Colonization of maize with *Fusarium* spp. and mycotoxin accumulation

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Chapter 1: General Introduction

Ear rots caused by fungi belonging to the species *Fusarium* is one of the most important diseases in maize worldwide. High losses of yield and quality are regularly reported in the last decades (D'Mello et al., 1999, Yiannikouris and Jouany 2002, Zimmer et al., 2008).

Infection of maize cobs with *Fusarium* spp. does not only cause yield losses, the highest damage is resulted by the ability of accumulation of mycotoxins (Logrieco et al., 2002). Mycotoxins are secondary metabolites, which are produced by different fungi. These substances lead to serious health damages or death in humans and animals. Outbreaks of diseases caused by intoxication of humans and livestock were consistently reported (Ross et al., 1992, Massart & Saggese, 2009). Humans can not only be affected by consuming contaminated maize products, some mycotoxins can also contaminate animal based products (D'Mello et al., 1999). Caused by this risks, the European community fixed a legal limit for the most important *Fusarium* mycotoxins, which are deoxynivalenol (DON), zearalenone (ZEA), fumonisins (sum of B1 and B2), as well as T-2 and HT-2 toxin (sum of T-2 and HT-2 toxin) (The Commission of the European communities, 2006).

There are two types of ear rots known, Red ear rot, caused by Fusaria belonging to the Discolor section, and Pink ear rot, caused by Fusaria belonging to the *Liseola* section (Munkvold 2003). *Fusarium graminearum* is the fungus mostly isolated from ears infected with Red ear rot (Logrieco et al., 2002), which can produce trichothecenes and ZEA (Richardson et al., 1985). Trichothecenes like DON and nivalenol (NIV) cause feed refusal, vomiting and reduced weight gain in swine and reduce the immune system in both livestock and humans (Korosteleva et al., 2009, Yazar & Omurtag 2008). ZEA is an estrogenic mycotoxin. It leads to fertility problems in sows (Kanora & Maes, 2009) and is suspected to cause precocious pubertal development in prepubertal exposed girls (Massart & Saggese, 2009). Pink ear rot is mainly associated with the occurrence of *Fusarium verticillioides* and *F. proliferatum*, and, to a lesser extend, *F. subglutinans* (Logrieco et al., 2002). *F. verticillioides* and *F. proliferatum* produce various toxins including fusaric acid, fusarins and fumonisins, while *F. proliferatum* and *F. subglutinans* are both producers of the mycotoxin moniliformin (Logrieco et al., 2002). Twenty-eight different types of fumonisins are known, which are divided in four groups A, B, C, and P series (Rheeder et al., 2002). Fumonisin B1 and B2 are the most important ones. The

levels of fumonisins B1 and B2 correlated with the incidence of esophageal cancer in South Africa (Sydenham et al., 1991, Marasas, 2001). Fumonisin B1 is known to be responsible for leukoencephalomalacia in horses and pulmonary edemas in swine (Hussein & Brasel, 2001). It is very likely that fumonisins B2 and B3 have the same effects. Moniliformin caused intoxication in broiler chicks (Ledoux, 2003), could be correlated with acute death in ducklings (Vesonder & Wu, 1998) and rats (Abbas et al., 1990), and was mutagenic in primary cultures of rat hepatocytes (Knasmüller et al., 1997).

Both types of ear rots are going along with accumulation of mycotoxins, which are highly toxic to humans and animals. Because the reduction of *Fusarium* infestation and as a consequence reduction of mycotoxin exposure with chemical tools is limited, avoidance of fungal infestation in the field is the method of choice.

Epidemiology of Red ear rot (*Gibberella* ear rot, GER)

Red ear rot primary occurs on the tip of the cob, developing a pink to reddish mycelium, which covers big parts of the cob. Sometimes the brownish perithecia can additionally be observed on infected husks. Perithecia are the teleomorphic form of *F. graminearum*, *Gibberella zea* (Logrieco et al, 2002), which is the main causing agent of Red ear rot in Europe. Next to this fungus, *F. culmorum* can also induce this disease (Logrieco et al. 2002, Munkvold, 2003).

F. graminearum forms chlamydospores, which overwinter in plant debris. Chlamydospores germinate in spring and produce perithecia, which leave ascospores in the air. This is the most important dispersal structure (Munkvold, 2003). Beside ascospores, macroconidia formed in sporodochia and hyphal fragments from host tissue and host debris are important inoculum sources (Sutton, 1982). Ascospores reach the silks via wind, while the macroconidia, which are the asexual dispersal structures, are mainly dispersed via splashes (Sutton 1982). Insect transmission was also observed. The spores germinate on the silks, form a germination tube and grow through the silks to the cob. The highest susceptibility was reported one to six days after silk emergence (Reid et al., 2002). GER mostly occurs in the cooler regions of Europe. High humidity during silk emergence, followed by rainfall during kernel development enhances the development of *Fusarium graminearum* and accumulation of mycotoxins (Sutton 1982).

The role of mycotoxins accumulated by *F. graminearum* is not completely understood. While DON seems to be a pathogenicity factor in wheat, which is especially important for the spread of the fungus from one spikelet to the next (Bai et al., 2001), results concerning the role of trichothecenes in the pathogenesis of maize are inconsistent. Harris et al. brought out in experiments with DON producing wild type strains and knock-out mutants of the TRI5-Gene that DON production is not essential for *F. graminearum* to infect maize tissues. Nevertheless DON-nonproducing strains of *F. graminearum* were usually less virulent than the DON producing strains (Harris et al., 1999). NIV-producer seem to be more aggressive in maize than DON-producer (Carter et al., 2002). Maier et al. also observed in experiments with knock-out mutants of the TRI5-Gene that disruption mutants of NIV producing strains were significant less aggressive than the wild type, while they could not find differences in aggressiveness of DON producing strains between wild type and knock-out mutants (Maier et al, 2006). In contrast, ZEA is known to occur relatively late in the pathogenesis (Doohan et al, 2003), so it does not seem to be a pathogenicity factor. In addition, the role of ZEA in the pathogenesis of GER remains unclear.

Epidemiology of Pink ear rot (*Fusarium* ear rot, FER)

Pink ear rot typically occurs on kernels randomly distributed on cobs as lightly pinkish mycelium. This disease is mainly caused by *Fusarium verticillioides* and *F. proliferatum*, but also *F. subglutinans* can be the causal agent (Munkvold, 2003). *F. verticillioides* occurs ubiquitous and is able to colonize maize plants systemically and asymptomatically (Reid et al., 1999, Munkvold, 2003).

F. verticillioides, F. proliferatum and *F. subglutinans* are heterothallic species, but in contrast to *F. graminearum*, sexual reproduction does not play a role as an important dispersal. *F. verticillioides* forms thickened hyphae, which can overwinter in plant debris. The most important inoculum for FER and also symptomless kernel infection are large amounts of the asexual microconidia and macroconidia formed in infected crop residues (Munkvold, 2003). They are translocated via wind, rain or insect transmission on the cobs. Silk infection seems to be the most important pathway for the fungus to enter the plant (Munkvold et al., 1997b). Insects do not only play a role for translocation of spores, but they injure the plants by feeding on it. These injuries can act as portals of entry for the fungus and therefore support colonization of the plants, as it is known for

the European corn borer (*Ostrinia nubilalis*) and other insects (Munkvold et al., 1997b, Farrar & Davis, 1991, Papst et al., 2005). *F. verticillioides* can also infect systemically from infected plant debris in the soil, and is also a seedborne pathogen (Munkvold et al., 1997b, Bacon & Williamsen, 1992). FER typically occurs in warmer, dryer areas (Munkvold, 2003, Miller, 2001, Munkvold et al., 1997a).

Pink ear rot pathogens have a wide range of host plants. Beside maize (Chulze, 1996), *F. proliferatum* as well as *F. verticillioides* are also able to colonize sorghum (Gonzalez, 1997), rice (Abbas et al., 1998, Wulff et al., 2010) wheat (Leslie 1992, Bottalico & Perrone, 2002), and asparagus (Logrieco, 1998, Elmer, 2000). Additionally, *F. proliferatum* was found on cotton (Osekre et al, 2009), onions and garlic (Stankovic et al., 2007), while *F. verticillioides* infected bananas (Mirete et al., 2004). Despite *F. proliferatum* plays an important role in the FER complex, and is found regularly together with *F. verticillioides* on symptomatic ears, little is known about the epidemiology and ecology of this fungus. Most of the work about epidemiology and ecology is done on the species *F. verticillioides*. Up to ten years ago, many authors still misidentified the species *F. verticillioides* as *F. moniliforme*. Furthermore the species called *F. moniliforme* sometimes included also the species *F. proliferatum* and others (Seifert et al., 2003). Because *F. verticillioides* and *F. proliferatum* share many morphological characteristics, it can be speculated that they also share a similar disease cycle (Munkvold & Desjardins, 1997).

The relationship between the development of symptoms, the fungal growth and the mycotoxin accumulation is still unclear. Ramirez et al. observed no correlation between fumonisin contamination and the level of infection with *Fusarium* species (Ramirez et al., 1996). In contrast, Pascale et al. found a good correlation-coefficient of 0.933 and 0.989 between ear rot symptoms and mycotoxin concentration after inoculation with *F. verticillioides* and *F. proliferatum*, respectively (Pascale et al., 2002). Fumonisin B1 does not seem to have any influence on plant development, when placed on maize seedlings (Bacon & Williamson, 1992). Indeed, FB1-nonproducing strains were able to infect ears after inoculation via injection in the silk channel, and produced high levels of ear rot (Desjardins & Plattner, 2000). Reid et al. found 1999 that *F. verticillioides* is able to colonize plants, which where artificial inoculated with *F. graminearum*, and to suppress this fungi. The accumulation of fumonisins was not higher than after inoculation with *F. verticillioides* alone. Therefore fumonisins even do not seem to be produced to suppress competitive fungi.

Diagnostic tests of ear rot causing pathogens and estimation of their performance

Several *Fusarium* species are known to be involved in ear rot. Detailed knowledge of the amount and the *Fusarium* species in host tissue is necessary for studies of interactions between different *Fusarium* species inside the host tissue, studies concerning the mycotoxin productivity and studies of the natural distribution of *Fusarium* species. Ergosterol analysis is a helpful tool to quantify the total amount of fungal biomass growing in plants, but it does not distinguish between the single fungal species (Seitz et al., 1979). In contrast, plating surface sterilized plant tissue on agar plates does not give any knowledge of the severity of the fungal colonization. Furthermore, distinction between the *Fusarium* species on the basis of morphological criteria is often difficult and requires wide experience.

