6506 DC *	4381 DE		76 F	10963 BC *
FG + FA + FP	18339 A		286 H	149 H	18774 AB	8275 BC *		283 F	53 F	8611 B-D *
FC + FA + FP		6532 FG	179 H	55 H	6766 C		4814 DE *	46 F	65 F	4925 D-F *
FG + FC + FA + FP	15848 AB	5619 G	271 H	251 H	21989 A	6423 DC *	2211 EF *	180 F	35 F	8849 B-D *
LSD	5412	2983	458	ns		5002	3645	351	ns	

[1] FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

[2] Inoculum concentration 5 x 10^4 conidia ml^{-1} per species in mixture

[3] Inoculum concentration constant (5 x 10^4 conidia ml^{-1})

[4] Means were separated by independent multivariate analyses for *Fusarium* isolates and total *Fusarium* colonization within inoculation (Duncan test, $p \leq 0.05$)

* Significantly different from variable inoculum concentration (t-test, $p \leq 0.05$)

LSD, least significant difference within columns (Duncan test, $p \leq 0.05$); NS, Not significant

2.2.5.5 Relationship among disease parameters

Disease severity, 1000-kernel weight reduction, frequency of *Fusarium* infected kernels and total fungal biomass were significantly correlated to each other with linear correlation coefficients summarized in Table 20.

Using microbiological assays for wheat ears inoculated with *F. poae*, this isolate could be detected only in a very limited number of kernels. On the other hand, the frequency of *F. avenaceum* infected kernels was moderate to low. Real-time PCR, however, was able to determine DNA of *F. poae* and *F. avenaceum* isolates at low to medium levels, respectively. The coefficients of determination, therefore, were low for these isolates. In most cases, correlation coefficients were poor or non-significant between the estimated parameters for *F. graminearum* and in some cases for *F. culmorum*.

Table 20: Coefficients of determination ($p \leq 0.001$) among *Fusarium* DNA content, frequency of infected wheat kernels, disease severity and kernel weight reduction

			Disease severity	Kernel weight	Infected kernels	Fungal DNA content			
						Total	FG	FC	FA	FP
Disease severity		2005[A]			0.77	0.64	0.15	ns	ns	ns
		2006[A]			0.77	0.64	ns	0.27	ns	ns
		2006[B]			0.70	0.39	ns	0.19	0.17	ns
Frequency of infected kernel	Total	2005[A]				0.64				
		2006[A]				0.67				
		2006[B]				0.45				
	FG	2005[A]	ns	ns			0.12			
		2006[A]	ns	ns			ns			
		2006[B]	ns	ns			0.45			
	FC	2005[A]	0.29	0.15				ns		
		2006[A]	0.60	0.74				0.22		
		2006[B]	0.39	0.34				0.28		
	FA	2005[A]	0.54	0.52					ns	
		2006[A]	0.73	0.45					ns	
		2006[B]	0.58	0.24					0.25	
	FP	2005[A]	0.19	0.12						ns
		2006[A]	0.36	0.12						ns
		2006[B]	0.74	0.41						0.11
Kernel weight		2005[A]	0.56		0.43	0.50	ns	ns	ns	ns
		2006[A]	0.60		0.62	0.53	ns	0.34	ns	ns
		2006[B]	0.55		0.42	0.31	ns	ns	ns	ns

[A] Variable concentration of inoculum

[B] Constant concentration of inoculum

[C] Total frequency of *Fusarium*-infected kernels

[D] ns, Not significant

FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

2.2.6 Field experiments

The preference of establishment of *Fusarium* isolates was studied in two consecutive inoculations with one-day interval in 2005. In 2006 wheat ears were inoculated at mid-flowering with one isolate or all possible combinations of four isolates in two approaches of inoculum concentrations were investigated. To monitor development of the isolates on wheat ears and at harvest, FHB severity, 1000-kernel weight, frequency of infected kernels and *Fusarium* species-specific amount of DNA were investigated.

2.2.6.1 Effect of single and mixed inoculations with *Fusarium* spp. on severity of *Fusarium* head blight (constant inoculum concentration)

In inoculation with *F. graminearum* alone and it mixtures with other isolates resulted in high disease severities, which did not differ significantly from each other (Table 21). This indicated that the disease severity was not directly proportional to the dilution factors of *F. graminearum* conidia in the mixtures and no additive effects in the mixtures to produce more disease. This was true for *F. culmorum* and its mixtures with low virulence isolates, except in combination with *F. poae*, where the amount of disease severity was significantly less than inoculation with *F. culmorum* alone. The disease severity of wheat ears in mixture including *F. avenaceum* and *F. poae* was similar to ears inoculated with these isolates alone.

2.2.6.2 Effect of single and mixed inoculations with *Fusarium* spp. on reduction of 1000-kernel weight

For variable and constant inoculum concentrations, inoculations with isolates alone or in mixtures, the kernel weight was hardly affected by inoculations (Table 21). Only kernel weight of plants, which had been inoculated with *F. graminearum* alone or in mixtures were significantly different from those of non-inoculated control plants. Only three combinations caused about 22% reduction in kernel weight in two inoculation approaches. In most cases, plant inoculated with constant and variable inoculum concentrations produced more and less similar kernel biomass. This is in contrast to greenhouse experiments, where the kernel weight was significantly reduced in inoculations with high virulent isolates.

Table 21: Visual ratings of *Fusarium* head blight severity (1 to 9) and kernel weight reduction after inoculation of wheat ears (cv. Taifun) with various combinations of *Fusarium* spp. at GS 65 (2006)

Inoculum [1]	1000-kernel weight		Disease severity ratings
	Variable conc. [2]	Constant conc. [3]	Constant conc. [3]
Nom-inoculated	36.5 AB	36.5 AB	
F. graminearum (FG)	28.8 CD	28.8 D	6.7 A
F. culmorum (FC)	32.0 A-D	32.0 CD	4.3 CD
F. avenaceum (FA)	36.6 A	36.6 AB	2.0 F
F. poae (FP)	36.4 AB	36.4 AB	1.0 F
FG + FC	29.6 CD	32.9 B-D	5.6 A-C
FG + FA	28.7 CD	31.7 CD	6.4 AB
FG + FP	30.1 CD	30.4 CD	5.9 B-C
FC + FA	31.6 B-D	33.5 A-C	4.6 CD
FC + FP	32.8 A-D	33.4 A-C	2.3 EF
FA + FP	35.9 AB	37.4 A	1.9 F
FG + FC + FA	30.7 CD	33.7 A-C	4.9 B-D
FG + FC + FP	28.3 D	31.5 CD	6.4 AB
FG + FA + FP	30.7 CD	31.6 CD	5.8 A-C
FC + FA + FP	33.2 A-C	33.7 A-C	3.7 DE
FG + FC + FA + FP	29.5 AB	33.2 A-C	7.0 A

[1] FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

[2] Inoculation with variable inoculum concentrations of (50,000 conidia ml $^{-1}$ per species)

[2] Inoculation with constant amount of inoculum (50,000 conidia ml $^{-1}$)

[3] Means with the same letters within a column are not significantly different (Duncan test, $p \leq 0.05$)

Data are the average of three disease severity ratings (7, 14 and 21 days after inoculation)

2.2.6.3 Effects of single and mixed inoculations with *Fusarium* spp. on disease development on spikelets

Spikelets from wheat plots inoculated at GS 65 with *Fusarium* isolates alone or in mixture of 2, 3 or 4 species had infection rates ranging from 15 to 50 %, 23.4 to 67.1% and 36.5 to 88.2% after 7, 14 and 21 days after inoculation, respectively (Table 22, 23 and 24). The number of infected spikelets increased during maturation of wheat ears.

The virulence of *Fusarium* isolates on the spikelets increased in the order *F. poae*, *F. avenaceum*, *F. culmorum* and *F. graminearum* when inoculated alone. In mixed inoculations, the frequency of infected spikelets reduced for all isolates due to the decrease in the number of spores per isolate. However, the degree of reduction was depended on the composition of the mixture. This effect became more pronounced in the low virulent isolate *F. poae* in the mixtures including three to four isolates. Mixtures including *F. graminearum* gave higher total frequency of infected spikelets than the mixtures without this isolate. The presence of *F. graminearum* in the mixtures strongly reduced the frequency of infected spikelets caused by the other isolates. The frequency of infected spikelets in mixture including *F. avenaceum* and *F. poae* was similar to ears inoculated with these isolates alone.

The number of infected spikelets increased during the milk ripening either in inoculation with one *Fusarium* isolate or in co-inoculations (Table 25). For example, in inoculation with one isolate, the number of infected spikelets increased 37.0, 33.5, 28.0, and 21.5 % for *F. graminearum*, *F. culmorum*, *F. avenaceum* and *F. poae*, respectively. As exemplified for the combination of *F. graminearum*, *F. culmorum* and *F. avenaceum* in Figure 11, For *F. graminearum*, the increase in the number of infected spikelets was independent whether this isolate had been inoculated alone or in mixture with other isolates. The presence of *F. graminearum* in the mixtures significantly reduced the increase in the number of infected spikelets by 78 %, 90 % and negatively increased, in co-inoculations with *F. culmorum, F. avenaceum* and *F. poae* respectively compared to when these isolates inoculated alone.

Disease development by *F. culmorum* on the spikelets was similar either when inoculated alone or in mixtures including *F. avenaceum* and/or *F. poae*. On the other hand, disease development attributed to these isolates was inhibited in the presence of *F. graminearum* in the mixtures. *F. poae* proved to be the weakest competitor among isolates and showed a decrease in the rate of infected spikelets in four out of seven combinations.

Table 22: Frequency of *Fusarium*-infected spikelets 7 days after inoculation of wheat ears (cv. Taifun) with various combinations of *Fusarium* species (2006, constant inoculum concentration)

Inoculum [1]	Infected spikelets [%]				
	FG	FC	FA	FP	Total [2]
FG (*F. graminearum*)	50.0 A [3]				50.0 A
FC (*F. culmorum*)		33.5 B-D			33.5 C
FA (*F. avenaceum*)			31.6 C-D		31.6 C
FP (*F. poae*)				15.0 I-N	15.0 D
FG + FC	33.3 B-D	12.6 K-O			44.7 A-C
FG + FA	40.9 B		13.7 J-O		48.3 AB
FG + FP	42.1 AB			5.3 O-R	44.5 A-C
FC + FA		27.2 C-F	21.4 E-J		37.1 A-C
FC + FP		25.5 C-G		10.3 L-Q	31.3 C
FA + FP			24.4 D-H	13.8 J-O	35.2 BC
FG + FC + FA	20.9 F-J	11.9 K-P	7.4 M-R		35.3 BC
FG + FC + FP	31.2 C-D	11.8 K-P		1.5 QR	35.4 BC
FG + FA + FP	34.3 BC		8.1 M-R	2.8 P-R	39.3 A-C
FC + FA + FP		26.7 C-G	16.1 H-M	6.2 N-R	37.6 A-C
FG + FC + FA + FP	23.7 E-I	9.0 M-R	9.7 L-R	0.3 R	40.0 A-C

[1] FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

[2] Total lower than sum of species because some spikelets infected by two species

[3] Means were separated by independent multivariate analyses for isolates and total *Fusarium* infected spikelets, respectively (Duncan test, $p \leq 0.05$)

Table 23: Frequency of *Fusarium*-infected spikelets 14 days after inoculation of wheat ears (cv. Taifun) with various combinations of *Fusarium* species (2006, constant inoculum concentration)

Inoculum [1]	Infected spikelets [%]				
	FG	FC	FA	FP	Total [2]
FG (*F. graminearum*)	63.6 A [3]				63.7 A-C
FC (*F. culmorum*)		48.4 B-D			48.4 E
FA (*F. avenaceum*)			36.9 EF		36.9 F
FP (*F. poae*)				23.4 GH	23.4 G
FG + FC	41.8 DE	16.8 H-K			51.7 C-E
FG + FA	55.9 AB		18.5 H-J		66.0 AB
FG + FP	51.8 BC			5.6 L-N	54.6 A-E
FC + FA		43.1 C-E	29.3 FG		55.4 A-E
FC + FP		42.4 DE		13.1 I-L	50.2 DE
FA + FP			38.1 EF	23.7 GH	54.3 B-E
FG + FC + FA	40.0 DE	17.8 H-J	10.2 J-M		60.2 A-E
FG + FC + FP	53.4 B	18.1 H-J		1.5 MN	67.1 A
FG + FA + FP	56.5 AB		12.5 J-L		62.1 A-D
FC + FA + FP		41.9 DE	21.2 G-I	7.8 N	53.9 B-E
FG + FC + FA + FP	41.2 DE	14.4 H-L	14.4 H-L	0.9 MN	55.0 A-E

[1] FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

[2] Total lower than sum of isolates because some spikelets infected by two isolates

[3] Means were separated by independent multivariate analyses for isolates and total *Fusarium* infected spikelets, respectively (Duncan test, $p \leq 0.05$)

Table 24: Frequency of *Fusarium*-infected spikelets 21 days after inoculation of wheat ears (cv. Taifun) with various combinations of *Fusarium* species (2006, constant inoculum concentration)