PCR with species-specific primers allows the qualitative detection of pathogens in plant material. Real-time PCR offers the possibility to observe the dynamic of the amplification process. The PCR cycle in which the PCR reaction reaches the exponential phase allows the determination of the starting quantity of target DNA (Schena et al., 2004). For fumonisin producing fungi, many PCR assays are developed (Murillo et al., 1998, Möller et al., 1999, Patino et al., 2004). During previous works, species-specific real-time PCR assays were established for *F. verticillioides* using primers developed 2004 by Mulè et al. (Nutz, 2006), as well as for *F. proliferatum* using primers designed 2006 by Jurado and co-workers (Döll, 2008).

In most publications, sensitivity of a Real-time PCR assay is defined as LOD (limit of detection), given in a certain amount of target DNA per reaction. Mainly, these values are empirically derived. However, Real-time PCR performs different from time to time, and these values may vary in different PCR runs. There are various methods to define a cut-off value, which is the ct-value above which a result is counted as negative. Calculating the mean plus two standard deviations (2SD) of the negative reference sample is a common method, but it does not respect the optimal ratio between sensitivity and specificity (Greiner et al., 2000).

The ROC curve analysis evaluates the performance of a diagnostic test, and the cut-off value can be evaluated which proofs the highest sensitivity while highest specificity is achieved. The aim of the ROC-curve analysis is to compare the differentiation between "diseased" and "not diseased" samples with results obtained with a second, well established test method, which is called “gold standard”, or with spiked samples.

The sensitivity is defined as the number of known positive samples, which are also detected positive at a certain PCR-cycle (Turechek et al., 2007). The specificity is defined as known negative samples, which are detected negative at a certain PCR cycle. Negative samples, which are detected as positive, the "false positive", are designated 1-specificity. The cut-off value is the PCR cycle above which a sample is considered to be negative (Turechek et al., 2007). In a reaction incorporating 40 PCR-cycles, 40 cut-off points are possible. If, for example, cycle 30 is chosen as cut-off point, the fraction of real positive samples which crossed the threshold cycle until PCR cycle 30 are the "true-positive-fraction". Negative samples, which are crossing the threshold line before the cut-off cycle 30, are the “false positive fraction” (Turechek et al., 2007). A higher cut-off point cycle, for example cycle 31, may lead to more known positive detected samples, which increases the sensitivity. On the other hand, the risk also increases that more false positive samples are detected. If this is the case, the specificity decreases.

This emphasizes that determination of the optimal cut-off cycle is crucial for obtaining the best discrimination between diseased samples and healthy samples.

Aims of the study

The goal of this work is to improve the knowledge of the coherences between the accumulation of mycotoxins in maize plants, development of fungal biomass and development of ear rot symptoms.

A reliable method for quantification of fungal biomass in different maize tissues was needed. Species-specific PCR-assays for *F. verticillioides* (Nutz, 2006) and *F. proliferatum* (Döll, 2008) were already established in previous works, and their performance in application to field samples was estimated using ROC-curve analysis.

Secondly, the distribution of mycotoxins and fungal biomass of different maize genotypes with *F. graminearum* had to be investigated. Special attention needed to be paid to the different harvest time points at silage and grain harvest to assess the risk of silage contamination resulting from contaminated stalks due to ear rot. Additionally, it should be investigated if different maize genotypes behave different in their ability to translocate fungal biomass and mycotoxins.

The third aim was the investigation of the dynamic of fungal growth and mycotoxin accumulation in maize in the field. Therefore a time course needed to be carried out,

with harvest time points every second week. This experiment was carried out with the two main ear rot causing pathogens in Europe, *F. verticillioides* and *F. graminearum* and with a mixture of both.

The adjusted data should be presented to the project partners in the “CEREHEALTH” consortium, a cooperative project founded by the Federal Ministry of Education and Research. The goal of this project was to develop new resistance breeding strategies against *Fusarium* head blight of wheat, *Fusarium* ear rot of maize and *Septoria tritici* blotch in wheat. It should be searched for combined resistence QTL and candidate genes for different pathosystems of the same crop and from different crops (broad-spectrum resistance). The best QTL or loci should be directly applied in the breeding programs of the industrial partners.

Lastly, the influence of different cropping systems on the infestation of maize with *Fusarium* spp. and the accumulation of mycotoxins was investigated. Twenty-five different maize genotypes were grown in three years on different locations under organic and conventional conditions, and the mycotoxin contend was assessed as well an the symptoms and the fungal biomass, to investigate which cropping system lead to reduced risk of mycotoxin contamination.

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Chapter 2

Determination of the LOQ in real-time PCR by receiver operating characteristic curve analysis: application to qPCR assays for *Fusarium verticillioides* and *F. proliferatum**

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Abstract

Real-time PCR (qPCR) is the principal technique for the quantification of pathogen biomass in host tissue, yet no generic methods exist for the determination of the limit of quantification (LOQ) and the limit of detection (LOD) in qPCR. We suggest to use Youden index in the context of the receiver operating characteristic (ROC) analysis for this purpose. LOQ was defined as the amount of target DNA that maximizes the sum of sensitivity and specificity. LOD was defined as the lowest amount of target DNA that was amplified with a false-negative rate below a given threshold. We applied this concept to qPCR assays for *Fusarium verticillioides* and *Fusarium proliferatum* DNA in maize kernels. Spiked matrix and field samples characterized by melting curve analysis of PCR products were used as the source of true positives and true negatives. Based on the analysis of sensitivity and specificity of the assays, we estimated the LOQ values as 0.11 pg of DNA for spiked matrix and 0.62 pg of DNA for field samples for *F. verticillioides.* The LOQ for *F. proliferatum* were 0.03 pg for spiked matrix and 0.24 pg for field samples. The mean LOQ values correspond to approximately 8 genomes for *F. verticillioides* and 3 genomes for *F. proliferatum*. We demonstrated that the ROC analysis concept, developed for qualitative diagnostics, can be used for the determination of performance parameters of quantitative PCR.

Keywords Real-time PCR - *Fusarium verticillioides* - *Fusarium proliferatum* - Receiver Operation Characteristic - limit of detection - limit of quantification

Introduction

Real-time PCR (qPCR) is the standard analytical method for quantifying pathogen biomass in the tissue of host organisms. Standard performance parameters of an analytical method are the limit of detection (LOD) and the limit of quantification (LOQ). The LOD is defined as the lowest amount of the analyte detectable in a single reaction. The LOQ is the lowest amount of analyte that can be quantified. The methods commonly used in chemical analysis for determining LOD and LOQ values [1 - 3] are unsuitable for qPCR.

We suggest that the LOD and LOQ can be determined by use of receiver operation characteristic (ROC) curve analysis, which is a method used to evaluate the sensitivity and specificity of diagnostic tests. ROC is based on a comparison of the outcome of a series of assays ("positive" and "negative") with the "true" status of the samples. The "true" status is either evaluated with a well-established test, which is called the "gold standard", or it is known a priori because the samples were prepared by spiking negative matrix with the target analyte. The central concept in ROC curve analysis is the cut-off point. The cut-off point is athreshold value of the analytical signals below which samples are regarded as negative and above which samples are regarded as positive. The ROC curve is a plot of the sensitivity (genuinely positive samples that are detected as positive, "true positives") against one minus specificity (negative samples that are detected as positive, "false positives") for different cut-off points [4]. In qPCR, the cut-off point is the threshold cycle above which a sample is considered to be negative. If a cycle number is chosen as a cut-off point, the fraction of positive samples that reached the threshold of fluorescence intensity before this cycle is the "true positive fraction". The fraction of negative samples that reached the threshold of fluorescence intensity before this cycle is the "false positive fraction". If a higher cycle number is chosen as a cut-off point, more samples are likely to be rated as positive, increasing the sensitivity. At the same time, the false positive rate is likely to grow and the specificity to decrease. An optimal cut-off point corresponds to the desired trade-off between true positive and false negative rates. To balance the demands for sensitivity and specificity

of a diagnostic assay, i.e., to determine the optimal cut-off point, Youden index is often used [23].

Using artificially prepared, spiked samples for estimating an optimal cut-off value guarantees that the assignment of samples to true positives and true negatives iscorrect. The drawback is that the properties of matrix spiked with target DNA may differ from the properties of samples obtained from the field. The optimal cut-off point determined with the help of spiked samples may therefore differ from the optimal cut-off point for field samples. In the current research, we investigated this dilemma by assigning field samples to true positive and true negative by melting curve analysis. We than compared cut-off values derived for field samples with those obtained for spiked matrix. As a model system, we used the fungal plant pathogens *Fusarium verticillioides* and *Fusarium proliferatum* in maize kernels.

Fusarium species are among the most important pathogens of maize worldwide. Infection with *Fusarium* spp. reduces grain yield and quality [5], and infected grain, when used for the production of food and feedstuff, is often contaminated with mycotoxins that endanger the health of consumers and livestock [6]. Illness of farm animals and less frequently of humans caused by *Fusarium* mycotoxins has regularly been reported [7 - 9].

Fusarium species cause two types of ear rot in maize: red ear rot (*Gibberella* ear rot) caused by *Fusarium* spp. belonging to the Discolor section, and pink ear rot (*Fusarium* ear rot or ear mold) caused by species of the Liseola section. *Fusarium* species isolated from cobs exhibiting pink ear rot symptoms are usually *Fusarium verticillioides*, *F. proliferatum*, and *F. subglutinans* [5]. Apart from maize [10] and asparagus [11], *F. proliferatum* has been found in wheat [12], sorghum [13], and rice [14], but only infection of the first two crops is considered economically relevant. *F. verticillioides* and *F. proliferatum* are producers of fumonisin mycotoxins. Fumonisins B1 (FB1) and B2 (FB2) are the most abundant fumonisins in maize, and levels of FB1 are generally higher than those of FB2 [15]. FB1 causes leukoencephalomalacia in horses and pulmonary edemas in swine [16], and it is very likely that fumonisins B2 and B3 have the same effects. Although toxicologically relevant amounts of fumonisins in maize are occasionally found in food products in countries with a highly developed agriculture, serious health impacts of fumonisin contamination are thought to occur in areas with suboptimal growing and storage conditions and a high maize consumption [17]. Indeed, levels of FB1 and FB2 in maize used as staple food in South Africa correlated with the

incidence of esophageal cancer [18]. Beside fumonisins, *F. verticillioides* produces the mycotoxins fusaric acid and fusarins, while *F. proliferatum* was reported to produce mycotoxins beauvericin, enniatins, fusaproliferin, and moniliformin [19].