Inoculum [1]	Infected spikelets [%]				
	FG	FC	FA	FP	Total [2]
FG (*F. graminearum*)	87.0 A [3]				87.0 A
FC (*F. culmorum*)		67.1 B-D			67.1 BE
FA (*F. avenaceum*)			59.6 DE		59.7 C
FP (*F. poae*)				36.5 GH	36.5 D
FG + FC	70.9 B-D	20.0 I-K			74.3 AB
FG + FA	67.8 B-D		18.1 I-K		75.0 AB
FG + FP	74.3 A-C			1.5 LM	74.3 AB
FC + FA		58.2 DE	41.8 GF		69.1 BC
FC + FP		61.5 B-D		16.8 J-K	67.5 BC
FA + FP			59.3 DE	19.6 I-K	67.4 BC
FG + FC + FA	61.6 B-E	22.9 I-K	12.5 K-M		76.2 AB
FG + FC + FP	75.2 AB	22.6 I-K		0.6 LM	88.2 A
FG + FA + FP	66.5 B-D		27.4 H-J	0.0 M	86.5 A
FC + FA + FP		49.6 EF	31.2 G-I	1.8 LM	66.4 BC
FG + FC + FA + FP	60.9 C-E	14.3 J-L	12.4 K-M	0.3 LM	76.8 AB

[1] FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

[2] Total lower than sum of isolates because some spikelets infected by two isolates

[3] Means were separated by independent multivariate analyses for isolates and total *Fusarium* infected spikelets, respectively (Duncan test, $p \leq 0.05$)

Table 25: Increase in the number of infected spikelets within two weeks after inoculation of wheat ears (cv. Taifun) with various combinations of *Fusarium* spp. at GS 65 (2006, constant inoculum concentration)

Inoculum [1]	Increase of infected spikelets within 14 days [%]				
	FG	FC	FA	FP	Total
FG (*F. graminearum*)	37.0 A-D [2]				37.0 A-C [2]
FC (*F. culmorum*)		33.5 A-E			33.5 A-C
FA (*F. avenaceum*)			28.0 A-G		28.0 BC
FP (*F. poae*)				21.5 C-I	21.5 C
FG + FC	37.6 A-C	7.4 H-L			29.6 BC
FG + FA	26.9 A-G		4.0 I-L		26.6 BC
FG + FP	32.3 A-F			-3.8 L	29.8 BC
FC + FA		29.0 A-F	19.1 E-J		31.9 A-C
FC + FP		36.0 A-E		6.6 H-L	36.1 A-C
FA + FP			35.0 A-E	5.9 H-L	32.2 A-C
FG + FC + FA	40.7 AB	11.0 G-L	5.1 I-L		40.9 A-C
FG + FC + FP	44.0 A	10.7 G-L		-0.9 KL	52.7 A
FG + FA + FP	32.2 A-F		19.4 E-J	-2.8 L	47.1 AB
FC + FA + FP		22.9 B-H	15.1 F-K	-4.4 L	28.7 BC
FG + FC + FA + FP	37.6 A-D	5.3 H-L	2.8 J-L	0.0 KL	36.7 A-C

[1] FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

[2] Means were separated by independent multivariate analysis for all isolates and total infection, respectively (Duncan test, $p \leq 0.05$)

Sampling was done 7, 14 and 21 days after inoculations

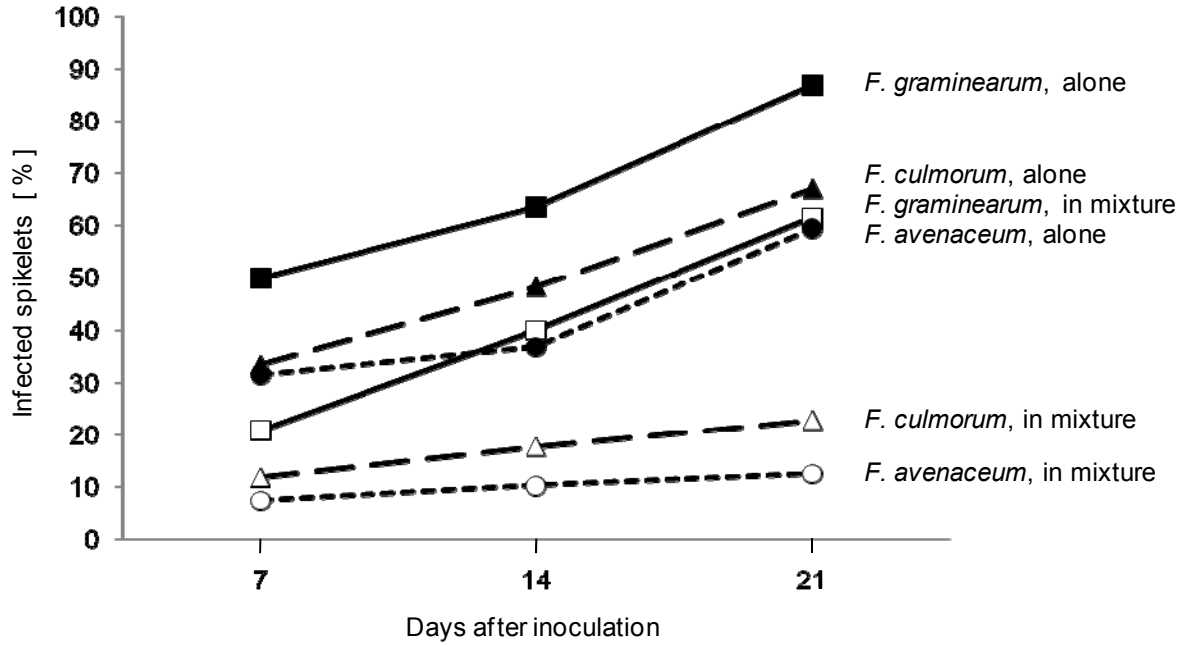

Figure 11: Increase in the frequency of wheat spikelets infected by *Fusarium graminearum, F. culmorum* and *F. avenaceum*, respectively, within 14 days when inoculated either alone (filled marks) or in a mixture of three isolates (open marks) at GS 65 (cv. Taifun; all treatments were inoculated with a total of 50,000 spore ml^{-1})
FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

2.2.6.4 Effects of inoculation with *Fusarium* spp. on frequency of infected glume, lemma, palea and kernel

From late flowering to milk ripening various parts of wheat flower parts were differentiated for their colonization by isolates (Table 26 and 27). In both years, the frequency of *Fusarium* infection decreased in the order lemma, palea, glume and developing kernel, respectively. However, in some cases there were no significant differences for the species on the wheat flower parts. *Fusarium graminearum* and *F. culmorum* were the predominant isolates on the different parts of spikelets in 2005 and 2006, respectively, while *F. poae* had the lowest infected levels.

Similar to the spikelets, the colonization of all parts of wheat flower increased with the time after inoculation (Table 28 and 29). The increase in the frequency of infection was more pronounced in most virulent isolates increasing with sampling times. For isolate with low virulence, the increase in infection of wheat flower parts in different sampling times was

similar in 2006, while in 2005 there were significant differences in frequency of infection among the sampling times. For *F. poae* there was no significant difference among the sampling times.

Table 26: Frequency of infected glume lemma, kernel and palea after consecutive inoculations of wheat ears (cv. Drifter) in one interval with *Fusarium* spp. (2005)

Fusarium species [1]	Frequency of infection [%]			
	Glume	Lemma	Palea	Kernel
F. graminearum	41.7 D [2]	54.6 C	47.1 D	45.8 D
F. culmorum	58.3 C	72.1 A	70.8 A	64.6 B
F. avenaceum	23.8 F	41.2 D	35 E	23.8 F
F. poae	5.0 G	7.5 G	4.6 G	5.8 G

Inoculation wheat ears with 91,000 conidia ml $^{-1}$ in two individual consecutive days

[1] FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

[2] Means separated by independent multivariate analysis (Duncan test, $p \leq 0.05$)

Data are the average of three times samplings (14, 21 and 35 d p.i.)

Table 27: Frequency of infected glume, lemma, palea and kernel of wheat ears (cv. Taifun) after inoculation with a combination of four *Fusarium* spp. at GS 65 (2006)

Fusarium species [1]	Frequency of infection [%]			
	Glume	Lemma	Palea	Kernel
F. graminearum	31.0 BC [2]	38.0 A	32.6 AB	26.6 CD
F. culmorum	13.0 F	22.3 D-F	18.6 E	9.6 FG
F. avenaceum	5.6 GH	10.0 FG	5.0 GH	2.6 H
F. poae	1.0 H	1.6 H	0.3 H	0.3 H

Inoculation wheat ears with 91,000 conidia ml $^{-1}$ in two individual consecutive days

[1] FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

[2] Means separated by independent multivariate analysis (Duncan test, $p \leq 0.05$)

Data are the average of three times samplings (7, 14 and 21 d p.i.)

Table 28: Frequency of *Fusarium* spp. within five weeks after consecutive inoculations of wheat ears (cv. Drifter) in one interval with *Fusarium* spp. at GS 65 (2005)

Fusarium species [1]	Frequency of infection [%]		
	7 d p.i.	21 d p.i.	35 d p.i.
F. graminearum	14.7 FG[2]	62.2 C	65.0 C
F. culmorum	19.3 F	85.3 B	94.7 A
F. avenaceum	10.3 GH	31.9 E	50.6 D
F. poae	6.9 HI	5.6 HI	4.7 I

Inoculation wheat ears with 91,000 conidia ml $^{-1}$ in two individual consecutive days

[1] FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

[2] Means separated by independent multivariate analysis (Duncan test, $p \leq 0.05$)

Table 29: Frequency of *Fusarium* spp. within two weeks after of wheat ears (cv. Taifun) after inoculation with a combination of four *Fusarium* spp. at GS 65 (2006)

Fusarium species [1]	Frequency of infection [%]		
	7 d p.i.	14 d p.i.	21 d p.i.
F. graminearum	9.5 E[2]	33.0 B	53.8 A
F. culmorum	5.3 E-G	15.8 D	26.8 C
F. avenaceum	2.0 F-H	6.8 EF	8.8 E
F. poae	0.8 GH	0.0 H	1.8 F-H

Inoculation wheat ears with 91,000 conidia ml $^{-1}$ in two individual consecutive days

[1] FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

[2] Means separated by independent multivariate analysis (Duncan test, $p \leq 0.05$)

2.2.6.5 Shift in the ratio among *Fusarium* species during *Fusarium* head blight development on wheat ears

The Chi-squared goodness of fit was applied to compare theoretical (expected) and observed frequency of infection and content of fungal DNA on wheat ears (Table 30). From wheat anthesis (the time of inoculation) to harvest, a shift in the composition of the *Fusarium* species colonization the ears could be observed. The ratio (frequency of infection and content of fungal DNA) among isolates using the test differed significantly from the ratio 1 : 1 : 1 : 1 expected for equal virulence and competitiveness the isolates.

The proportion of *F. graminearum* either in inoculation alone or in mixtures increased during the growth period and reached the highest value for the DNA content of kernels (reached 80 % of total amount of fungal DNA in the four-isolate mixture). For example, in a four-isolate mixture, the frequency of *F. graminearum* infected spikelets increased from 55.0 to 69.3 % within two weeks after inoculation.With reduction in the number of conidia in mixtures by a factor of 2, 3, and 4, the infection ratios increased for *F. graminearum* and in some cases *F. culmorum*, while the infection ratios for *F. avenaceum and F. poae* reduced and resulted in a infection ratio of almost 0 for *F. poae* in a three isolate-combination (Fa, Fg with Fp).

For the combination of *F. avenaceum, F. culmorum*, and *F. poae*, for instance, not only the overall colonization of spikelets increased with the incubation time, but also the proportion of *F. culmorum*, the most aggressive species in this mixture (Figure 12). The proportion of *F. culmorum* increased during the growth period and reached the highest value for the DNA content of kernels. In two-isolate combinations, the observed and expected frequencies were significantly different. Nevertheless, *F. avenaceum* and *F. culmorum* proved are not significantly different in their capability to colonize the wheat spikelets after anthesis; this was also true for *F. avenaceum* and *F. poae* in the first stages of disease development.

The ratios increased corresponding to the virulence and competitive abilities of the isolates. When wheat ears were inoculated with the mixtures including of *F. avenaceum* and/or *F. poae*, the ratios of these species on the spikelets were higher than those obtained by frequency and intensity of kernel colonization, which indicated differences in development of the isolates in the spikelets and kernels.