The relationship between the development of symptoms, the amount of fungal biomass in the plant tissue, and the production of mycotoxins is incompletely understood. Ramirez [20] found that fumonisin contamination and the level of infection for *Fusarium* species of the Liseola-section did not correlate. In contrast, Pascale [21] found that fumonisin contamination was highly correlated with earrot symptoms after inoculation of maize with *F. verticillioides* or *F. proliferatum*. Clarifying the relationship between the accumulation of fungal biomass in the plant, development of symptoms, and mycotoxin production requires a speciesspecific method to reliably quantify *F. verticillioides* and *F. proliferatum* biomass in plant tissue.

Real-time PCR is useful for quantifying fungal colonization of crops while distinguishing among species. Species-specific PCR primers have been developed for most *Fusarium* species that cause ear rot (e.g., [22 - 26]).

In this work, we evaluate qPCR assays for quantification of *F. verticillioides* and *F. proliferatum* in maize kernels. Furthermore, we examine the use of the Youden index in the framework of ROC curve analysis for estimating the LOD and LOQ of qPCR assays.

Material and Methods

Fungal cultures

Fungal strains we used are listed in Table 1. Fungal cultures for DNA extraction were grown in 100 ml potato dextrose broth (PDB, Scharlau, Barcelona, Spain, 24 g l^{-1}) at room temperature without shaking. The mycelium was harvested after 14 days by filtration and freeze-dried.

DNA isolation from pure fungal cultures grown in liquid media

A variant of the CTAB method as described by [27] was used, and the quality and quantity of DNA was estimated by electrophoresis in 0.8 % (w/v) agarose gels (Cambrex, Rockland, ME, USA) prepared in TAE buffer (40 mM Tris, 1 mM EDTA, pH adjusted to 8.5 with acetic acid). The electrophoresis was carried out at 4 V cm-1 for 90 min. The gel was stained with ethidium bromide (2 mg l-1) and documented with a

digital imaging system (Vilber Lourmat, Marne la Vallee, France). The densitometry was performed using Multi Analyst-Software (BioRad, Hercules, CA, USA). The concentration of fungal DNA was calculated by comparing a dilution series with defined amounts of DNA of lambda phage (methylated, from *Escherichia coli* host strain W3110).

DNA extraction from maize field samples

Maize kernels were dried at 60°C for 24 h and ground in a cross hammer mill (Cross Beater Mill SK 1; bottom sieve 1 mm; Retsch, Haan, Germany). The DNA extraction from 1 g of maize meal was carried out following an upscaled protocol for DNA extraction from plant material as described by Brandfass [28]. The quality and concentration of DNA were determined by agarose gel electrophoresis as described above. Total DNA from 1 g of starting material was dissolved in 200 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH adjusted to 8.0). The DNA solution was diluted tenfold, and 1 µl was used as template for each reaction.

Primers

The primers used for *F. verticillioides* were VER1 (CTTCCTGCGATGTTTCTCC) and VER2 (AATTGGCCATTGGTATTATATATCTA), which were designed by Mulè et al. [25] based on the coding sequence of the calmodulin gene; these primers amplify a DNA fragment of 587 bp. The primers used for *F. proliferatum* were Fp3-F (CGGCCACCAGAGGATGTG) and Fp4-R (CAACACGAATCGCT TCCTGAC), which were designed by Jurado et al. [26] based on the intergenic sequence of the ribosomal RNA gene cluster; these primers amplify a DNA fragment of 230 bp.

Real-time PCR assays

The optimized conditions for qPCR assays were as follows: The reaction mixture for *F. verticillioides* (25 µl) contained reaction buffer amended with NH4 (67 mM Tris-HCl, 16 mM (NH4)2SO4, 0.01% (v/v) Tween-20, pH 8.8 at 25°C; Bioline, Luckenwalde, Germany), 2.5 mM MgCl2, 0.1 mM of each of the four deoxynucleoside triphosphates (Bioline, Luckenwalde, Germany), 0.3 µM of each primer, 0.75 units of Taq DNA polymerase (BIOTaq, Bioline, Luckenwalde, Germany), 10 nM fluorescein (used for the calculation of well factors, see below), 0.1x SYBR Green I (Invitrogen, Karlsruhe,

Germany), and 1 µl of template DNA. The reaction mixture for *F. proliferatum*-specific PCR was identical except for the following components: 2 mM MgCl2, 0.6 µM of each primer, and 0.4 units of Taq DNA polymerase.

Real-time PCR was performed in an iCycler thermocycler (BioRad, Hercules, CA, USA). The amplification for *F. verticillioides* consisted of an initial denaturation at 95°C for 1.5 min, during which the well factors were collected (compensation for differences among optical properties of individual wells), followed by 40 cycles of 50 s denaturation at 94°C, 50 s annealing at 62°C, and 1 min elongation at 72°C. The final elongation step was performed for 7 min at 72°C. Fluorescence was measured in each cycle during the annealing phase. Melting curve analysis was performed after each PCR: Samples were heated to 95°C for 1 min, cooled to 55°C for 1 min, and heated to 65°C, and subsequently the temperature was ramped up from 65°C to 95°C in steps of 0.5°C each 10 sec. Fluorescence was measured at each step.

The PCR for the quantification of *F. proliferatum* DNA was performed according to the following protocol: Initial denaturation for 1.5 min at 95°C; followed by 35 cycles with 35 s at 95°C, 30 s at 64°C, and 30 s at 72°C, with fluorescence measurement during the annealing step of each cycle; and a final elongation of 5 min at 72°C. The melting curve analysis was performed as described above.

Calibration curves and PCR efficiency

Dilution series were prepared containing purified fungal DNA in amounts of 0.05, 0.15, 0.5, 1, 5, 10, and 50 pg mixed with maize DNA. For *F. proliferatum*, two additional standards (1.5 pg and 15 pg of fungal DNA) were used. Every set of standards was analyzed 10 times. Standard curves were generated by plotting threshold cycle (Ct) values against the logarithm of starting DNA quantities. The slopes of the standard curves were used to calculate the reaction efficiency *E* of PCR assays, using the following equation:

$$E = [10(-1/\text{slope})] - 1$$

These samples were also used as spiked positive samples for ROC curve analysis (see below).

Specificity of PCR primers

The specificity of both PCR assays was determined with DNA extracted from pure cultures of 81 fungal isolates (14 *Fusarium* species and 20 isolates of 12 other fungal

species, Table 1). Samples were classified as positive when the melting point was identical with the melting point of the standard with a tolerance of 0.5°C.

Sensitivity, specificity, ROC curves and optimal cut-off points

ROC-curve analysis was used for estimating the performance of qPCR assays [29]. ROC curves were constructed as plots of sensitivity versus (1 - specificity) for a set of positive and negative samples. Sensitivity (Se) is the fraction of true positive samples that score positive. *Se* was calculated for each PCR cycle by dividing the number of true positive samples with equal or lower Ct value by the total number of true positive samples. Specificity (Sp) is the fraction of true negative samples that score negative. *Sp* was calculated for each PCR cycle by dividing the number of true negative samples with higher or equal Ct value by the total number of true negative samples. ROC curves show the relationship between sensitivity and specificity. They facilitate visual evaluation of the performance of an assay. The area under a ROC curve can be regarded as an aggregate quality indicator for a diagnostic assay.
Youden index *J* is defined as [29]:

$$J = Se + Sp - 1$$

The optimal cut-off point is the PCR cycle with the highest value of Youden index:

$$\text{optimal cut-off point} = \max ct \{J\}$$

Samples with a threshold cycle (Ct) larger than the chosen cut-off point are classified as negative while samples with threshold cycle lower than the cut-offpoint are classified as positive [30]. ROCs, areas under ROC curves (AUROC), and Youden indices were calculated with the ROC-module of the package "Sigma Plot 11.0" (Systat Software, Inc., San Jose, USA). The same software was used to generate graphics.

Determination of LOQ and LOD

LOQ was determined as the amount of DNA corresponding to the threshold cycle at which the sum of specificity and sensitivity of the assay was maximized. For this purpose, Youden index *J* was calculated for each PCR cycle. The cycle for which *J* reached the maximum was selected as the optimal cut-off point. LOQ was determined as DNA amount corresponding to the optimal cut-off point in the calibration curve.

LOD was determined as DNA amount corresponding to the threshold cycle at which at most 5% of true positive samples scored negative (*Se* of 0.95).

Determination of mycotoxin production

Polished rice (25 g) and 35 ml of tap water were autoclaved in 100-ml Erlenmeyer flasks and inoculated with a 100-µl spore suspension of the fungal strains. The cultures were incubated at 25°C for 2 weeks. A 4-g portion of the colonized substrate (water content 15–20%) was extracted with 40 ml of acetonitril. A 1-ml volume of the extract was dried in a vacuum, and the residue was dissolved in 1 ml of methanol/water (1:1), defatted with 1 ml of cyclohexane, and diluted 20- times with methanol/water (1:1). HPLC was performed on a reverse-phase C18 column (Kinetex, 50.0 x 2.1 mm, particle 2.6 µm; Phenomenex) with a gradient of methanol in water with 7 mM acetic acid at flow rate of 0.2 ml/min. The analytes were ionized by electrospray and detected by MS/MS with an ion trap detector (500 MS, Varian, Darmstadt, Germany).

Table 1 Fungal strains used in this work. Source code: A: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; B: Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; C: E. Möller, University of Hohenheim, Germany; D: H. Nirenberg (BBA Berlin, Germany) via E. Möller, University of Hohenheim, Germany; E: Department of Crop Sciences, University of Göttingen, Germany; F: International Center for Agricultural Research in the Dry Areas, Aleppo, Syria; G: J.F. Leslie (Kansas State University, Manhattan) via E. Möller, University of Hohenheim, Germany; H: National Institute for Agricultural Research, Paris, France; I: Th. Miedaner (State Plant Breeding Institute) via E. Möller, University of Hohenheim, Germany; J: FRC Pennsylvania USA IA; K: A. Desjardins, USA, Mexico, via E. Möller; L: E.J.A. Blakemore, via E. Möller; M: Mykothek FAP (W. Winter), via E. Möller N. A. Szecsi, Budapest, Hungary via E. Möller; O: Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, via E. Möller, University of Hohenheim, Germany; P: P. Battilani, Faculty of Agriculture, Università Cattolica del Sacro Cuore, Piacenza, Italy, via T. Miedaner, State Plant Breeding Institute, University of Hohenheim, Stuttgart, Germany.