Table 30: Comparison of expected and measured ratios of *Fusarium* infections of spikelets (7, 14, and 21 days after inoculation) and kernels at harvest (frequency and species-specific amount of *Fusarium* DNA) after inoculation of wheat ears (cv. Taifun) at GS 65 (2006)

Fusarium species	Expected ratio	Actual frequency and content of fungal DNA									
		Spikelets						Kernels at harvest			
		7 dpi.		14 dpi.		21 dpi.		Frequency		DNA content	
FG + FC	1:1	1.45:0.55	*	1.43:0.57	*	1.56:0.44	*	1.59:0.41	*	1.52:0.48	*
FG + FA	1:1	1.50:0.50	*	1.50:0.50	*	1.58:0.42	*	1.81:0.19	*	1.96:0.04	*
FG + FP	1:1	1.78:0.22	*	1.80:0.20	*	1.96:0.04	*	1.94:0.06	*	1.98:0.02	*
FC + FA	1:1	1.12:0.88	ns	1.19:0.81	Ns	1.17:0.83	ns	1.53:0.47	*	1.60:0.40	*
FC + FP	1:1	1.42:0.58	*	1.53:0.47	*	1.57:0.43	*	1.91:0.09	*	1.80:0.02	*
FA + FP	1:1	1.28:0.72	ns	1.23:0.77	Ns	1.50:0.50	*	1.77:0.23	*	2.00:0.00	*
FG + FC + FA	1:1:1	1.56:0.89:0.55	*	1.76:0.79:0.45	*	1.91:0.71:0.39	*	2.10:0.79:0.11	*	2.07:0.87:0.05	*
FG + FC + FP	1:1:1	2.01:0.80:0.10	*	2.19:0.74:0.06	*	2.29:0.69:0.02	*	2.63:0.35:0.02	*	2.09:0.87:0.04	*
FG + FA + FP	1:1:1	2.28:0.54:0.19	*	2.46:0.54:0.00	*	2.12:0.88:0.00	*	2.84:0.16:0.00	*	2.98:0.02:0.00	*
FC + FA + FP	1:1:1	1.63:0.99:0.38	*	1.77:0.89:0.33	*	1.80:1.13:0.07	*	2.26:0.68:0.06	*	2.44:0.41:0.14	*
FG + FC + FA + FP	1:1:1:1	2.22:0.84:0.91:0.03	*	2.32:0.81:0.81:0.05	*	2.77:0.65:0.56:0.01	*	2.85:0.91:0.22:0.02	*	3.21:0.67:0.09:0.04	*

Inoculation with 50,000 conidia ml $^{-1}$ for all treatments FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

* Expected and actual ratio significantly different (Chi-square test on goodness of fit, $p \leq 0.05$) ns, not significant

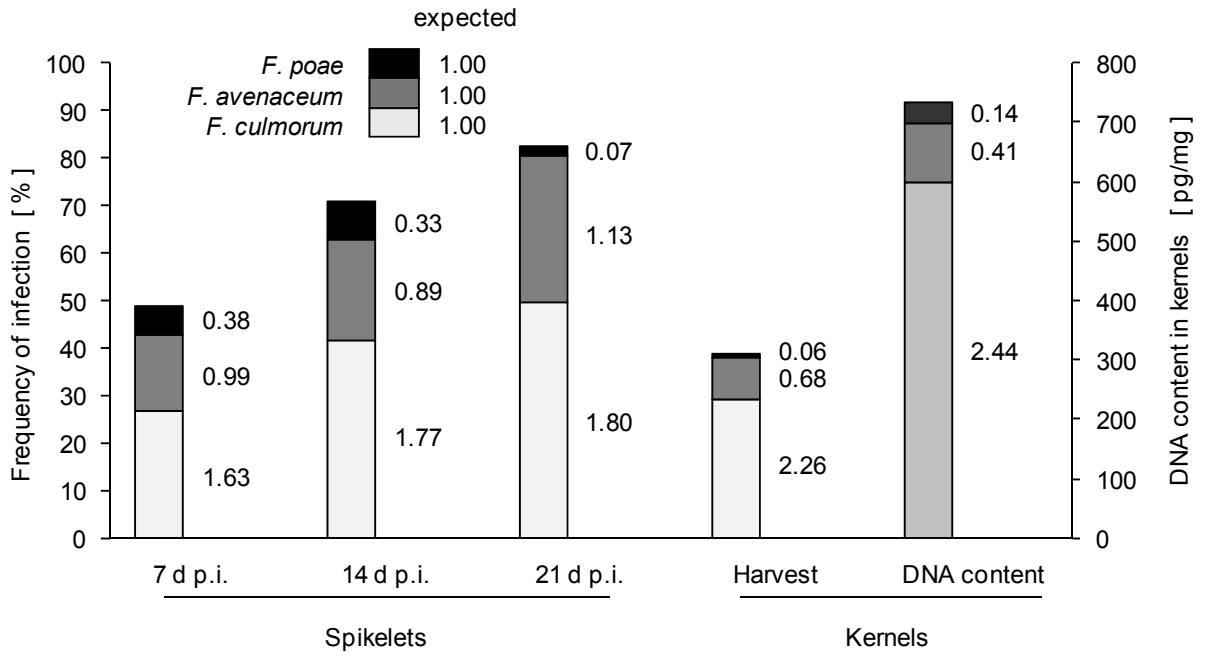

Figure 12: Shift in the ratio of *Fusarium* isolates colonizing wheat spikelets 7, 14, and 21 days after inoculation, and kernels at harvest, respectively, after ear inoculation at GS 65 with a mixture of *F. poae*, *F. avenaceum*, and *F. culmorum* (cv. Taifun; total concentration of 50,000 conidia ml^{-1}; 2006)

2.2.6.6 Effect of inoculation of with *Fusarium* spp. on frequency of spikelets and kernels infected by more than one *Fusarium* species

Especially the spikelets of wheat ears proved to be infected by more than one isolate when inoculated in mixtures (Table 31). The frequency of single spikelets infected by two *Fusarium* isolates increased with the time and reached 1.6 (Fg with Fp) to 30.9 % (Fc with Fa) after 21 days after inoculation. Most of the combinations including *F. poae* showed only a slight increase over time, whereas mixtures with *F. avenaceum* and *F. culmorum* resulted in the highest percentage of kernels colonized by two isolates. In contrast to the overall increase in co-colonization of spikelets, the percentage of kernels bearing two *Fusarium* isolates was relative low at harvest (up to 2.5 %). In the combination Fa with Fp – total frequency of infected kernels only 12 % - no co-colonization was detected, whereas other mixtures including *F. avenaceum* resulted in the highest frequencies.

In consecutive inoculations only two-isolate combinations including Fa with Fc, Fa with Fg as well as Fc with Fg resulted in double-infected kernels. Inoculation of *F. avenaceum* in the second day resulted in the highest frequency of infected kernels with *F. graminearum* and *F. culmorum.*

Table 31: Shift in the frequency of spikelets and kernels infected by at least two *Fusarium* species after inoculation of wheat ears 65 (cv. Taifun) with various combinations of *Fusarium* spp. at GS (2006, constant inoculum concentration)

Inoculation [1]	Plant part infected by two *Fusarium* spp. [%]			
	Spikelets			Kernel
	7 d p.i.	14 d p.i.	21 d p.i.	harvest
FG + FC	1.3 D [2]	6.9 B	16.6 BC	0.3 C
FG + FA	6.3 BC	8.4 B	10.9 CD	2.5 A
FG + FP	2.9 CD	2.8 B	1.6 E	0.3 C
FC + FA	11.5 A	17 A	30.9 A	2.0 A-C
FC + FP	4.5 CD	5.3 B	10.9 CD	0.8 BC
FA + FP	3.0 CD	7.5 B	11.6 CD	0 C
FG + FC + FA	4.9 CD	7.8 B	20.9 B	0.5 C
FG + FC + FP	9.1 AB	5.9 B	10.3 CD	0.3 C
FG + FA + FP	5.9 BC	6.9 B	7.5 DE	1.0 A-C
FC + FA + FP	11.4 A	17.0 A	16.3 BC	1.3 A-C
FG + FC + FA + FP	2.7 CD	16.0 A	11.3 CD	2.3 AB

[1]FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8.

[2] Means were separated by independent multivariate analyses for each time and kernel at harvest (Duncan test, $p \leq 0.05$)

2.2.6.7 Effect of inoculation with *Fusarium* spp. on the frequency of *Fusarium*-infected kernels

Simultaneous inoculation

As expected from infections of spikelets, *F. graminearum* proved to be most virulent isolate on wheat kernels in single isolate inoculations alone. The ranking in virulence among isolates became even more obvious when inoculated in mixtures. For variable inoculum concentrations, the infection rates with *F. graminearum* did not affect by the presence of *F. avenaceum* and/or *F. poae* in mixtures, while mixtures including *F. culmorum* significantly reduced *F. graminearum* infection frequency (Table 32).

Table 32: Frequency of *Fusarium*-infected wheat kernels (cv. Taifun) after inoculation of ears with variable combinations of *Fusarium* species at GS 65 (2006, variable inoculum concentrations)

Inoculum [1]	Infected kernels [%]				
	FG	FC	FA	FP	Total
F. graminearum (FG)	62.5 A[2]				62.5 BC[2]
F. culmorum (FC)		45.3 D			45.3 DE
F. avenaceum (FA)			12.3 F		12.3 F
F. poae (FP)				5.0 G-J	5.0 F
FG + FC	52.5 C	10.3 FG			62.5 BC
FG + FA	66.0 A		4.5 G-J		68.0 AB
FG + FP	66.8 A			0.8 IJ	66.3 AB
FC + FA		40.0 D	7.8 F-H		44.0 DE
FC + FP		51.0 C		2.0 H-J	51.8 CD
FA + FP			26.0 E	4.0 G-J	35.5 E
FG + FC + FA	54.8 C	10.0 FG	3.3 H-J		62.5 BC
FG + FC + FP	52.0 C	13.3 F		0.5 J	62.5 BC
FG + FA + FP	56.3 BC		7.3 F-I	0.0 J	60.0 BC
FC + FA + FP		40.3 D	8.5 F-H	0.0 J	46.0 DE
FG + FC + FA + FP	61.5 AB	13.5 F	2.8 H-J	0.0 J	76.5 A

[1]FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8.

[2] Means were separated by independent multivariate analyses for isolates and total *Fusarium* infected kernels, respectively (Duncan test, $p \leq 0.05$)

Mixtures generally gave lower frequency of *F. culmorum*, *F. avenaceum* and *F. poae* infected kernels than when these isolates were inoculated alone. The total infection rates in the mixtures were contributed by the isolate with high virulence and were significantly lower than the sum derived from single inoculations demonstrating significant interactions between or among the isolates during kernel colonization.

In inoculation with constant inoculum concentration, *F. graminearum* clearly dominated kernel infection suppressing not only *F. poae* but also *F. avenaceum* and *F.*

culmorum. Although diluted in mixtures by a factor of 2 to 4, *F. graminearum* resulted in 60 – 90 % (mixtures of 2 species), 57 - 83% (mixtures of 3 species), and 62% (mixture of four species), respectively, of the infection frequency when inoculated alone (Table 33).

Table 33: Frequency of *Fusarium* infected kernels after inoculation of wheat ears (cv. Taifun) with various combination at GS 65 (2006, constant amount of inoculum)

Inoculum [1]	Infected kernels [%]				
	FG	FC	FA	FP	Total
FG (*F. graminearum*)	62.5 A[2]				62.5 A[2]
FC (*F. culmorum*)		45.3 C-F			45.3 C-F
FA (*F. avenaceum*)			12.3 JK		12.3 GH
FP (*F. poae*)				5.0 L-N	5.0 H
FG + FC	38.5 F-H	9.8 KL			48.0 B-E
FG + FA	47.5 CD		5.0 L-N		50.0 B-E
FG + FP	58.8 AB			1.8 L-N	60.3 AB
FC + FA		31.0 HI	5.5 L-N		34.5 F
FC + FP		40.0 D-G		1.8 L-N	41.0 D-F
FA + FP			9.5 KL	2.5 L-N	12.0 G
FG + FC + FA	35.5 G-I	13.3 JK	1.8 L-N		50.1 B-E
FG + FC + FP	46.8 C-E	6.3 K-N		0.3 MN	53.1 A-D
FG + FA + FP	52.3 BC		3.0 L-N	0.0 N	54.3 A-C
FC + FA + FP		29.5 I	8.8 K-M	0.8 MN	37.8 EF
FG + FC + FA + FP	39.0 E-G	12.5 KJ	3.0 L-N	0.3 MN	52.5 A-E

[1]FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

[2] Means were separated by independent multivariate analyses for *Fusarium* spp. and total *Fusarium* infected kernels, respectively (Duncan test, $p \leq 0.05$)

Infection of kernels by *F. poae* was very low in most combinations, and reduced – to the single inoculation - by at least 50 % in the mixture with *F. avenaceum*. Although *F. culmorum* was the second most successful isolate when inoculated alone, competition from *F. graminearum* strongly suppressed its development in mixture. In mixtures, the frequency

of infected kernels with *F. graminearum*, *F. culmorum* and *F. avenaceum* were inhibited to different degrees ranging from 6 to 43 %, 35 to 86 % and 28 to 85 %, respectively, compared to when these isolates inoculated alone. This indicated reduction in the number of conidia in the mixtures by a factor of 2, 3, and 4 and significant interactions among the isolates on wheat ears. The frequency of *F. poae* infected kernels was similar either in single or mixed inoculations. In mixed inoculations, the total frequency of *Fusarium*-infected kernels was similar to the frequency of individual isolates with high virulence. This indicated that most of the infection could be contributed to the high virulence isolates.

Consecutive inoculations

As expected, the frequency of infected kernels with two time single isolate inoculations in two consecutive days increased by 52, 62 and 26 % for *F. graminearum*, *F. culmorum* and *F. avenaceum*, respectively compared to one time inoculation (Table 34). However, the infection rates were not significantly different among the isolates. The frequency of *F. poae*-infected kernels was similar in one or two time inoculations with one-day interval.

The frequency of *F. graminearum* infected kernels was not affected when *F. avenaceum* was inoculated either in the first or second day, compared to one time inoculation with *F. graminearum* alone. In Fg with Fc combination, the frequency of infected kernels reduced for the two isolates when they were inoculated either before or after each other. This effect was more pronounced in *F. culmorum.* Establishment of *F. culmorum* or *F. avenaceum* on the wheat ears reduced the colonization of the kernels by each other when inoculated in the second day by 42 and 47 % respectively demonstrating different competitive abilities of the species in time and space. The presence of *F. poae* did not affect the infection rates by *F. graminearum* and *F. culmorum*, while the frequency of *F. avenaceum*-infected kernels reduced in the presence of *F. poae*. When *F. poae* was inoculated either alone or in combination with other isolates, there was no significant difference in the *F. poae* infection rates.

Table 34: Frequency of *Fusarium*-infected kernels (cv. Drifter) after two consecutive inoculations with two-species combinations of *Fusarium* species at GS 65 (2005)

The first day	The second day	Infected kernels [%]			
		FG	FC	FA	FP
(FG) *F. graminearum*	FG	95.3 A [1]			
	FC	39.3 I	53.3 D-F		
	FA	47.7 FG		56.0 DE	
	FP	51.3 E-G			1.3O
(FC) *F. culmorum*	FG	30.7 JK	67.3 C		
	FC		94.0 A		
	FA		71.3 C	30.7 JK	
	FP		54.0 D-F		1.3O
(FA) *F. avenaceum*	FG	28.0 K	9.3 LM	52.0 E-G	
	FC		41.3 HI	58.6 D	
	FA			92.0 A	
	FP			90.6 A	0.0P
(FP) *F. poae*	FG	59.3 D			3.3M-P
	FC		82.0 B		0.0P
	FA			51.3 E-G	2.0N-O
	FP			6.6 L-P	8.0L-N
FG	Water	46.0 GH			
FC			36.0 IJ	10.0 L	
FA				68.0 C	
FP					6.6L-O

Inoculation with 91,000 conidia ml $^{-1}$ in two individual consecutive days

FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

[1] Means were separated by independent multivariate analyses for *Fusarium* spp. (Duncan test, $p \leq 0.05$)

2.2.6.8 Effects of inoculation of *Fusarium* species on fungal biomass measured as species-specific DNA content

Simultaneous inoculation

Similar to greenhouse experiments, the ranking among *Fusarium* species became even more pronounced when assessing fungal biomass of kernels as measured by the species-specific *Fusarium* DNA content (Table 35 and 36). For inoculations with one isolate, *F. graminearum* gave by far the highest amount of fungal DNA, followed by *F. culmorum*, *F. avenaceum* and *F. poae* with 36 %, 7 %, and 3 %, respectively, of the fungal DNA content in kernels as compared to *F. graminearum*.

With variable inoculum concentration the amounts of *F. graminearum* DNA was similar in all combinations, except in Fc with Fg and Fa, Fc with Fg combinations where DNA content was significantly reduced by 47 % and 38 % respectively relative to single inoculation. The amount of *F. culmorum* DNA in mixed inoculations was 26 to 78 % lower than when this isolate was inoculated alone even though the differences were not significant. The inherent low DNA content of *F. avenaceum* and *F. poae* was further reduced in mixtures with the other isolates to a lower degree. The total amount of *Fusarium* DNA in co-inoculations of all isolates was similar to those produced by the most virulent isolate *F. graminearum* demonstrating significant interactions among the isolates on wheat ears.

With constant inoculum concentration, the content of *F. graminearum* DNA was not directly proportional to the dilution factors of its conidia in the mixtures. The reduction of DNA content of *F. graminearum* was ranging from 14 to 84 % in mixtures with *F. culmorum* and *F. avenaceum*, and *F. poae*, respectively (Table 36). For example, in combination with *F. poae*, *F. graminearum* resulted in almost the same biomass despite of the fact that only half of the conidia had been inoculated for this isolate. *F. graminearum* DNA was reduced by *F. culmorum* and to a lesser extent also by *F. avenaceum*. The content of *F. culmorum* DNA reduced in combination with other isolates especially in mixtures with *F. avenaceum* and/or *F. poae* even thought; there were no significant differences for *F. culmorum* DNA in the mixtures.

Overall, the total *Fusarium* DNA was negatively affected by interactions among the isolates in mixtures relative to inoculation with one isolate. This demonstrated competition between these isolates resulting in an overall low *Fusarium* biomass in wheat kernels. For example, in Fa, Fc with Fg and Fa, Fc with Fp combinations, the total *Fusarium* biomass was 76 % and 86 % lower than single inoculations with the most virulent isolates in the mixtures. In two or three isolate-mixtures including *F. culmorum*, *F. avenaceum* and /or *F. poae*, the

total *Fusarium* DNA were similar to the low virulence isolates. In the combination of all isolates, *F. graminearum* accounted for 81 % of total *Fusarium* biomass and where it was a quarter of the total spore concentration, while the total fungal biomass was 45 % of inoculation with *F. graminearum* alone.

Table 35: Content of fungal DNA in wheat (cv. Taifun) kernels after inoculation with different combination of *Fusarium* spp. at GS 65 (2006, variable inoculum concentrations)

Inoculum [1]	Fungal biomass [ng/mg kernel dry weight]				
	FG	FC	FA	FP [2]	Total
FG (*F. graminearum*)	11.8 A [3]				11.8 A [3]
FC (*F. culmorum*)		4.3 C-E			4.3 B-D
FA (*F. avenaceum*)			0.8 DE		0.8 D
FP (*F. poae*)				359.6 E	0.4 D
FG + FC	5.6 B-D	1.9 DE			7.4 A-C
FG + FA	10.8 A		0.06 E		10.9 A
FG + FP	12.1 A			0.0 E	12.1 A
FC + FA		2.9 DE	0.3 E		3.2 CD
FC + FP		2.9 DE		0.0 E	2.9 CD
FA + FP			0.5 E	0.0 E	5.3 D
FG + FC + FA	4.5 C-E	1.0 DE	0.02 E		5.5 B-D
FG + FC + FP	7.9 A-C	1.3 DE		16.5 E	9.3 AB
FG + FA + FP	10.5 A		0.07 E	110.0 E	10.7 A
FC + FA + FP		3.2 C-E	0.07 E	300.5 E	3.6 CD
FG + FC + FA + FP	9.8 AB	1.4 DE	0.3 E	0.0 E	11.5 A

[1] FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

[2] DNA content in pg/mg kernel dry weight

[3] Means were separated by independent multivariate analyses for isolates and total amount of *Fusarium*-DNA of infected kernels (Duncan test, $p \leq 0.05$)

Table 36: Content of fungal DNA in wheat (cv. Taifun) kernels after inoculation with different combination of *Fusarium* spp. at GS 65 (2006, constant inoculum concentrations)

Inoculum [1]	Fungal biomass [ng/mg kernel dry weight]				
	FG	FC	FA [2]	FP [2]	Total
FG (*F. graminearum*)	11.8 A [3]				11.8 A [3]
FC (*F. culmorum*)		4.3 C-E			4.3 E
FA (*F. avenaceum*)			778.6 F		0.8 G
FP (*F. poae*)				359.6 F	0.4 G
FG + FC	4.7 CD	1.5 F			6.2 D
FG + FA	8.8 B		245.2 F		8.8 C
FG + FP	10.2 AB			62.2 F	10.4 B
FC + FA		0.8 F	186.0 F		0.9 G
FC + FP		0.7 F		76.0 F	0.8 G
FA + FP			123.7 F	0 F	0.1 G
FG + FC + FA	1.9 EF	0.8 F	50.4 F		2.8 F
FG + FC + FP	2.4 D-F	1.0 F		49.4 F	3.4 EF
FG + FA + FP	5.6 C		41.6 F	0 F	5.7 D
FC + FA + FP		0.6 F	53.4 F	35.0 F	0.6 G
FG + FC + FA + FP	5.3 C	1.1 F	145.1 F	60.0 F	6.5 D

[1] FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

[2] DNA content in pg/mg kernel dry weight

[3] Means were separated by independent multivariate analyses for isolates and total amount of *Fusarium*-DNA of infected kernels (Duncan test, $p \leq 0.05$)

Consecutive inoculations

In consecutive inoculations, wheat ears which had been inoculated two times in one day interval with individual isolates resulted in two and three times increase in the amount of species-specific DNA than one time inoculation for *F. graminearum*, and *F. culmorum* and *F. avenaceum*, respectively (Table 37). One time inoculation with *F. graminearum* either alone or in combination with other isolates resulted in similar amount of fungal DNA by this isolate.

Table 37: Content of fungal DNA in wheat (cv. Drifter) kernels after two consecutive inoculations with a 1-day interval with *Fusarium* spp. at GS 65 (2005)

The first day	The Second day	Fungal biomass [ng/mg kernel dry weight]			
		FG	FC	FA	FP
F.graminearum (FG)	FG	9.5 A [1]			
	FC	3.6 C-F	2.6 EH		
	FA	4.3 B-E		0.9 HI	
	FP	4.7 B-D			0.05 I
F.culmorum (FC)	FG	3.5 C-G	4.1 C-E		
	FC		6.1 B		
	FA		3.8 C-F	1.0 HI	
	FP		3.3 D-G		0.03 I
F. avenaceum (FA)	FG	1.8 G-I	0.7 I	0.3 I	
	FC		4.0 C-E	1.0 HI	
	FA			1.3 HI	
	FP			0.5 I	0.05 I
F. poae (FP)	FG	3.7 C-F			0.01 I
	FC		5.3 BC		0.0 I
	FA			0.4 I	0.04 I
	FP			0.4 I	0.002 I
FG	Water	4.5 B-E			
FC			1.9 F-I	0.3 I	
FA				0.4 I	
FP					0.09 I

Inoculation with 91,000 conidia ml $^{-1}$ in two individual consecutive days

[1] Means were separated by independent multivariate analyses for isolates (Duncan test, $p \leq 0.05$)

FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

This indicated inoculation with other isolates before or after establishment of *F. graminearum* on wheat ears, does not affect development of *F. graminearum* DNA content kernels. Inoculation with *F. graminearum* one day before *F. culmorum* resulted in significant reduction of the amount of *F. culmorum*. While, the content of *F. culmorum* DNA were similar when *F. avenaceum* and *F. poae* inoculated either before or after this isolate.

2.2.6.9 Effects of inoculation of *Fusarium* species on kernel mycotoxin contents

For the quantification of mycotoxins in the grain samples using LC/MS/MS the peak area correlated with a known amount of mycotoxins. For all mycotoxins, standard curves were prepared based on ten and two fold serially diluted mycotoxins in two replications. The standard curves were used for the quantification of mycotoxins in unknown kernel samples. Standard curves for some important mycotoxins are presented in Figure 13-15.