Species	Strain	Source*
Fusarium acuminatum	ICARDA 93803	F
Fusarium acuminatum	ICARDA 92099	F
Fusarium acuminatum	ICARDA 93682	F
Fusarium acuminatum	ICARDA 93831	F
Fusarium avenaceum	Fa95	C
Fusarium avenaceum	Fa23	E
Fusarium avenaceum	Fa21	E
Fusarium avenaceum	Fa39	E
Fusarium avenaceum	Fa5-2	E
Fusarium avenaceum	Fa7	E
Fusarium concolor	Fconc1	E
Fusarium concolor	Fconc2	E
Fusarium crookwellense	BBA 63558 DSM 8704	D
Fusarium crookwellense	BBA 64483	D

Fusarium crookwellense	BBA 64545	D
Fusarium culmorum	Fc15	I [27]
Fusarium culmorum	Fc2	D [27]
Fusarium culmorum	Fc22	I [27]
Fusarium culmorum	CBS 251.52	A
Fusarium culmorum	FcH69	E
Fusarium graminearum	DSM 62217	B [27]
Fusarium graminearum	DSM 62722	B [27]
Fusarium graminearum	DSM 64848	B [27]
Fusarium graminearum	DSM 67638	B [27]
Fusarium graminearum	DSM 4528	B [27]
Fusarium graminearum	DSM 1096	B
Fusarium oxysporum	FO 125	E
Fusarium oxysporum	SAGW 124	E
Fusarium oxysporum	Foxy121	E
Fusarium oxysporum	Foxy436	E
Fusarium oxysporum	Foxy119	E
Fusarium oxysporum	Foxy6	E
Fusarium poae	DSM 62376	B
Fusarium poae	FP 2	I
Fusarium poae	Fpoae 369	E
Fusarium poae	Fpoae 365	E
Fusarium poae	Fpoae 517	E
Fusarium proliferatum	DSM 764	B
Fusarium proliferatum	DSM 840	B
Fusarium proliferatum	DSM 62267	O
Fusarium proliferatum	DSM 62261	O
Fusarium proliferatum	DSM 63267	O
Fusarium proliferatum	FPRO1	N [23]
Fusarium proliferatum	FPRO2	N [23]
Fusarium proliferatum	FPRO3	N
Fusarium proliferatum	FPRO4	N
Fusarium proliferatum	FPRO5	N
Fusarium proliferatum	FPRO8	N
Fusarium proliferatum	FPRO9	N
Fusarium proliferatum	FPRO11	N
Fusarium proliferatum	FPRO12	N
Fusarium proliferatum	D00502	G [12, 40]
*Fusarium sacchari (*former *subglutinans)*	B03852	G [40, 41]
*Fusarium sacchari (*former *subglutinans)*	B03853	G [41]
Fusarium solani	Fsol1	E
Fusarium subglutinans	B00278	G [12]
Fusarium subglutinans	B00281	G [12]
Fusarium subglutinans	B01722	G [40]
Fusarium subglutinans	B01728	G [40]
Fusarium subglutinans	B038J	G
Fusarium subglutinans	B03819	G
Fusarium subglutinans	B03820	G
Fusarium subglutinans	B03821	G
Fusarium subglutinans	B03828	G [40]
Fusarium subglutinans	E02192	G [12]
Fusarium tricinctum	FT1	E
Fusarium tricinctum	FT2	E
Fusarium tricinctum	FT3	E
Fusarium verticillioides	Jan 51	M [23]
Fusarium verticillioides	EJAB,21/1BA	L [23]

Fusarium verticillioides	FRC M-7358	K [42]
Fusarium verticillioides	FRC M-7362	K [42]
Fusarium verticillioides	FRC M-7367	K [42]
Fusarium verticillioides	FRC M-7370	K [42]
Fusarium verticillioides	FRC M-7437	K [42]
Fusarium verticillioides	FRC M-7363	K [42]
Fusarium verticillioides	FRC M-8114	J [39, 42]
Fusarium verticillioides	FV 234/1	P [39]
Fusarium verticillioides	Jan 34	M [23]
Fusarium verticillioides	F01377	G [12, 40]
Fusarium verticillioides	A00102	G [12]
Fusarium compactum	ICARDA 93823	F
Acremonium chrysogenum	AC1	E
Acremonium chrysogenum	AC2	E
Acremonium longisporum	AL	E
Acremonium ochraceum	AO	E
Acremonium polychromum	AP	E
Alternaria alternata	A 4.1.1	E
Cladosporium herbarum	CH 3	C
Cladosporium herbarum	CH 4	E
Drechslera sorokiniana	D 3.1	E
Microdochium nivale	GN 7	I
Microdochium nivale	GN 25	I
Microdochium nivale	GN 35	I
Microdochium nivale	GN 36	I
Pseudocercosporella herpotrichoides	C39A	E
Pseudocercosporella herpotrichoides	PHA 20/3	C
Rhizoctonia cerealis	INRA 161	H
Rhizoctonia cerealis	SAGW J7	E
Rhizoctonia cerealis	SAGW J5	E
Septoria nodorum	7n/II/2	E
Ustilago maydis	DSM 3121	B

*Relevant reference given in brackets

Results

The first amplifications were performed under conditions for end-point PCR as described by Mule et. al [25] and Jurado et. al [26]. To improve the sensitivity, we reduced the reaction volume to 25 µl and optimized the following: the concentrations of dNTPs, MgCl2, and primers; the activity of *Taq* DNA polymerase; and the cycling parameters for qPCR conditions. For *F. verticillioides*, the most important changes in the conditions for PCR concerned the concentrations of dNTPs and MgCl2, which were increased from 50 to 100 µM and from 1.5 to 2.5 mM, respectively, as compared to the original publication. In contrast, the amount of *Taq* DNA polymerase could be reduced from 1.25 to 0.75 units. An annealing temperature of 62°C yielded specific products, in contrary to the annealing temperature of 56°C, which was suggested by the designers

of the primers [25]. In the *F. proliferatum* assay, the amount of each primer could be reduced from 0.8 mM to 0.6 mM, dNTPs could be reduced from 1 mM to 100 µM, and *Taq* DNA polymerase could be reduced from 1.0 to 0.4 units per reaction. The annealing temperature was lowered from the recommended temperature of 69°C [26] to 64°C.

The optimized conditions were used for the ROC curve analysis with artificially prepared samples and field samples. Artificial negative samples consisted of nontarget DNA and blank plant matrix and artificial positive samples consisted of plant matrix spiked with known quantities of target DNA (0.05 pg to 50 pg). A total of 226 artificial samples for *F. verticillioides* assay and 224 samples for *F. proliferatum* assay were used. Field samples originated from monitoring and field trials carried out from 2005 to 2008 in Germany and Italy; 994 field samples for *F. verticillioides* assay and 436 field samples for *F. proliferatum* assay were used. Melting curve analysis was used as the "gold standard" for classification of field samples as positive or negative. Unknown samples generating products with melting temperatures ±0.25°C above/below the mean melting temperature of the standards and positive controls for a given PCR run were ranked as positive. Over a period of three years, the melting temperature among PCR runs fluctuated between 90.0°C and 91.5°C for F*. verticillioides* and between 91.5°C and 92.5°C for *F. proliferatum*. Within a single PCR run, melting temperatures for standards and positive controls were constant within a range of 0.5°C. Calibration curves generated with spiked matrix revealed a linear relationship between Ct values and the logarithm of DNA amount down to at least 0.05 pg for *F. proliferatum* and 0.15 pg for *F. verticillioides* (Fig. 1). The average PCR efficiency of the assays was 0.92 for *F. verticillioides* and 0.98 for *F. proliferatum*. The Ct values for *F. proliferatum* DNA were consistently about four cycles lower than the values for the same amount of *F. verticillioides* DNA. With all 13 *F. verticillioides* isolates (formerly *F. moniliforme*) and 15 *F. proliferatum* isolates (Table 1), we obtained PCR products with the expected melting temperatures. As a confirmation of the taxonomic affiliation of these strains, we determined which mycotoxins they produced. Ten strains labeled as *F. verticillioides* and 12 strains labeled as *F. proliferatum* were grown in rice for two weeks. With one exception, only *F. proliferatum* strains produced *F. proliferatum*-specific depsipeptide beauvericin (Table 3). Furthermore, neither species produced enniatins, and all strains except one produced fumonisins.

Pure maize DNA and all isolates of 18 nontarget fungal species tested negatively (87 isolates for the *F. proliferatum* assay and 89 isolates for the *F. verticillioides* assay). Samples of nontarget fungal DNA generated no amplification products or unspecific products with melting temperatures lower than those of the target products by at least 4°C (Fig. 2).

ROC curve analysis was performed for spiked maize matrix and field samples (Fig. 3). For both fungi, areas under ROC curves were slightly higher for spiked matrix than for field samples. The ROC curves were used to determine the LOQs of the assays. We defined the LOQ of the PCR assays as DNA amounts that maximized the sum of sensitivity and specificity. The corresponding Ct values (optimal cut-off points) were determined by maximizing the Youden index. Calibration curves (Fig. 1) were used to determine LOQs for both assays using these Ct values (Table 2).

For a given threshold of the false-positive rate, the LOD was defined as the lowest amount of target DNA that was amplified with a false-negative rate below or equal to this threshold. We selected a maximal acceptable false-negative rate of 5% and then used this threshold to determine the LOD values (Table 2).

Table 2: Performance parameters of qPCR assays

Samples	Positive[a]	Negative[b]	Optimal cut-off point	Sensitivity at optimal cut-off	Specificity at optimal cut-off	LOD [pg]	LOQ [pg]
F. verticillioides							
Spiked matrix	112	114	36	0.96	0.97	0.021	0.11
Field samples	796	198	33	0.85	0.95	---	0.62
F. proliferatum							
Spiked matrix	92	132	30	0.99	0.96	0.016	0.03
Field samples	379	57	27	0.94	0.96	---	0.24

[a]Spiked matrix: number of samples spiked with target DNA; field samples: number of samples that generated products with melting temperatures differing by less than 0.25°C from target DNA. [b]Spiked matrix: number of samples consisting of matrix with non-target DNA only; field samples: number of samples that generated melting curves different from those of target DNA.