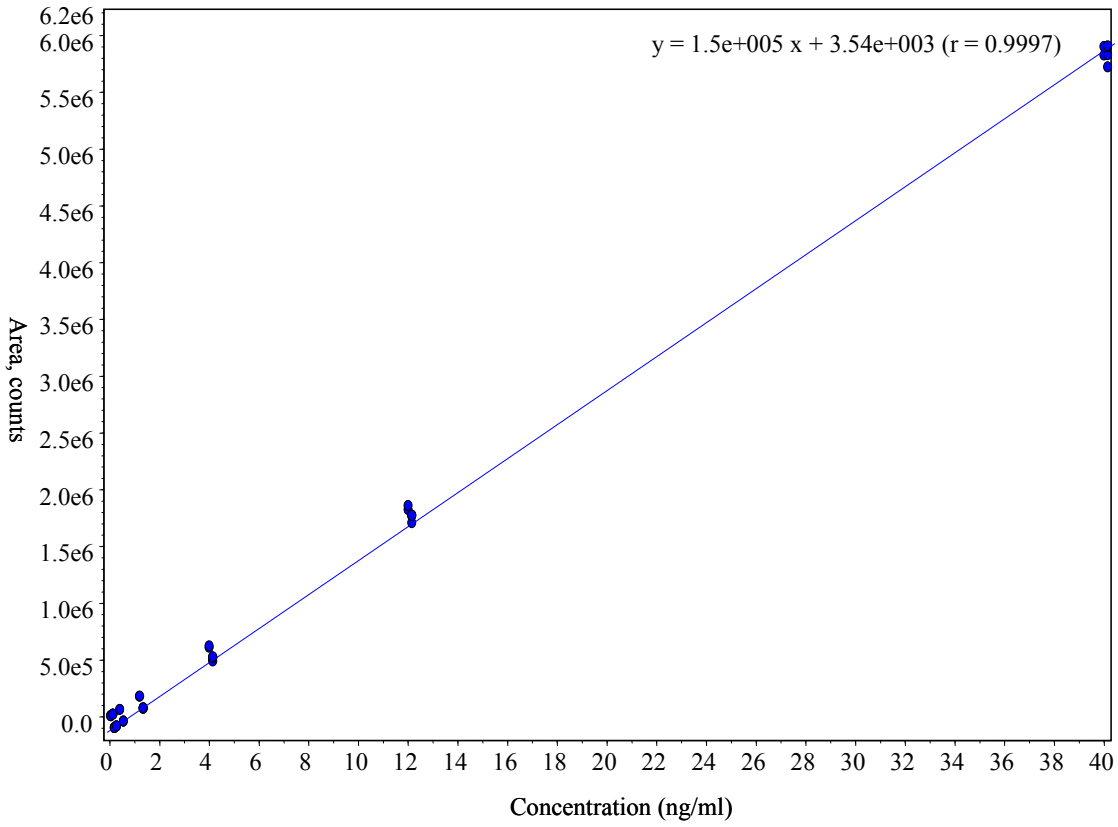

Figure 13: Standard curve used for quantification of enniatin B

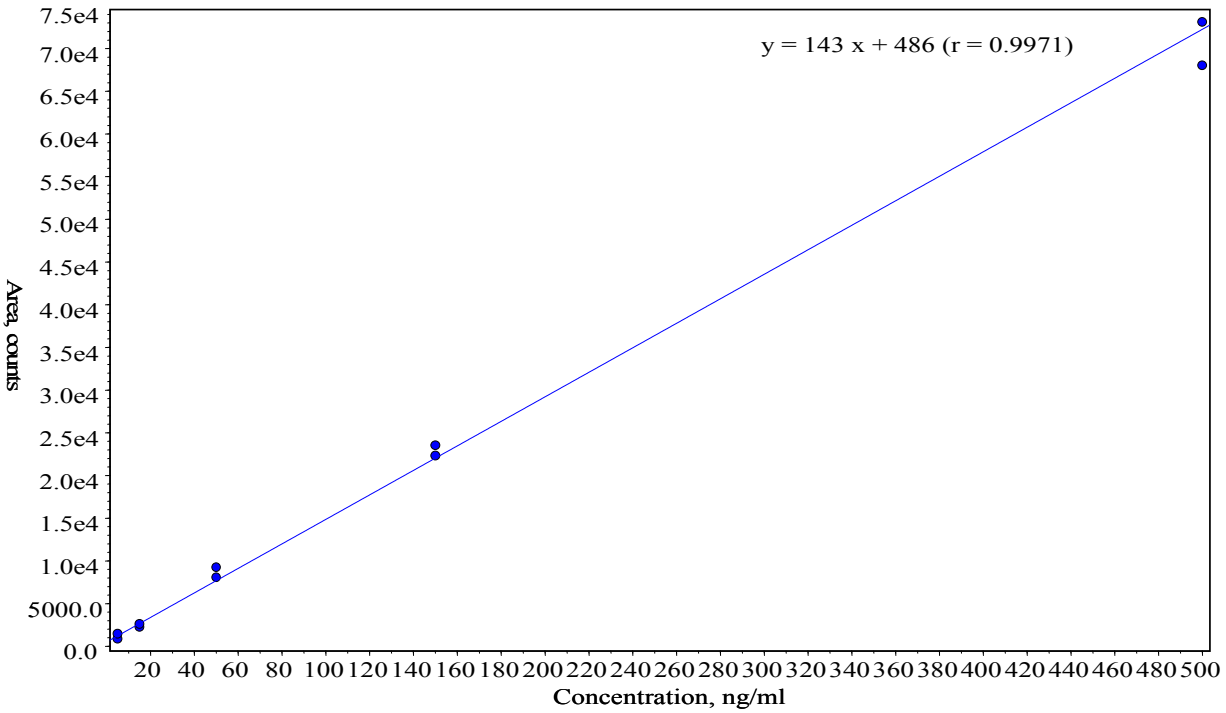

Figure 14: Standard curve used for quantification of 15-Acetyl-deoxynivalenol

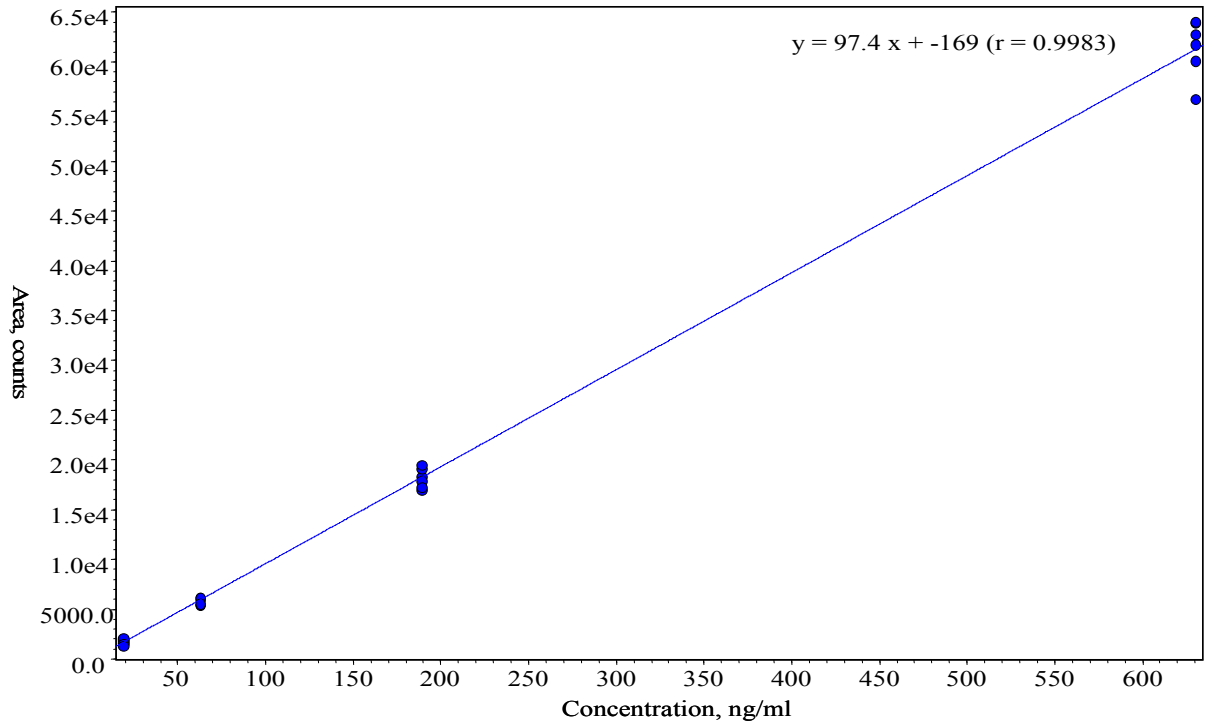

Figure 15: Standard curve used for quantification of deoxynivalenol

Simultaneous inoculation

As expected from microbiological and molecular assays, *F. graminearum* resulted in the highest content of mycotoxins in the kernels in either single inoculation or co-inoculations with other isolates. In single-isolate inoculations, *F. graminearum* produced both derivates of deoxynivalenol, while *F. culmorum* produced 3-ACDON. The deoxynivalenol and Deoxynivalenol-3-glucoside productivity of *F. graminearum* was seven and five times, respectively higher than for *F. culmorum* when these isolates were inoculated alone (Table 38).

In inoculation with constant inoculum concentration, *F. graminearum* clearly contributed to the mycotoxin contents of kernels. The amount of deoxynivalenol was similar in inoculation with *F. graminearum* alone or in mixtures, which included this isolate (Table 38). However, in co-inoculations with *F. avenaceum*, production of deoxynivalenol was two times higher than inoculation with *F. graminearum* alone. Although *F. graminearum* diluted in mixtures by a factor of 2 to 4, the amount of deoxynivalenol was not significantly different, which demonstrate existence of significant interactions in the mixtures resulting in an increase in the ability of mycotoxin production by the isolates.

For *F. culmorum*, the frequency and intensity of infected kernels reduced in two-isolate combinations with *F. avenaceum* and *F. poae* and the conidia concentration by a factor of 2 in two-isolate mixtures, the production of deoxynivalenol was two and four times higher in co-inoculations with *F. avenaceum* and *F. poae*, respectively than inoculation with *F. culmorum* alone.

For Deoxynivalenol-3-glucoside and derivatives of deoxynivalenol, the results were very similar to deoxynivalenol in co-inoculations of the isolates. However, there were no significant differences in the amounts of derivatives of deoxynivalenol.

The presence of *F. graminearum* in the mixtures had negative effects on production of enniatin A1, B, B1, and moniliformin, while in mixtures including *F. culmorum* and *F. poae*, there was an increase in production of these mycotoxins. This effect was more obvious for moniliformin in mixtures including *F. graminearum.* For example, in Fa with Fp the production of enniatin A1, B, B1, and moniliformin increased by 4, 3, 3 and 25 times higher than inoculation with *F. avenaceum* alone, respectively even though with reduction of the conidia in the mixtures by factors 2, 3 and 4. This indicates that significant interactions among the isolates in the mixtures. *F. poae* was the only isolate produced nivalenol in low amounts in some combinations, which were not significantly different.

Table 38: Amount of detected mycotoxins in wheat (cv. Taifun) kernels after inoculations with constant inoculum concentrations of *Fusarium* species at GS 65 in 2006

Inoculum	Fungal mycotoxin (ng/g kernel dry weight)									
	DON	D3G	ZEAR	3-ACDON	15-ACDON	ENNA1	ENNB1	ENNB	MON	NIV
F. graminearum (FG)	18343	1876	4	120	223					
F. culmorum (FC)	2488	366	3	102						
F. avenaceum (FA)						5	68	153	5	
F. poae (FP)							1	72		26
FG + FC	20050	2443	24	283	199	1	9	52		
FG + FA	27535	3610		165	144	5	37	106		
FG + FP	37000	3930	2	188	329					
FC + FA	5716	483	4	207	16	8	80	262	143	
FC + FP	9816	878	14	337			3	9		26
FA + FP						22	231	517	128	9
FG + FC + FA	15910	2027	1	215	123		6	20		
FG + FC + FP	14315	1757		90	116		1	2		
FG + FA + FP	16960	1712	8	295	216	4	37	55		
FC + FA + FP	2980		2	47		17	166	463	134	16
FG + FC + FA + FP	17175	2330		145	156	1	13	254		7
No-inoculation			1			2	13	28	48	
LSD	13050	1334	ns	ns	ns	ns	128	302	ns	ns

FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8; LSD, Least significant differences within columns; DON, deoxynivalenol; D3G, Deoxynivalenol-3-glucoside; 3-AcDON,3-acetyldeoxynivalenol; ZON, zearalenone; Alpha -ZOL, alpha-zearalenol; MON, moniliformin; ENN, enniatin; NIV, nivalenol; least significant differences within each column.

In inoculation with variable inoculum concentration, ranking in mycotoxin production by the isolates became even more obvious when inoculated in mixtures. Although, the infection rates and fungal biomass of *F. graminearum* were not affected by the presence of *F. avenaceum* and/or *F. poae* in mixtures, the mycotoxin content of kernels dramatically increased (Table 39). This demonstrated the existence of significant interactions in the mixtures resulting in an increase in the ability of mycotoxin production by *F. graminearum.* In Fc with Fg combination, the biomass of both isolates was reduced to different degrees while the ability of deoxynivalenol production increased by a factor of 1.5 and 12 times higher than inoculation with *F. graminearum* and *F. culmorum* alone, respectively.

The ranking in deoxynivalenol, Deoxynivalenol-3-glucoside and derivatives of deoxynivalenol was more obvious in inoculation with *F. culmorum* alone or in co-inoculations with *F. avenaceum* and/or *F. poae*. For example, the ability of deoxynivalenol production in co-inoculations with *F. avenaceum* and/or *F. poae* increased by 3 to 7 times higher than when this isolate was inoculated alone. In two-isolate mixtures, *F. culmorum* with *F. avenaceum* or *F. poae*, the amount of detected deoxynivalenol was higher than when this isolate was inoculated in three isolate mixture with *F. avenaceum* and *F. poae* isolates.

Similar to constant inoculum concentration approach, the production of enniatins increased in mixed inoculations with two or three isolate mixtures including *F. avenaceum* or/and *F. poae* and *F. culmorum.* The production of enniatins and moniliformin was reduced in mixtures including *F. graminearum* and in some mixtures. This effect was more pronounce for the moniliformin in those mixtures including *F. graminearum.* In Fa with Fp combination, the production of enniatin A1, B, B1, and moniliformin increased by 11, 11, 8 and 39 times respectively than inoculation with *F. avenaceum* alone. Similar to constant inoculum concentration approach, nivalenol was detected in the same levels only in inoculation with *F. poae* either alone or in mixtures. In co-inoculation with *F. avenaceum,* the amount of this mycotoxin was 3 times higher than single inoculation with *F. poae*, but the differences were not significant.