Table 3: Production of mycotoxins by selected *Fusarium* strains

Mycotoxin [µg/g rice culture]*						
Strain	Fumonisin B1	Beauvericin	Enniatin B	Enniatin B1	Enniatin A	Enniatin A1
F.verticillioides						
1.51	90	<LOD	<LOD	<LOD	<LOD	<LOD
FRC M-7358	154	<LOD	<LOD	<LOD	<LOD	<LOD
FRC M-7362	240	<LOD	<LOD	<LOD	<LOD	<LOD
FRC M-7367	93	<LOD	<LOD	<LOD	<LOD	<LOD
FRC M-7370	5.2	<LOD	<LOD	<LOD	<LOD	<LOD
FRC M-4737	5.4	<LOD	<LOD	<LOD	<LOD	<LOD
FRC M-7363	116	<LOD	<LOD	<LOD	<LOD	<LOD
FRC M-8114	265	<LOD	<LOD	<LOD	<LOD	<LOD
1.34	53	1.2	<LOD	<LOD	<LOD	<LOD
Fv234/1	114	<LOD	<LOD	<LOD	<LOD	<LOD
F.proliferatum						
DSM 62267	<LOD	518	<LOD	<LOD	<LOD	<LOD
DSM 62261	141	678	<LOD	<LOD	<LOD	<LOD
DSM 63267	29	2.6	<LOD	<LOD	<LOD	<LOD
Fpro1	226	135	<LOD	<LOD	<LOD	<LOD
Fpro2	218	10	<LOD	<LOD	<LOD	<LOD
Fpro3	233	5.7	<LOD	<LOD	<LOD	<LOD
Fpro4	200	424	<LOD	<LOD	<LOD	<LOD
Fpro5	150	277	<LOD	<LOD	<LOD	<LOD
Fpro8	52	2.0	<LOD	<LOD	<LOD	<LOD
Fpro9	75	309	<LOD	<LOD	<LOD	<LOD
Fpro11	27	186	<LOD	<LOD	<LOD	<LOD
Fpro12	26	637	<LOD	<LOD	<LOD	<LOD

*LOD values were 5 ng/g for beauvericin, enniatin B, B1, A1, and fumonisin B1; 10 ng for enniatin A

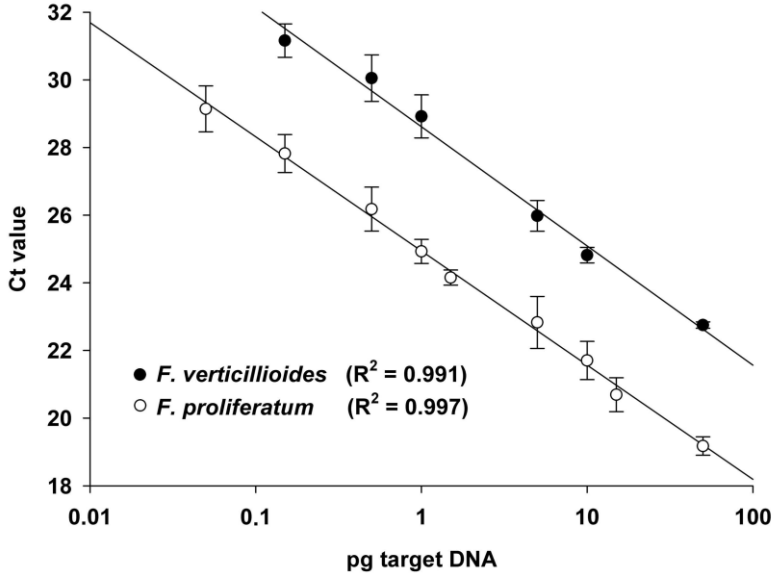

Figure 1. Linear standard curves obtained from dilution series of *F. verticillioides* DNA (filled symbols) and *F. proliferatum* DNA (open symbols) in a range from 0.05 to 50 pg, with five to 10 replications per quantity. The quantity of 0.05 pg of *F. verticillioides* DNA was excluded from the standard curve because of low reproducibility. The threshold cycle (Ct) is plotted against the decadic logarithm of starting DNA quantity in grams. Error bars represent standard deviation.

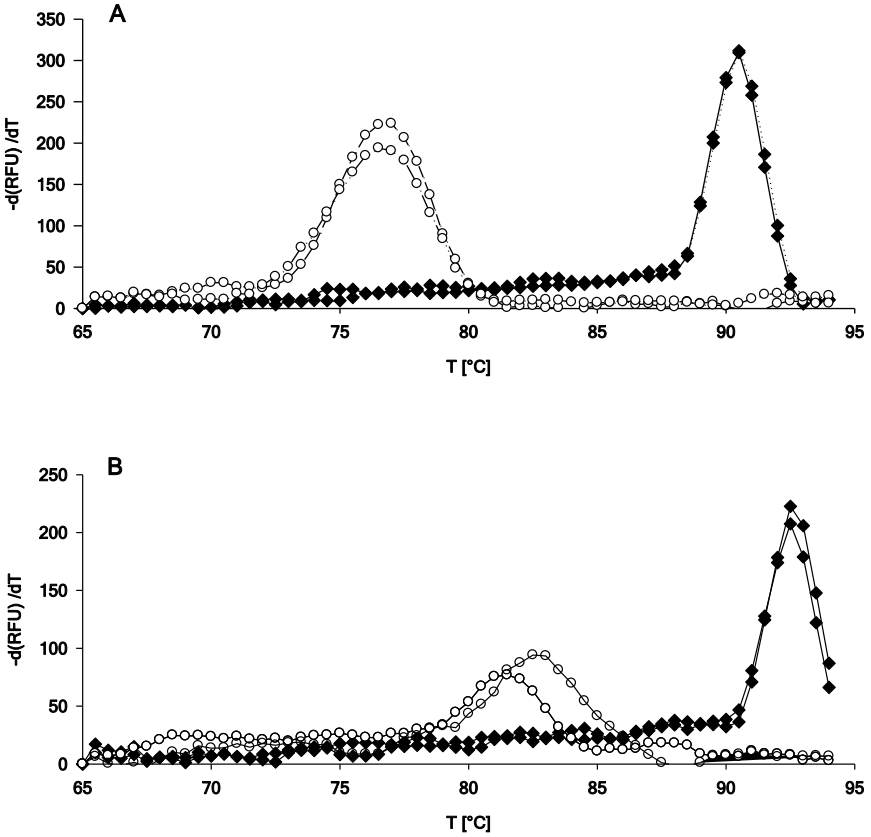

Figure 2. Melting curve analysis of PCR products obtained with primers specific for *F. verticillioides* (a) and *F. proliferatum* (b). Filled symbols indicate the negative first derivation of SYBR Green fluorescence for PCR products heated from 65°C to 94°C. Open symbols indicate melting curves of PCR products of negative controls (water and non-target DNA).

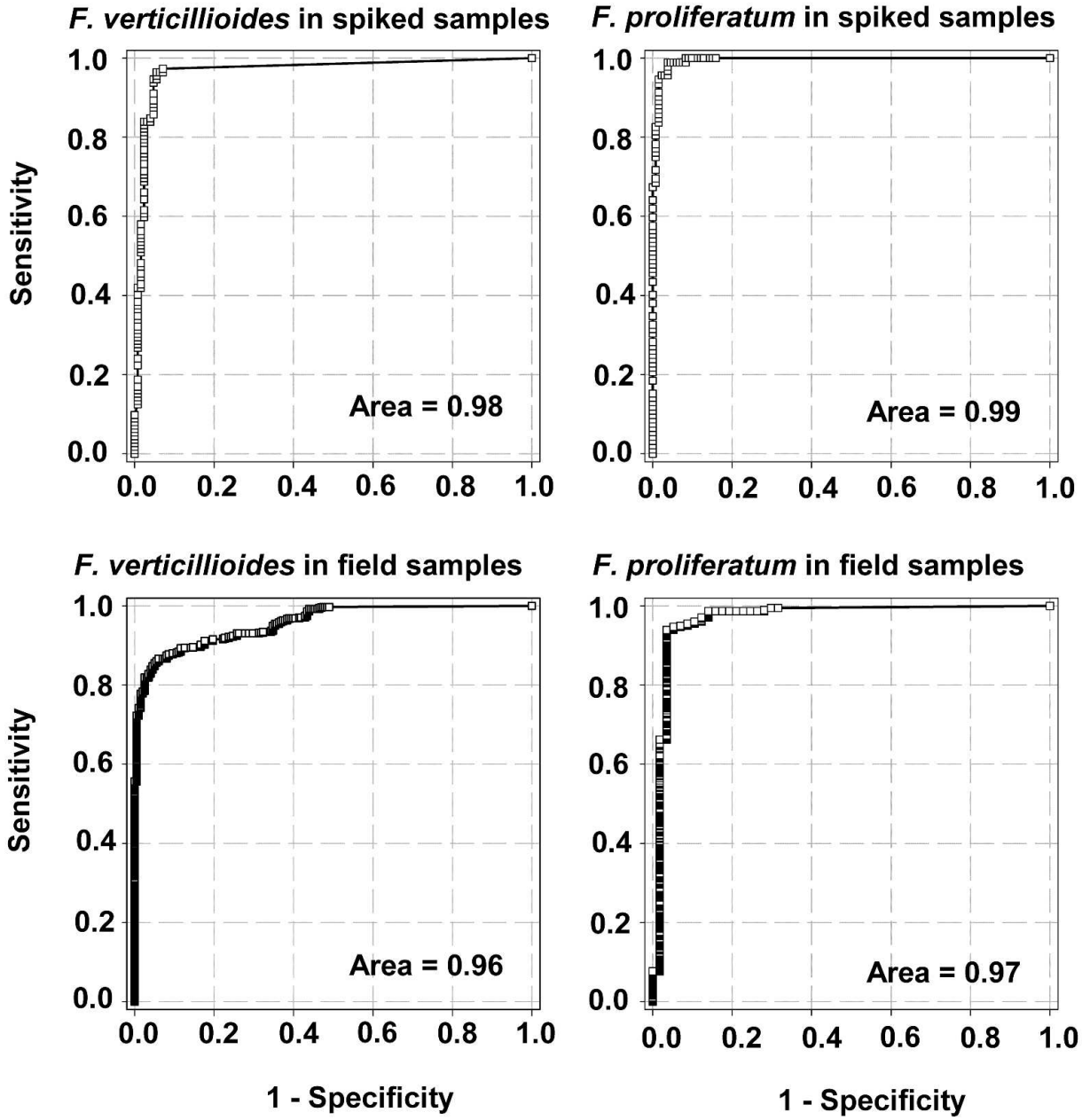

Figure 3. ROC curves for qPCR for *F. proliferatum* and *F. verticillioides*. The upper panels show the ROC curves resulting from maize flour spiked with *F. verticillioides* DNA (n = 226) and *F. proliferatum* DNA (n = 224). The lower panels show the ROC curves for field samples for *F. verticillioides* DNA (n = 994) and *F. proliferatum* DNA (n=436).