Table 39: Amount of detected mycotoxin in wheat (cv. Taifun) kernels after inoculations with variable inoculum concentrations of *Fusarium* species at GS 65 in 2006

Inoculum	Fungal mycotoxin (ng/g kernel dry weight)									
	DON	D3G	ZEAR	3-ACDON	15-ACDON	ENNA1	ENNB1	ENNB	MON	NIV
F. graminearum (FG)	18343	1876	3.7	120	223					
F. culmorum (FC)	2488	366.4	2.9	102						
F. avenaceum (FA)						5	68	153	5	
F. poae (FP)							1	72		26
FG + FC	28475	2216	11	258	314					
FG + FA	37350	2932	2	253	443	6	54	4		
FG + FP	39250	3200		239	397			158		11
FC + FA	11005	1248	50	266		5	50	43	15	
FC + FP	16150	1784	7	322			0.0	118		
FA + FP						56	521	770	195	77
FG + FC + FA	26230	820	4	264	254	1	7	316		11
FG + FC + FP	21675	3084	0.6	153	273			1		25
FG + FA + FP	45700	890	83	332	393	3	28	45	12	
FC + FA + FP	8805	1672	12	291	14	16	165	247	46	
FG + FC + FA + FP	28650	4348	3	239	314	3	24	126		
LSD	14390	1404	ns	183	172	14	132	299	84	ns

FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8; LSD, Least significant differences within columns; DON, deoxynivalenol; D3G, Deoxynivalenol-3-glucoside; 3-AcDON,3-acetyldeoxynivalenol; ZON, zearalenone; Alpha -ZOL, alpha-zearalenol; MON, moniliformin; ENN, enniatin; NIV, nivalenol; LSD, least significant differences within each column.

Consecutive inoculation

Similar to microbiological and molecular assays, with two time single isolate inoculations in two consecutive days, the amount of deoxynivalenol significantly increased by 46 and 30 % for *F. graminearum* and *F. culmorum*, respectively compared to one time inoculation (Table 40).

When *F. graminearum* was inoculated with other isolates either before or after, the amount of deoxynivalenol for Fg with Fc was significantly higher than the combinations with other isolates. Production of deoxynivalenol by *F. graminearum* was not affected when *F. avenaceum* or *F. poae* were inoculated in the first day, compared to one time inoculation with *F. graminearum* alone. However, inoculation with *F. graminearum* in the first day resulted in significant increase of deoxynivalenol.

In most cases, where *F. culmorum* was inoculated with other isolates resulted in the same amount of deoxynivalenol similar to inoculations with this isolate alone. Overall, the amount of zearalenone were similar in most combinations. Only two-time inoculations with *F. graminearum* and in Fa with Fg combination, where *F. avenaceum* was inoculated one day after *F. graminearum*, the amount of zearalenone were higher than the other inoculations.

The moniliformin and enniatin B1 productivity of *F. avenaceum* increased when this isolate was inoculated two times with one-day interval or in combinations with other isolates compared to one-time inoculations. For example, wheat ears which had been inoculated two times with one-day interval with *F. avenaceum* isolate resulted in 6 and 3.5 times increase in the amount of moniliformin and enniatin B1, respectively. However, inoculation with *F. poae* one day after *F. avenaceum* reduced the production of the two mycotoxins.

Table 40: Amount of detected mycotoxins in wheat kernels (cv. Drifter) after two consecutive inoculations with 1-day interval with *Fusarium* spp. at GS 65 (2005, inoculation with 91,000 conidia ml $^{-1}$ in two individual consecutive days)

The first day	The second day	Fungal mycotoxin [ng/g kernel dry weight]					
		DON	3-AcDON	HT2-toxin	ZON	MON	ENNB1
F. graminearum (FG)	FG	3203	357		211		
	FC	4395	161		88		
	FA	-	-	-	-	-	-
	FP	2267		11	366		
F. culmorum (FC)	FG	4356	188		89	10	
	FC	6917	309		44	16	
	FA	4171	279		74	242	2400
	FP	3435	180		35		
F. avenaceum (FA)	FG	1885	55		82	127	1276
	FC	6404	260		124	112	1536
	FA		45			432	3685
	FP			12		43	218
F. poae (FP)	FG	1599	46		77	23	
	FC	4799	175		43	18	
	FA	124	48			207	2374
	FP				14		
FG	Water	1725					
FC		4804					
FA						70	1066
FP							
No-inoculation		116		12	14		304
LSD (least significant differences within each column)		392	85	4	47	17	269

Inoculation with 91,000 conidia ml $^{-1}$ in two individual consecutive days; FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8; LSD, Least significant differences within columns; DON, deoxynivalenol; 3-AcDON,3-acetyldeoxynivalenol; ZON, zearalenone; Alpha -ZOL, alpha-zearalenol; MON, moniliformin; ENNB1, enniatins B1

3. Discussion

Fusarium head blight (FHB) of small grains was first described over a century ago and considered a major threat to wheat and barley during the early years of the last century. In recent years, FHB has again increased worldwide with great concerns arising from mycotoxin contamination and increase in the risk of human and animal health (Stack and McMullen, 1985; McMullen *et al*., 1997).

There is little information on interactions between or among *Fusarium* species. To understand and identify such interactions a study was carried out using microbiological and molecular assays based on available species-specific primers. This study involved consecutive and simultaneous inoculations of wheat ears with four *Fusarium* species either alone or in a mixture of two to four species. Experiments were carried out using single isolates for each species under field and greenhouse conditions. Therefore, interpretation of the results on content of fungal biomass, disease severity, kernel weight and frequency of infected kernels have been generalized to the isolates even though in some cases the term species is used.

Several species-specific fragments in different *Fusarium* species have been identified (Parry and Nicholson, 1996; Turner *et al*., 1998; Chelkowski *et al*., 1999; Nicholson *et al*., 2003; Waalwijk *et al*., 2003; Demeke *et al*., 2005; Waalwijk *et al*., 2004; Jurado *et al*., 2005; Leisova *et al*., 2006). These fragments have no similarity with each other to design unique species-specific primers for selective amplification of individual *Fusarium* species in a complex of non-target DNA. Different primers were used in this study, which selectively amplified specific fragments corresponding to the individual species in a mixture of different fungal or plant DNA either in conventional PCR or in real-time PCR assays.

The results confirmed the specificity of the primers as reported by Waalwijk *et al*. (2004) Parry and Nicholson (1996). In most cases, the results of identification based on morphology of *Fusarium* species were certified with species-specific primers.

In a few cases, contradictory results were obtained by conventional PCR assays as compared to morphological identification in isolates of *F. poae*. These isolates had been miss-identified as *F. poae* based on morphological identification. These isolates were later identified as *F. langsethiae* and *F. sporotrichioides*. The results demonstrated that molecular identification using species-specific primers is a superior tool to confirm *Fusarium* species.

Detection, quantification and amplification of a specific part of DNA fragment allowed monitoring phytopathogenic fungi for epidemiological studies over time and space. For FHB disease complex, it is necessary to study the spectrum, role and contribution of individual species to the disease using molecular tools. The SYBR green real-time PCR

proved to be highly specific for individual detection of the species in a mixture including fungal and plant DNA. Existing DNA of plant materials or other fungal DNA, even with other DNA of FHB pathogens, did not affect the assays.

This method can be applied in monitoring of a large number samples for identification and quantification of *Fusarium* species in a complex matrix. The results of validation assays indicated that amplifications using real-time PCR were highly reproducible within replicates of the same sample DNA preparations as well as between different sample preparations.

The dynamic range for detection of *Fusarium* species varied from 9000 to 0.9 pg the pathogens. When the subsequent dilution of 0.09 pg was used, the results could be obtained in some of the real-time PCR experiments. Waalwijk *et al*. (2004) reported that the concentration of 0.09 pg could be used to calculate the detection limit of the real-time PCR assays. The genome sequence of *F. graminearum* (Whitehead Institute, 2003) predicts a genome size of 36 Mb (equal to 0.04 pg). Therefore, the detection limit of the assays can be calculated to be less than five genome equivalents.

The DNA extraction methods produced different yields of fungal DNA. High quality DNA was obtained using CTAB and CTAB + DNeasy plant mini kit methods. However, CTAB method was much more efficient than the CTAB + DNeasy plant mini kit method. This method was therefore routinely used to extract DNA in further experiments. The procedure described here works well for extracting high quality DNA from all wheat plant tissue and the mycelium of *Fusarium* species tested. High quality purified DNA using the CTAB method was extracted from a matrix of plant and fungal DNA. Reproducibility among replicates of the grain samples using real-time PCR confirmed the reliability of this protocol for quantification of FHB pathogens using real-time PCR.

In many other studies either routine DNA extractions or comparison of different DNA extraction methods, CTAB method has been involved and mentioned as one of the best methods (Chen and Ronald, 1999; Drabkova *et al*., 2002; Drabkova, 2004; Sharma *et al*., 2003).

A simple and rapid spore production method was developed for some *Fusarium* species. All species produced a large amount of mycelium in PDB (**P**otato **D**extrose **B**roth**)**. Mucilaginous colonies with large quantities of conidia characterized by great reduction in mycelia formation were formed within 2 days after transferring the mycelia onto LSPDA, SNA or WA (**L**ow **S**trength **P**otato **D**extrose **A**gar, **S**ynthetic **N**utrient **A**gar, **W**ater **A**gar). As the presence of liquid medium in the culture prevented species from producing conidia, it was important to dry the culture plates before incubation.

In the conventional method, all species produced mycelia, conidia or visible sporodochia, even though mycelia were the main biomass. Most isolates of *F. graminearum* did not produce high amounts of spores with the most common method. *F. graminearum* showed high variability in spore production even among the plates within a single isolate.

The extreme change from liquid to solid and dry media is likely to trigger response mechanisms by the *Fusarium* species/isolates to form mucilaginous colonies with large quantities of conidia in culture. This may be explained by the fact that *Fusarium* species produce a high amount of mycelium in PDB. Therefore, a high amount of mycelia in liquid medium depleted of nutrients and extreme changes in environmental conditions – dryness and aeration - may shift the metabolism and ontogenesis of the fungi to exponential production of phialids and conidia in mucilaginous colonies. Many filamentous fungi conidiate poorly or not at all in submerged culture even though such proliferation may be abundant during growth on open surfaces (Kølmark, 1984).

In tests on the virulence of conidia produced with both methods, the frequency of kernel infection, FHB severity and *Fusarium* biomass of kernels as measured by *Fusarium* DNA content were not significantly different. This indicated that the pathogenic potential of conidia produced with the new method was similar to those conidia produced with the conventional method. For highly virulent species, symptoms were observed within 1 to 2 days after inoculation which indicated the efficiency of conidia to produce disease. For both methods, *F. graminearum* resulted in the highest rates of infection and colonization as well as FHB severity, followed by *F. culmorum*, *F. avenaceum* and *F. poae,* respectively.

The method has been successfully applied to produce inoculum in various experiments, for example on maize ear rot caused by *F. graminearum, F. culmorum, F. verticillioides* and *F. proliferatum* and interactions among the major FHB pathogens on wheat ears. The method described here facilitates studies on different aspects of *Fusarium* species and their conidia.

F. graminearum was the most virulent *Fusarium* isolate from Rhineland area, Germany, infecting wheat spikelets and kernels at harvest, followed by *F. culmorum*, *F. avenaceum* and *F. poae*, respectively. This is in agreement with reports from Miedaner *et al*. (1997), Muthomi *et al*. (2000), and Fernandez and Chen (2005), Xu *et al*. (2007). In contrast, Wong *et al*. (1995) and Mihuta-Grimm and Forster (1989) observed higher virulence for *F. culmorum* than for *F. graminearum*, whereas Fernandez and Chen (2005) reported no difference in virulence between these two species. Large variation in virulence among the isolates of *Fusarium* species as reported by Miedaner and Schilling (1996), Muthomi *et al.*

(2000), Miedaner *et al*. (2004), and Fernandez and Chen (2005) may explain differences between these studies.

F. graminearum also proved to be more competitive than the other isolates under greenhouse and field conditions. Similar to virulence, the competitiveness of isolates decreased in the order *F. graminearum, F. culmorum*, *F. avenaceum* and *F. poae*. This indicates the competitive ability of an isolate may be associated to virulence. This implies that an isolate with high competitive ability could be more virulent than its low competitive counterpart. In co-inoculations other isolates was out-competed by the presence of *F. graminearum* in the mixtures. This effect was more pronounce under greenhouse experiments. In the two-party mixture, for example, the amount of fungal DNA of *F. graminearum* and *F. culmorum* was 30 % and 48 % lower than in single inoculations, while kernel colonization was lowered by 16 % and 62 %, respectively. These two isolates exhibited co-suppression even though *F. graminearum* was more suppressive to *F. culmorum* on wheat ears. The most likely explanation for this case is that the virulence of isolates in co-inoculation is reduced by competition. The effect of interaction and especially competition may have resulted in the fungus expending more energy in production of inhibitors for competitors and hence reduced virulence.

Four out of the six possible two-party interactions were competitive - *F. graminearum* x *F. culmorum*, *F. avenaceum* x *F. culmorum*, *F. culmorum* x *F. poae*, *F. avenaceum* x *F. poae* - and two combinations - *F. graminearum* x *F. avenaceum*, *F. graminearum* x *F. poae* - showed amensalism. Competitive interactions between isolates were more disadvantageous to the less-virulent isolates and did not confer any advantage to the more virulent affiliate in the mixture which was not able to produce more disease than when was inoculated alone. These interactions were detrimental to both isolates - although to a different degree. These results are similar to the observations of Simpson *et al*. (2004), who reported that in mixed inoculations, *F. culmorum* suppressed the growth of *Microdochium* species on wheat seedlings. However, when *M. majus* became established on the seedlings, it was able to co-suppress colonization by *F. culmorum*.

Competitive interaction among pathogenic fungi in any stages of their life cycle in the same ecological niches may be due to exploitation and interference mechanisms (Carroll and Wicklow, 1992). Competitive exploitation is an indirect interaction associated with the ability of an organism to consume available resources (without reducing access of the other organisms to the same resource pool). FHB pathogens may interact in different way during growing plant parts. Early germination, fast-growing germ tubes and mycelia associated with

fast absorption of nutrients (Wagacha *et al*., unpublished) and rapid growth on different parts of spikelets may have an effect on the competitiveness of isolates during the infection process. The conidia of *Fusarium* spp. germinated on the surface of wheat flower parts after 6-12 h p.i., but germ-tubes did not penetrate into host tissue immediately (Kang and Buchenauer, 2002; Kang *et al*., 2005). The researchers also showed a dense hyphal network developed on the inner surface of wheat flowers during the first days after inoculation and infection was observed after development of the network. Therefore, interactions can occur during the first stages of infection such as early interactions (spore germination and penetration) using various mechanisms to occupy the ecological niches. Such interactions resulted in predominance of one isolate while the others occurred in low levels.

Interference competition is a direct interaction and involves chemical or behavioural mechanisms that influence the access of fungi to resources. Many fungi produce secondary metabolites such as mycotoxins that may influence competitive outcomes. Mixed inoculation showed that competitive interactions among the major FHB pathogens may or may not lead to reduction of fungal biomass, while the mycotoxin production dramatically increased (Xu *et al*., 2007). Deoxynivalenol (DON), 3-acetyl-DON and nivalenol have been shown to be detectable in early stages of infection and are discussed to be important for differences in virulence at the very beginning of infection (Kang and Buchenauer, 2002; Maier *et al*., 2005).

This may also explain differences in competitiveness of the most virulent isolate compared to isolates with moderate to low virulence. The effect of mycotoxins on other fungi, however, is largely unknown. Among four isolates of *F. culmorum*, Miedaner *et al*. (2004) observed superior competitive ability of two DON-producing isolates as compared to NIV-producing isolates. Xu *et al*. (2007) mentioned that co-inoculations of wheat with the major FHB pathogens resulted in dramatic increase of mycotoxins.

F. graminearum suppressed the frequency and intensity of kernel colonization by *F. avenaceum* and/or *F. poae;* however, these isolates had no effect on *F. graminearum*. These results indicated that asymmetrical competitive interactions (amensalism) may also occur during wheat ear colonization. Similarly, the presence of *F. proliferatum* or *F. moniliforme* had limited inhibitory effect on the population of *F. graminearum* on maize (Velluti *et al*., 2000).

With variable inoculum concentrations of conidia in single and mixed inoculations, neither the amount of *F. graminearum* DNA nor the frequency of kernels infected by this isolate significantly differed, except in the two-party combination with *F. culmorum*. By now we have no explanation for the lack of competition of *F. culmorum* in mixtures of three or

four species; maybe in these cases *F. avenaceum* and/or *F. poae* reduced the effect of *F. culmorum* on *F. graminearum.*

In field experiments, *F. culmorum* and *F. graminearum* were the predominant species in 2005 and 2006, respectively while *F. poae* was the lowest. This may be explained by differences in environmental conditions since the mean daily temperatures in 2005 in most days were less than 15 ºC, while in 2006 were more than 25 ºC (Figure 16 and 17). *F. culmorum* is a major component of FHB in wheat growing areas with cool weather conditions (Mills, 1989; Birzele *et al*., 2002; Bottalico and Perrone, 2002). Reid *et al.* (1999) reported that dry and warm conditions during growth of maize strongly affect the infection of ears by *F. moniliforme* and *F. graminearum*. *Fusarium moniliforme* has at least one competitive advantage over *F. graminearum*, because of a broader response to temperature that confers direct and indirect benefits. Marin *et al*. (1998) showed that *F. moniliforme* and *F. proliferatum* are able to dominate several other common maize-contaminating fungi over a wide range of temperature and water availability conditions – e.g. *Aspergillus* spp. and *Penicillium* spp.

The Chi-square test on goodness of fit demonstrated a shift in the composition of the *Fusarium* isolates colonizing wheat ears from anthesis through harvest. The ratio (in the frequency of infected spikelets) differed significantly from the ratio 1 : 1 : 1 : 1 expected for equal aggressiveness and competitiveness of isolates. The differences in the proportions of frequency and intensity of colonization on spikelets and kernel at harvest were more obvious in low virulent isolates *F. avenaceum* and *F. poae* in mixtures with high virulence one. This indicated difference in ability of the isolates to colonize spikelets and kernels. On the other hand, the infection ratios proved existence of significant interactions among the isolates on spikelets and kernels. In most cases, one isolate out-competed the other ones resulting in an increase of its proportions in the kernels at harvest. This indicates the isolates varied in the competitiveness and virulence to host plant. The infection proportion of *F. graminearum* increased from anthesis to harvest in all inoculations. This was demonstrated by reaching the highest value for its DNA content in kernels, while the proportions of other isolates decreased in co-inoculations with *F. graminearum*. While other isolates showed reduction depending on the composition of mixtures including *F. graminearum*. The proportionate content of fungal DNA in mixed inoculations indicated that the ability of the isolates to colonize kernels is different. The differences in virulence and competitive abilities of the isolates may explain differences in the ratios test on the spikelets and kernels, which decreased in the order *F. graminearum*, *F. culmorum*, *F. avenaceum* and *F. poae.*

The number of infected spikelets and wheat flower parts increased from anthesis through early ripening in inoculation either alone or in mixtures. The degree of increase was dependent on the isolates and composition of the mixtures and decreased in the order *F. graminearum*, *F. culmorum*, *F. avenaceum* and *F. poae*, respectively. Several factors may explain differences in the frequency of infected spikelets in three times sampling. After inoculation, part of the inocula is not able to establish and penetrate the host. This means that not all inocula have the ability to produce disease. This may imply differences in potential of inoculum which depends on cultivar resistance, defence mechanisms of the plant and plant-pathogen interactions. The disease-producing ability of *Fusarium* spp. on the abaxial and adaxial surfaces of wheat flower parts (susceptibility) is different. Differences in the development of *Fusarium* spp. on the abaxial and adaxial surfaces of wheat spikelet were observed by Kang and Buchenauer (2002) and Kang *et al*. (2005). They showed that relatively little hyphal network developed on the outer surface of the glume, lemma, palea and rachis and no direct penetration of the abaxial surfaces of the spikelet was observed 3-4 days after inoculation. The researchers also showed that penetration of host tissues occurred by infection hyphae on the inner surfaces of lemma, glume and palea, and on the upper part of the ovary. No infection was observed after inoculating the outer surface of glume using a conidia suspension of *F. graminearum* (Strange and Smith, 1971). This may explain differences in susceptibility of surfaces of wheat floret. The frequency of infected spikelets by low virulence isolates was reduced by co-inoculation with high virulence ones. This indicated existence of different degrees of competitive interactions between isolates in turn affected the frequency of infected spikelets from anthesis through early ripening. For example, there was a negative increase of *F. poae* in the presence of higher aggressive species in three-species combinations. In contrast, it has been shown that after initial infection, *Fusarium* species are able to move in vascular tissue and into rachis by hyphal growth inside of plant tissue. Kang and Buchenauer (2000) and Guenther and Trail (2005) showed that FHB pathogens reach the rachis and systemically grow upwards and downwards inter- and intra-cellularly in vascular bundles and cortical parenchyma tissue of the rachis. There was similarity in increase of *F. graminearum* infected spikelets from anthesis to ripening either when inoculated alone or in mixtures. This may be explained by high competitiveness of *F. graminearum* in the mixtures with other isolates and linked to high virulence to host plant and high amount of deoxynivalenol in the kernels. Trichothecenes production has been shown to contribute to virulence of *G. zeae* on head blight (Proctor *et al*., 2002). The mycotoxins have also been shown to play a role in hyphal growth of the pathogen from one infected floret to neighboring

ones through the rachis internodes. Jansen *et al*. (2005) showed that in the absence of trichothecene production, the *Fusarium* fungus is prevented in wheat to move into the rachis by the development of strong cell wall fortifications in the rachis node.

In both years, the frequency of infected wheat flower parts decreased in the order lemma, palea, glume and developing kernel, respectively. This is in agreement with reports from Kang and Buchenauer (2000), Kang *et al*. (2005) and Wanyoike *et al*. (2002), who showed differences in structure of epidermal cells of wheat florets. The adaxial surface of wheat glumes has thick-walled epidermal and hypodermal cells, while the adaxial epidermal cell walls of lemma and palea are thin-walled. They also showed that inside of the floret cavities greatly facilitates hyphal growth and penetration of the adaxial surfaces of lemma and palea by FHB pathogens. At flowering stage, the open floret (the extruded anthers and the crevices between palea and lemma) also present an avenue for FHB conidia to reach the interior of spikelets either under artificial inoculation or natural infection. Lewandowski and Bushnell (2001) showed that under warm and mist-irrigated field conditions, colonies of *F. graminearum* on the exterior surface of the palea (near the keel) and on interior surfaces of the palea and lemma facing the floret mouth serve as starting points for floret invasion.

While the spikelets demonstrated high frequency of infection with more than one *Fusarium* isolate, kernels showed low infection frequency, especially under greenhouse experiments. This indicated that in inoculations with multiple isolates, only one isolate is fast grower and resulted in kernel colonization. On the other hand, early interactions between or among the isolates occur during the infection process on different wheat flower parts and result in an increase in the single species colonization. The observation may show the aggregation of FHB pathogens on the spikelets. This is because of multiple isolates inoculations and spreading the infection to the neighboring ones, however the rate of infection depends on the weather conditions and composition of the mixtures. Xu *et al*. (2004) observed the aggregation of FHB infected spikelets on one wheat ear. They mentioned that aggregation of the inoculum landed on individual ears and subsequent colonization of healthy spikelets may explain the aggregation of FHB infected spikelets on one wheat ears. Overall, the presence of *F. poae* in the mixtures showed no or only a slight increase in infected spikelets and kernels at harvest over time, whereas mixtures with *F. avenaceum* resulted in the highest percentages of kernels colonized by two isolates. The ability to produce or negate inhibitors by the isolates as well as the speed of growth on wheat flower parts may explain differences in the frequency of infection with more than one isolates. For example, under stress conditions or multi-species inoculations the isolates shifted to produce varied secondary

metabolites or more mycotoxins. Xu *et al*. (2007) reported that the amount of mycotoxins dramatically increased when FHB pathogens were inoculated as mixtures.

In greenhouse experiments, kernel weight was greatly reduced in single or mixed inoculations, where *F. graminearum* was in mixture with the other isolates, for example, 50 % and 42 % reduction in single inoculations of *F. graminearum* compared to non-treated control plants in 2005 and 2006, respectively. However, in field experiments, inoculations with *Fusarium* species alone or in mixtures, hardly affected kernel weight. For example, in inoculations wheat ears with *F. graminearum* and its mixtures in most cases the kernel weight was similar with non-inoculated control plants, in contrast to high fungal biomass and frequency of infected kernel. Weather conditions, infection process, the content of fungal biomass and method of harvesting may explain differences in kernel weight under greenhouse and field experiments. For example, in inoculation with *F. graminearum* and its mixtures under greenhouse conditions, symptoms on the spikelets develop within 10 days after inoculation, while symptoms development under field experiments required more time. Under field conditions where harvesting was done using a combine harvester, kernels with low density were discarded unlike in greenhouse experiments where manual harvesting was applied thereby retaining low-weight-kernels that were highly colonized. This may also explain differences for fungal biomass under greenhouse and field experiments. The kernel weight of plants inoculated with *F. avenaceum* and/or *F. poae* was not significantly different from that of non-inoculated plants. Low fungal biomass production by these species under field and greenhouse conditions did not affect yield. Additionally, the mode of infection and colonization by these species may differ from the most virulent species (Doohan *et al*., 1999).

Comparison of frequency and intensity of kernel colonization proved that there exist differences in the virulence and development of the isolates in the kernels. For example, under greenhouse conditions in inoculation with one species, the frequency of infection ratios of *F. graminearum* was 1.34, 2.0 and 14.5 and the amount of its DNA was 1.67, 19.74 and 85.11 times higher than for *F. culmorum, F. avenaceum* and *F. poae*, respectively.

Overall, mycotoxin production dramatically increased in co-inoculations compared to inoculations with one isolate alone. This represented a significant increase in mycotoxin productivity (per unit of fungus) with a corresponding the reduction or no-reduction of the biomass of isolates in mixed inoculations under interactions.

F. graminearum had the highest ability of mycotoxin production under field conditions. Similar to other estimated parameters, the ability of mycotoxin production of the isolates decreased in the order *F. graminearum, F. culmorum*, *F. avenaceum* and *F. poae*.

This indicated the competitive ability and virulence of an isolate may be associated to mycotoxin production or the ability of mycotoxin production.

Although the low biomass of *F. poae* further reduced in mixed inoculations, its presence in mixtures increased ability of mycotoxins production by other isolates. This indicated the consequence of competitive interaction among the isolates resulted in negative and synergistic effects on the fungal biomass and mycotoxin production, respectively. This may be explained by the fact that under stress conditions the isolates may produce more mycotoxins, i. e. competition for nutrients and space. Mycotoxins are secondary metabolites, which suggest that where *Fusarium* is present, production of mycotoxins may be more important in acquiring and retaining an ecological niche.