Discussion

Using published PCR primers for *F. verticillioides* [25] and *F. proliferatum* [26], we developed qPCR assays for the quantification of the DNA of these species in maize kernels. Mule and co-workers [25] evaluated the specificity of their primers for *F. verticillioides* by testing 21 strains of *F. verticillioides*, 12 strains of *F. proliferatum*, and 6 strains of *F. subglutinans,* in addition to single isolates of *F. graminearum,*

F. poae, A. flavus, and *A. strictum*. Jurado et al. [26] tested the specificity of primers for *F. proliferatum* against 12 strains of *F. graminearum*, 7 strains of *F. culmorum*, 5 strains of *F. poae*, 6 strains of *F. sporotrichioides*, and one or two strains of 8 other *Fusarium* species and 5 other fungal species. The use of only one strain of *F. verticillioides* and *F. subglutinans* in the test of primers for *F. proliferatum* [26] appeared insufficient. We therefore extended the specificity tests for both primer pairs with an additional 12 isolates of *F. verticillioides,* 15 isolates of *F. proliferatum,* 12 isolates of *F. subglutinans*, 42 isolates of non-target *Fusarium* species, and 20 isolates of other fungal species. These tests, performed under qPCR conditions, generated positive signals only for the target species. Primer pairs Fp3-F/Fp4-R [26] and VER1/VER2 [25] can therefore be regarded as species-specific in real-time mode for *F. proliferatum* and *F. verticillioides*, respectively. The qPCR assays described here are suitable for the estimation of *F. verticillioides* and *F. proliferatum* DNA in maize flour with an LOQ of 0.11 pg and 0.032 pg, respectively, which correspond to 3.8 µg and 1.05 µg of DNA/kg of flour, respectively. The mean LOQ values for field and spiked samples correspond to 8.5 genomes for *F. verticillioides* and 3.2 genomes for *F. proliferatum*, assuming that the genome size of both species is approx. 40 Mbp. The amount of genomic DNA determined by qPCR can be used as a measure of fungal content in studies of the relationships between *Fusarium* infection, mycotoxin production, and disease symptoms. Relative to classical end-point PCR, the sensitivity of the detection was increased significantly for both *F. verticillioides* and *F. proliferatum*. Furthermore, the costs of the modified assays were reduced because optimized PCR uses less Taq polymerase and a lower concentration of dNTPs than classical end-point PCR.

Ct values for *F. proliferatum* DNA were consistently lower than those for the same amount of *F. verticillioides* DNA. This observation is reasonable because the primers for *F. proliferatum* were derived from a multi-copy sequence [26] while the primers for *F. verticillioides* were based on a single-copy calmodulin gene [25]. The difference in the copy number of targets also explains why the *F. proliferatum* assay was more sensitive than the *F. verticillioides* assay.

ROC curve analysis of a dilution series of target DNA and non-target DNA generated AUROC values of 0.98 for the *F. verticillioides* assay and 0.99 for the *F. proliferatum* assay, which are close to the optimal value of 1. Occasionally, non-target DNA caused unspecific amplification. Based on cut-off points calculated according to the Youden index (Table 2), the sensitivity was 97% for the *F. proliferatum* assay and 94% for the

F. verticillioides assay, while the specificity was 97% in the *F. verticillioides* assay and 96% in the *F. proliferatum* assay. Therefore, automatic processing of the results based merely on Ct values (without melting curve analysis) is possible. Melting curve analysis is recommended when the content of target DNA approaches LOQ values. Adejumo et al. [31] compared PCR analysis to an agar plating method for detection of *F. verticillioides* in maize samples from a Nigerian market. They found that only 71% of the maize samples that were positive for *F. verticillioides* by agar plating were confirmed positive by species-specific PCR. Part of this contradiction can probably be explained by the morphological similarity between *F. verticillioides* and *F. proliferatum*, highlighting the difficulty in distinguishing between these species based on morphology. Another work by these authors [32] demonstrated an even greater difficulty in differentiating between *F. verticillioides* and *F. proliferatum* based on morphology: *F. verticillioides* was found to be the dominant species in Nigerian maize, followed by eight other *Fusarium* species but *F. proliferatum* was not found. It is likely that *F .proliferatum* isolates were confused with *F. verticillioides* in this work and that 29% of isolates morphologically identified as *F. verticillioides* but not confirmed by PCR were *F. proliferatum*. The use of PCR for differentiating *F. proliferatum* from *F. verticillioides* is therefore highly recommended [33].

To confirm the taxonomical affiliation of strains used in this work, we determined the production of beauvericin, enniatins, fumonisins, and moniliformin by 12 isolates each of *F. verticillioides* and *F. proliferatum*. While fumonisins are produced by both *F. verticillioides* and *F. proliferatum*, moniliformin is produced only by *F. proliferatum* [19] and beauvericin is produced by *F. proliferatum* but is not produced or is produced in only low amounts by *F. verticillioides* [34-36]. That *F. proliferatum* produces enniatins was affirmed in an authoritative review [19] but denied in other publications [37-38]. We did not find enniatins in any of the *F. verticillioides* or *F. proliferatum* cultures in the current study.

Our laboratory has extensively used the qPCR assays described here for quantifying *F. verticillioides* and *F. proliferatum*. Thus, we have used qPCR to analyze maize kernels artificially infected with *F. verticillioides* or *F. proliferatum*, naturally infected samples from the field, and maize cobs inoculated with mixtures of *F. verticillioides*, *F. proliferatum*, and other fungal species in the greenhouse.

ROC analysis was developed for the assessment of qualitative diagnostic assays. Turechek et al. [44] used ROC analysis to compare the performance of PCR primers

[45]. Inspired by their work, we used the ROC concept to establish performance parameters for quantitative PCR assays. LOQ and LOD, which have been fundamental parameters in analytical chemistry, thus became available for quantitative PCR.

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Chapter 3

Distribution of trichothecenes and zearalenone in maize plants after artificial inoculation with *Fusarium graminearum* at two harvest times

Abstract

Fusarium graminearum is one of the main causal agents of *Gibberella* ear rot (GER) disease in maize. This pathogen produces mycotoxins which lead to health risk for humans and animals. Maize is often feed as silage to livestock, for which whole maize plants are used. We inoculated maize cobs artificially via silk-channel injection and investigated the spread of fungal mycelia and mycotoxins in the stem below and above.
In a field study, twenty maize inbred lines were inoculated with a conidia suspension containing $1x10^5$ conidia of *Fusarium graminearum* per ml. The plants were monitored for visual symptoms of cob infection and the whole plants were harvested either at dough stage (46 dpi) or at grain maturity stage (77 dpi). The cob and the internodes below and above the infected ob were analyzed separately for the content of zearalenone (ZEA) and the trichothecenes deoxynivalenol (DON), 3-acetyl-deoxynivalenol, and 15-acetyl-deoxynivalenol with HPLC-MS/MS. The amount of *Fusarium graminearum* DNA was quantified with real-time PCR.
The mycotoxin accumulation was substantially higher in the cobs than in the stems at both harvest time points. A strong increase of the mycotoxin content was observed in the kernels between silage harvest, were an average of 275 mg/kg DON was detected, to grain maize maturity, were an average of 534 mg/kg was found. The amount of ZEA in the kernels has grown nearly tenfold from 0.4 mg/kg at 46 dpi to 3.6 mg/kg at 77 dpi. Mycotoxins did hardly spread in the stems near the cob until the silage harvest. At grain maize harvest, in the stems above the infected cob higher DON concentrations with an average of 1 mg/kg were measured, compared to 0.5 mg/kg detected in the stems below it. ZEA was found only in little amounts, and was more often detected in the stems below than above the infected cobs. Fungal biomass was found in the stems above and below the infected cobs, but while it was more often detected in the stems

above the cob, the average amount of fungal DNA was higher below it. Visual monitoring of cob infection did not correlate with mycotoxin content in the stem. No correlation was found between ear rot symptoms in the cob and mycotoxin contamination in the stems. Genotypes differed in their ability of translocation of toxins in the stems. Nevertheless some genotypes showed low toxin concentrations in the kernels as well as in the stems.

Introduction

The maize pathogen *Fusarium graminearum* causes yield losses and impairs the quality of maize and wheat. In the last decades, the importance of the pathogen for maize and wheat increased in Germany (Logrieco et al, 2002). *Fusarium* infection in maize does not only impair the yield, it is also responsible for the contamination of food and feedstuff with mycotoxins. Therefore the Commission of the European communities defined a legal limit for these mycotoxins in cereals for human consumption (750 µg/kg deoxynivalenol (DON) and 200 µg/kg zearalenone (ZEA) (The commission of the European communities, 2006).

DON and its derivates 3-acetyl-deoxynivalenol (3-ADON), 15-acetyl-deoxynivalenol (15-ADON) as well as ZEA belong to the most problematic *Fusarium* mycotoxins occurring in maize and wheat in northern Europe. For silage as ruminant feed whole maize plants are used and *Fusarium* species could regularly be found on maize stalks and in silage (Saß et al., 2007, Mansfield & Kuldau, 2007, Dorn et al., 2009). Xue et al. found an increase of *F. graminearum* incidence in kernels of silage maize with decreasing moisture at harvest time (Xue at al., 2005). Mansfield et al. found that DON levels decreased during silaging process (Mansfield et al., 2005). Nevertheless DON and other mycotoxins are still found in maize silage (Whitlow & Hagler, 2002).

The distribution of mycotoxins and fungal biomass in maize ears which are infected with *F. graminearum* is not completely known. Reid et al. studied the distribution of DON in infected maize ears, and found the highest concentration of DON in the rachis, the second most in the symptomatic kernels, and the least concentration in the symptomless kernels (Reid et al., 1996). Perkowski et al. confirmed these results (Perkowski et al., 1997). Additionally they observed a translocation of trichothecenes in different parts of the stem above and below the infected ear. These results differed between the investigated maize varieties. Because fungal growth was not studied in

both publications, it remains unclear if only the mycotoxins were transported or if the fungus itself translocates from the cob into different regions of the plant.

It is still unknown how to solve the problem with contamination of maize and maize products with mycotoxins. Resistance breeding seems to be the only opportunity to face this problem, and genotypic variances showed sufficient genetic variation of ear rot resistance to *F. graminearum* and *F. verticillioides* in breeding material (Löffler et al., 2010).

The overall aim of this work was the investigation of the spread of fungal mycelium and the presence of mycotoxins in the stems below and above artificially inoculated cobs in different maize genotypes. We compared the situation at two harvest times: grain maize harvest and silage harvest.

Materials and Methods

Field experiment

Twenty maize inbred lines were planted in four repetitions in the growing season 2007 in an experimental field of KWS SAAT AG in Einbeck, Lower Saxony, Germany. Inbred lines were composed of breeding material of KWS SAAT AG, Germany. Each plot consisted of one row with 20 plants each. Five to seven days after flowering ten plants per plot were inoculated consecutively, beginning with the second plant in a row. Inoculation was carried out via silk channel inoculation with 1.0 ml of a conidia suspension containing $1x10^5$ conidia per ml of *Fusarium graminearum* strain IFA66 (M. Lemmens, Institute of Biotechnology in Plant Production, Tulln, Austria, via T. Miedaner, State Plant Breeding Institute, University of Hohenheim, Stuttgart, Germany) by injection into the silk channel. Flowering times and harvest times were determined separately according to the growth stages of the individual maize genotypes. At dough stage (46 dpi) and grain maturity stage (77 dpi) the plants were monitored for visual symptoms of cob infection. Rating was done by estimating the percentage of visibly infected kernels on the ear surface after dehusking. Subsequently the whole plants were harvested by hand. The harvested plants were separated in three parts: the infected cobs, the stems above the cob up to the next node and the stems below the infected cob until the next node. Samples were dried in a drying oven (Hareus Christ, Osterode, Germany) at 45 °C for 7 days and pooled within each row. The cobs were

hand-shelled and the maize kernels were milled with a cross hammer mill by using a 1 mm sieve (Retsch, Haan, Germany).