This is true for the mixed inoculations with *F. avenaceum*, *F. poae* and *F. culmorum.* For other mixtures including *F. graminearum*, *F. avenaceum* and *F. poae*, the results indicated that in mixed inoculations the biomass of *F. graminearum* was similar to inoculation with this isolate alone, while the ability of mycotoxin production was increased. This means other isolates may have stimulatory or synergistic effects on mycotoxin productivity by *F. graminearum*. Previous studies showed that the effects of fungal interactions on mycotoxin production might result in decrease, increase or remain at constant level similar to the no-competition. For example, presence of *F. graminearum* in the mixtures inhibited enniatins and moniliformin production by *F. avenaceum* and in some mixtures, its presence resulted in no detection of moniliformin. The results are similar to the finding of Velluti *et al*. (2000), who revealed different behaviour in fumonisin B1 production by *F. moniliforme* and *F. proliferatum* in the presence of *F. graminearum*. They also mentioned that fumonisin B1 production by *F. proliferatum* was inhibited under all the conditions tested. On the other hand, *F. graminearum* was able to inhibit fumonisin B1 production by *F. moniliforme* at 15 ºC, while at 25 ºC fumonisin B1 was significantly increased.

Previous studies have shown that there is direct relation between aggressiveness of *Fusarium graminearum* and *F. culmorum* and their deoxynivalenol and nivalenol-producing capacity. It has been shown that more highly aggressive isolates produced more severe infection, more fungal biomass, more ergosterol and more deoxynivalenol (Alexander *et al*., 1997; Gang *et al*., 1998; Muthomi *et al*., 2000; Mesterházy, 2002). The results of Gang *et al*. (1998) indicated that head blight rating and deoxynivalenol content of grain were significantly correlated in the field experiments. Alexander et al. (1997) also mentioned that trichothecenes are not necessary for pathogenicity, although increase the extent of the disease.

It has been shown that deoxynivalenol was detectable 48 h after inoculation (Mirocha *et al*., 1997). The amount of deoxynivalenol in this very early phase of pathogenesis may be more important for differences in virulence at the very beginning of infection (Kang and Buchenauer, 2002; Maier *et al*., 2006). Mesterházy *et al*. (1999) reported that a close relationship was usually found between the aggressiveness of an isolate and its deoxynivalenol production.

It has been reported that population of *Fusarium culmorum* consists of different individuals or lines having different pathogenic characters including toxin-producing ability (Mesterházy, 2002). However, the deviations are not large and the very close correlations show the basic trends that is, higher isolate aggressiveness results in higher FHB severity, higher *Fusarium* damage kernel, higher yield loss and higher deoxynivalenol production. This may explain high virulence of *F. graminearum* and *F. culmorum* isolates which could be linked to their high disease severity, fungal biomass, frequency of infected kernels and amount of deoxynivalenol in the kernel samples.

Regarding increase in the amount of mycotoxins in co-inoculations, it can be stated that under field conditions or in most natural head blight epidemics where there are more than one isolate this should result in higher mycotoxin accumulation in grain.

Higher biodiversity within FHB pathogens living in the same ecological niche seems likely to reduce the overall resulting disease intensity; no synergism effects detected among *Fusarium* species infecting wheat kernels. However, previous studies showed that interaction may result in reduction of fungal biomass while the ability to produce mycotoxins can be varied causing either an increase or a decrease (Velluti, *et al*., 2000; Xu *et al*., 2007). Although these results do not explain the composition of the *Fusarium* head blight complex in the Rhineland, high frequency of the less virulent isolates of *F. avenaceum* and *F. poae* in the presence of *F. graminearum* and *F. culmorum* has been reported in various studies in the region. It is likely that a large spectrum of competitors for nutrients and space is less dangerous to food and feed production than the predominance of the highly virulent and competitive *F. graminearum* in other regions. The prevalence of *F. avenaceum* and *F. poae* may be due to specific inoculum sources, differences in optimum incubation conditions – temperature, relative humidity – and lower sensitivity to fungicides applied for the control of other diseases.

Although these two species have been reported in high frequency under field conditions, their biomass was found to vary from low to very low. Based on mycotoxin data it can be concluded that mycotoxin contamination of wheat is closely correlated to the *Fusarium*

biomass in kernels. The toxicity of mycotoxins produced by the *Fusarium* species, however, largely varies and it is known that other mycotoxins are more toxic to mammals than the well-known deoxynivalenol produced by *F. graminearum* and *F. culmorum* (Bhatnagar *et al*., 2002).

The present study demonstrated the existence of interactions between FHB pathogens on wheat ears. Competition for nutrients and infection sites played an important role in disease incidence, fungal biomass and as a consequence - mycotoxin production. This could be concluded from the reduction in disease severity, incidence and fungal biomass with a corresponding increase in the content of mycotoxins in kernels. However, it should be considered that these conclusions are drawn from studies based on single isolates of the four *Fusarium* species.

In addition to the present study, several studies showed that the *Fusarium* species varied in the spectrum and amount of mycotoxins, virulence to host plant and response to environmental conditions. It would be interesting to investigate intra- and inter-species interactions between or among FHB pathogens under different environmental conditions. Additionally, the effects of interactions among FHB pathogens on the amount and spectrum of mycotoxins produced on host plants with different genotypes should be investigated. It is also necessary to carry out studies on the possible interaction outcomes of high and low mycotoxin-producing isolates on wheat.

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6. Appendices

Table 41: Frequency of infected kernels by *Fusarium* spp. after two consecutive inoculations of wheat ears (cv. Drifter) with a 1-day interval at GS 65 (2005, FHB field)

The first day	The Second day	Infected kernels [%]			
		FG	FC	FA	FP
F. graminearum (FG)	FG	94.8 AB[1]			
	FC	51.1 GI	48.0 HI		
	FA	84.0 B-D		18.8 L-N	
	FP	81.1 CD			6.8 NO
F. culmorum (FC)	FG	24.9 KL	67.4 EF		
	FC		97.2 A		
	FA		82.6 B-D	20.0 LM	
	FP		78.0 DE		3.0 O
F. avenaceum (FA)	FG	35.6 JK	26.5 KL	43.4 IJ	
	FC		83.8 B-D	20.4	
	FA			81.7 CD	
	FP			56.9 F-H	3.5 O
F. poae (FP)	FG	84.5 B-D			0.5 O
	FC		92.4 A-C		5.2 O
	FA			82.0 B-D	1.5 O
	FP				14.5 L-O
FG	Water	75.6 DE			
FC			76.1 DE		
FA				62.3 FG	
FP					11.0 M-O

Inoculation with 91,000 conidia ml $^{-1}$ in two individual consecutive days

Wheat ears with FHB symptoms were randomly sampled

[1] Means were separated by independent multivariate analyses for isolates (Duncan test, $p \leq 0.05$)

FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

Table 42: Content of fungal DNA in wheat (cv. Drifter) kernels after two consecutive inoculations with a 1-day interval with *Fusarium* spp. at GS 65 (2005, FHB field)

The first day	The Second day	Fungal biomass [ng/mg kernel dry weight]			
		FG	FC	FA	FP
F. graminearum (FG)	FG	75 A[1]			
	FC	13 F	6 HI		
	FA	44 CD		1 K-M	
	FP	20	1 J-M		0.0 M
F. culmorum (FC)	FG	8 GH	11 FG		
	FC		23 E		
	FA		5 IJ	0.4 LM	
	FP		4 I-L		1 J-M
F. avenaceum (FA)	FG	55 B		2 J-M	
	FC		2 J-M	0.3 M	
	FA			3 I-M	
	FP			0.3 M	1
F. poae (FP)	FG	46 C			2 J-M
	FC		3 I-M		2 J-M
	FA			0.4 LM	0.0
	FP				1 K-M
FG	Water	42 D			
FC			8 GH		
FA				1 K-M	
FP					1 K-M

Inoculation with 91,000 conidia ml $^{-1}$ in two individual consecutive days

Wheat ears with FHB symptoms were randomly sampled

[1] Means were separated by independent multivariate analyses for isolates (Duncan test, $p \leq 0.05$)

FG, *F. graminearum* isolate 5.1; FC, *F. culmorum* isolate 3.11; FA, *F. avenaceum* isolate 1.7; FP, *F. poae* isolate 7.8

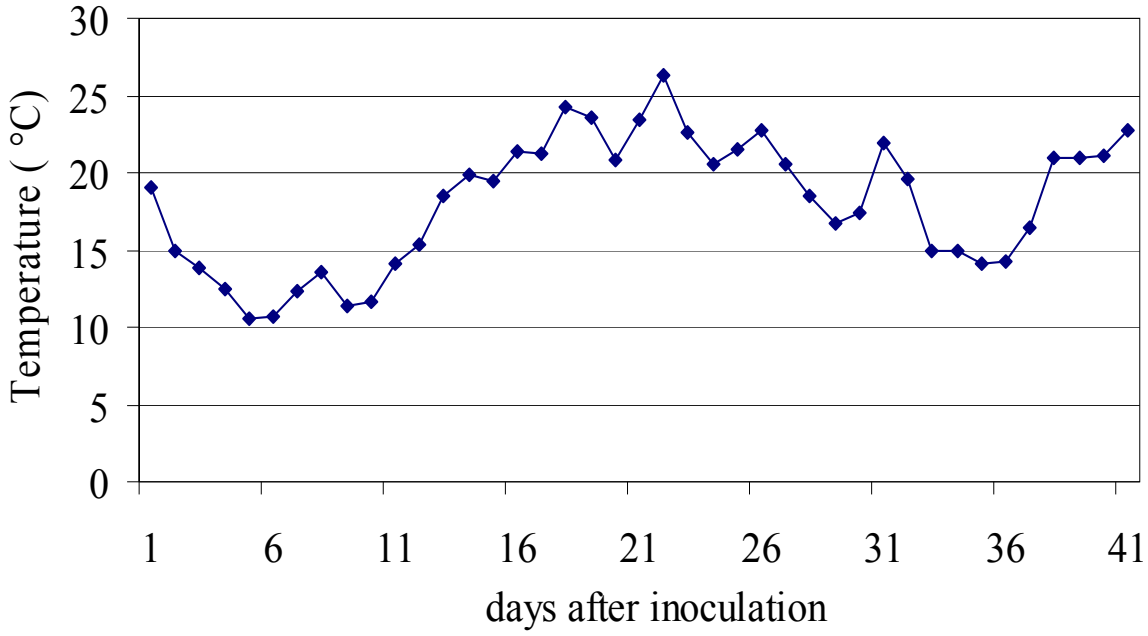

Figure 16: Mean daily temperature at the Poppelsdorf experimental station, University of Bonn, Germany, in 2005

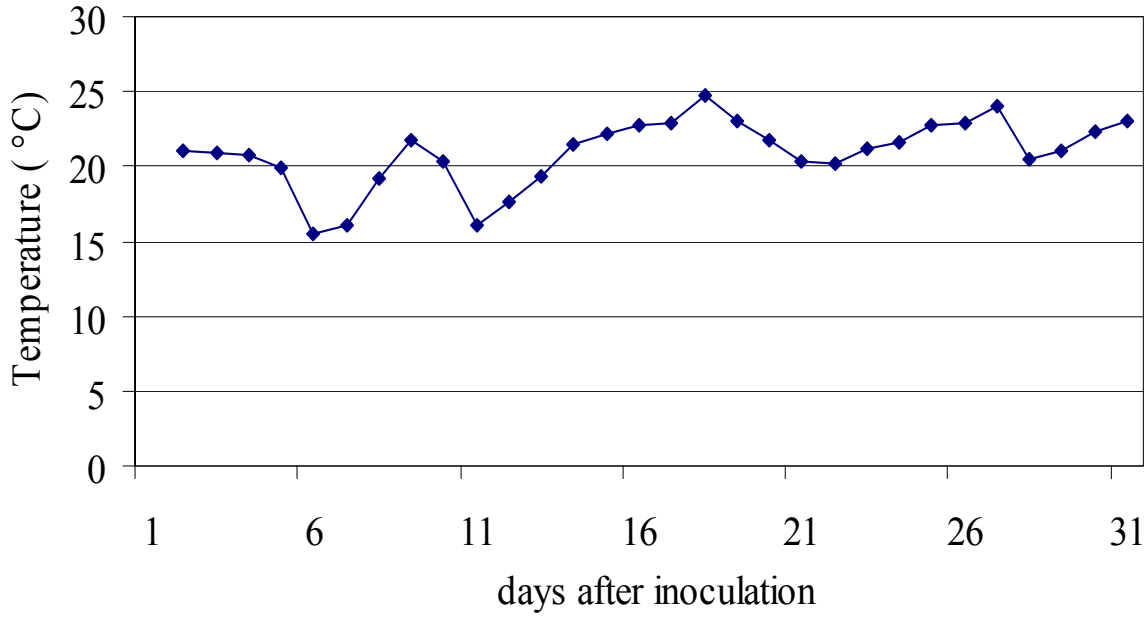

Figure 17: Mean daily temperature at the Poppelsdorf experimental station, University of Bonn, Germany, in 2006