Toxin quantification

Two grams of plant material were shaken for one hour with 40 ml acetonitril/water 80:20. Afterwards the samples were centrifuged at 4500 rpm for 10 min, and one ml of supernatant was transferred to 2 ml reaction tubes. Aliquots were dried in a vacuum concentrator (Martin Christ, Osterode, Germany), resuspended in 1 ml eluent (50% methanol, 5% acetonitrile, 5mM ammonium acetate, pH 4.5 adjusted with formic acid) and defatted with 500 µl hexane. The samples were analyzed for the content of deoxynivalenol, 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol and zearalenone with HPLC-MS/MS. The HPLC-MS/MS system consisted of ProStar 210 pumps, column oven Mistral 510, separating column Polaris C 18 Ether (100 x 2 mm, 3 µm), and 1200 LC Triple Quadrupole mass spectrometer with electrospray ionisation-interface (all Varian, Darmstadt, Germany). Standards for kernel samples were prepared with spiked blank samples, which were extracted as described above. Standards for stem samples were prepared in methanol/water 50%, because no matrix effects were observed with maize stems.

Quantification of fungal biomass

Total DNA was extracted with a CTAB method (Brandfass & Karlovsky, 2008), modified as follows: 500 mg stem material were extracted with 800 µl CTAB-buffer and 500 mg kernel flour were extracted with 500 µl CTAB-Buffer. Subsequently samples were diluted with double distilled water by a factor of ten before analyzing them in a real-time PCR. For amplification and quantification of *F. graminearum*-DNA in maize samples the iCycler System (BioRad, Hercules, CA, USA) was used. Real-time PCR assay and preparation of standards were carried out according to the real-time PCR assay for *F. graminearum* published by Brandfass & Karlovsky (2008) modified as follows: *F. graminearum*-specific PCR consisted of 1x ABsolute Blue QPCR SYBR Green Fluorescein Mix (containing Thermo Start ^{TM} DNA-Polymerase, 3 mM $MgCl_2$, dNTP Mixture, SYBR Green I, and 10 nM Fluorescein, Abgene Limited, United Kingdom). Initial denaturation was extended for 13.5 min as required for the activation of the polymerase.

Statistical analysis

Statistical analyses were performed with the Software Package Statistica 8.0 (StatSoft. Inc., Tuba, OK, USA).

Results

The mean ear rot incidence of the cobs was 56 % at silage harvest, and 81 % at grain maize maturity. As expected, highest toxin- as well as fungal DNA concentrations were in the kernels at both stages.

Comparison of different organs and harvest times

Except for ZEA, the toxin concentrations doubled between silage and grain maize harvest in the kernels. The amount of ZEA in the kernels increased between 46 dpi and 77 dpi by a factor of ten. At 46 dpi, toxins were found rarely neither in the stems above nor below the infected cob, and if so, only in low amounts. At 77 dpi, stems above the cob were higher contaminated with toxins than stems below, but the differences were not significant (Figure 1).

F. graminearum-DNA in the stems above the cob was found in equal amounts at both harvest times. In the stems below the infected cobs, the DNA amount was doubled between silage and grain maize harvest (Figure 1).

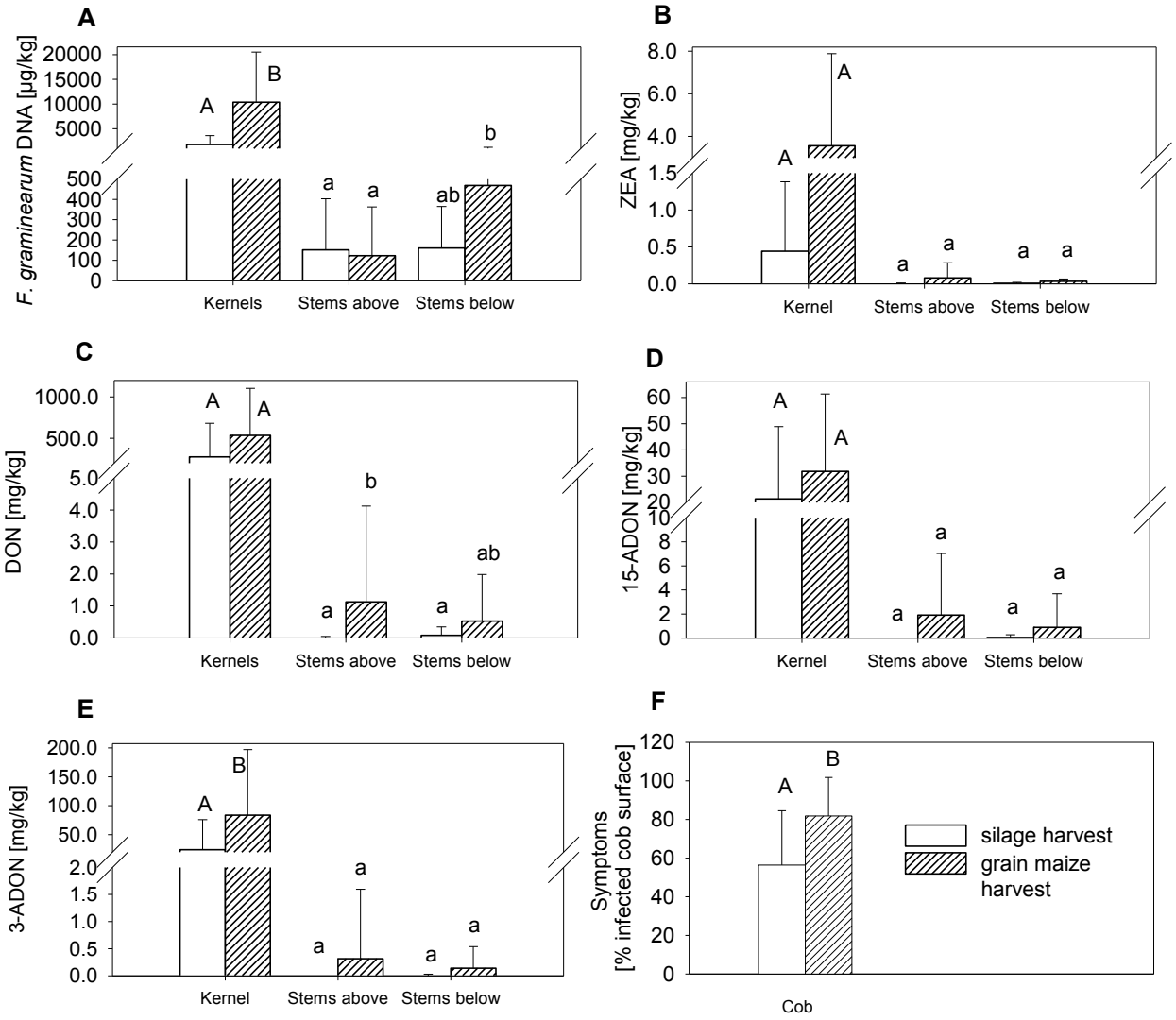

Figure 1: Parameters collected of different harvest time points and organs. Means (columns) and standard deviations (error bars) from different organs of the amount of *F. graminearum* DNA [µg/kg] (A), ZEA [mg/kg] (B), DON [mg/kg] (C), 15-ADON [mg/kg] (D) and 3-ADON [mg/kg] (E) of all tested genotypes are shown. The means (columns) and standard deviations (error bars) of the ear rot symptoms are given in % infected cob surface (F). Results at 46 dpi are shown in white columns, those at 77 dpi are shown in patterned columns. Different lowercase letters label different homogeneous groups within the stems, different capital letters label different homogenous groups within the kernels (p=0.05, Tukey test).

Correlation between symptoms and fungal infestation

The mean of fungal DNA in the kernels of both harvest times correlated positively (R=0.70) with the mean of symptoms at both harvest times (Figure 2). The concentration of the trichothecenes DON, 3-Acetyl-DON and 15-Acetyl-DON correlated with the symptoms with a correlation coefficient of R=0.61, R=0.55 and R=0.62, respectively (Figure 3). ZEA did not correlate with ear rot symptoms or DON concentration (Table 2). Genotypes differed in toxin accumulation in their stems, but lower amounts of toxins in the kernels did not necessarily lead to low toxin levels in the stems. Nevertheless some genotypes with low toxin concentrations in the grains also showed low toxin concentrations in the stems (Figure 4).

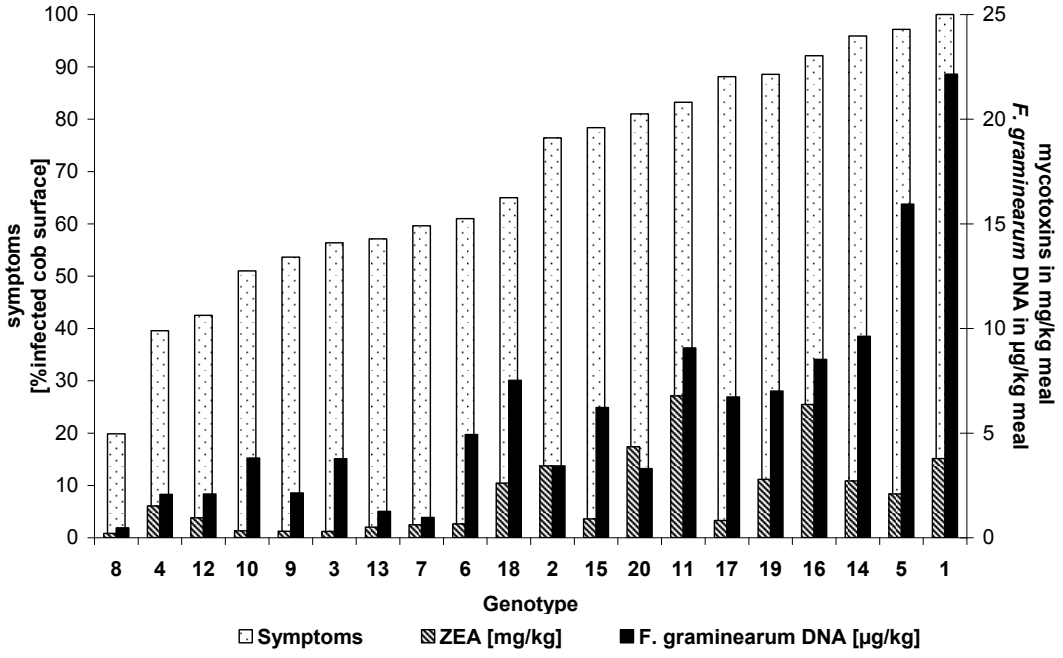

Figure 2: Amount of ZEA and *F. graminearum*-DNA in kernels (right ordinate). The average of both harvest times is shown. Genotypes are sorted by severity of cob symptoms (mean of both harvest times, left ordinate). Correlation coefficient between ear rot severity and amount of fungal DNA was R=0.69.

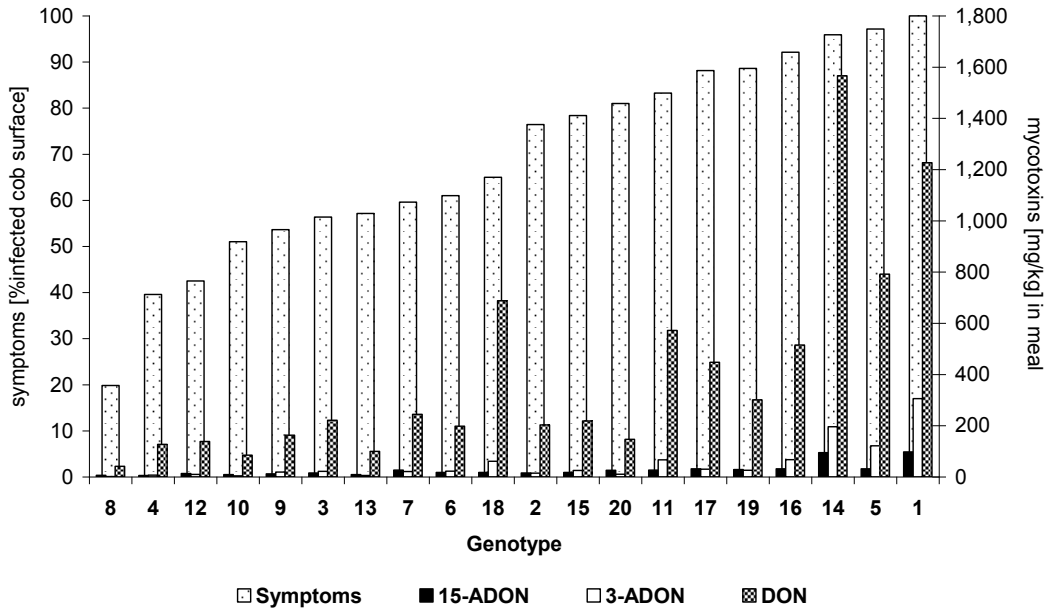

Figure 3: Trichothecenes in kernels (right ordinate). The average of both harvest times is shown. Genotypes are sorted by severity of cob symptoms (mean of both harvest times, left ordinate). The amount of DON correlated with GER symptoms (R=0.61), as well as 3-ADON and 15-ADON (R=0.55 and R=0.62, respectively).

The correlation between ear rot symptoms and DON concentration in the kernels was much higher at silage harvest (R=0.88) than at grain maize harvest (R=0.56, Figure 5). The correlation at grain maize was much better at DON concentrations lower than 500 mg/kg (R=0.66, Figure 6A) than at DON concentrations higher than 500 mg/kg (R=0.34, Figure 6B). This is due to the fact that symptoms were determined as the percentage of infected cob surface. This leads to a maximum value of GER symptoms of 100 %, while the DON concentration can still increase.

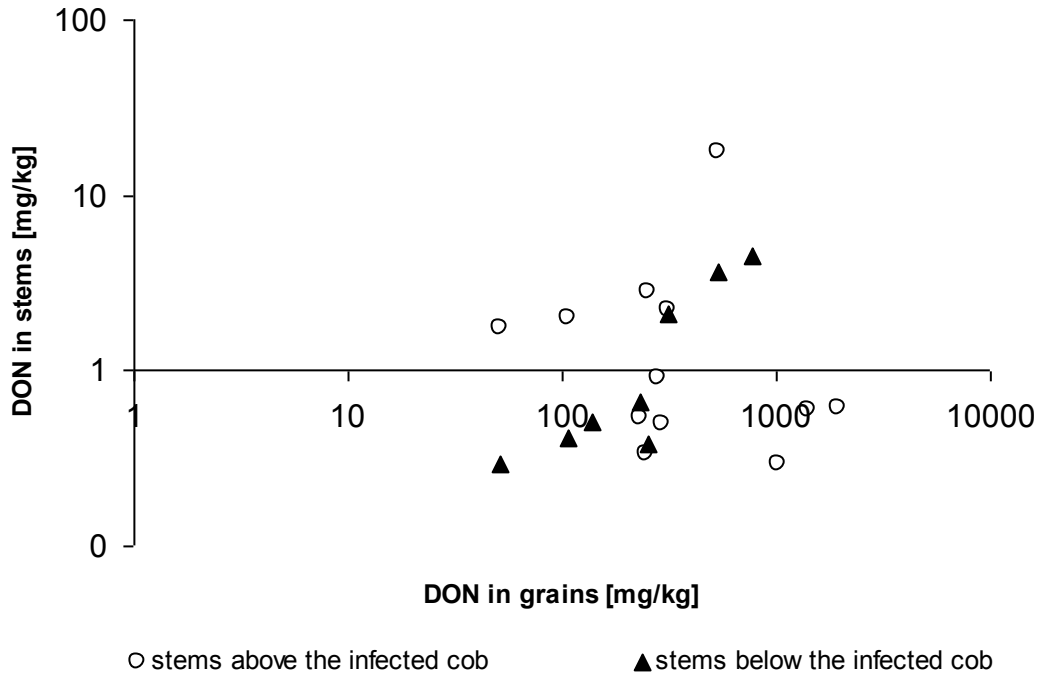

Figure 4: Scatter plot of DON amounts at grain maize harvest. DON levels in stems above the infected cob (white circles) and below the infected cob (black triangles) were plotted against DON levels in grains. Although no significant correlation was found, there are still genotypes with low DON levels both in stems above and below the infected cob and in grains.

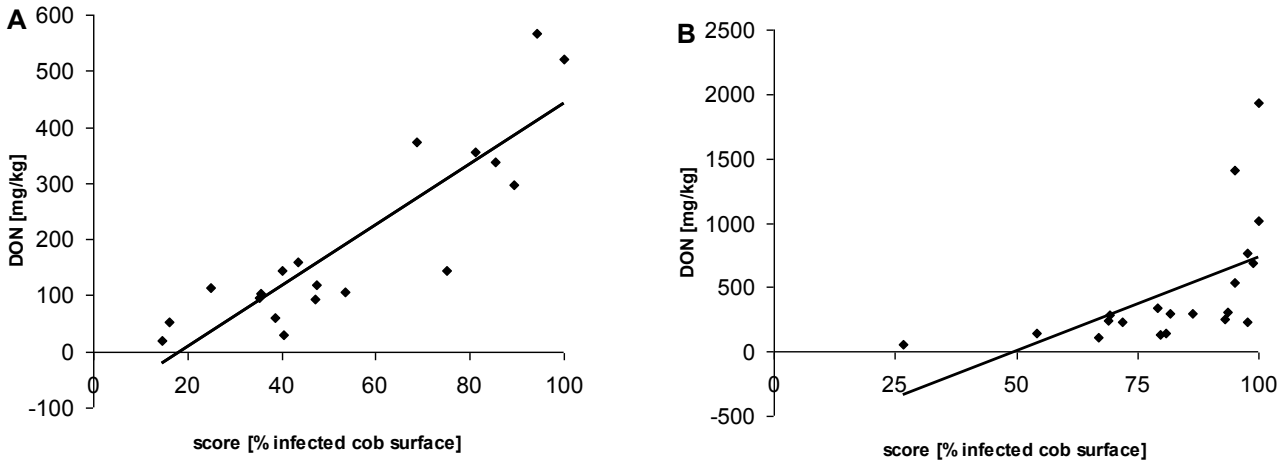

Figure 5: Scatter plots of concentration of DON in the kernels of 20 tested maize inbred lines against GER symptoms of the cobs at silage harvest (47 dpi, A) and grain maize harvest (77 dpi, B), The linear correlation was very high at silage harvest (R=0.88), while it was much weaker at grain maize harvest (R=0.56, $p<0.05$).

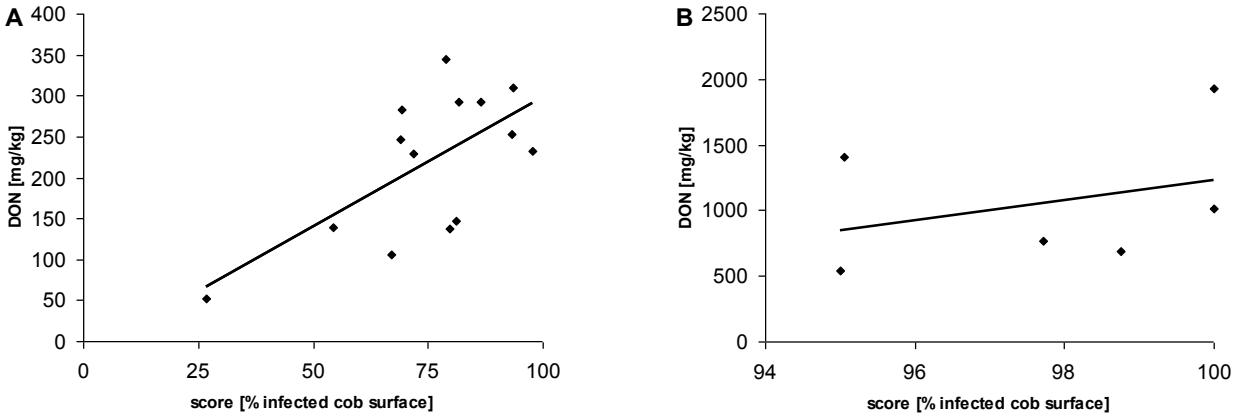

Figure 6: Scatter plots of the DON concentration in the kernels of 20 tested maize inbred lines against GER symptoms of the cobs at grain maize harvest (77 dpi) separately for DON values lower than 500 mg/kg DON (A) and values higher than 500 mg/kg DON (B). While a clear linear correlation could be found between DON-values lower than 500 mg/kg and GER symptoms (R=0.66, $p<0.05$), it was much weaker between higher DON-values and GER symptoms (R=0.34).

Table 1: Means, standard deviations and incidences of the amount of *F. graminearum* DNA and mycotoxins of the different organs at different harvest timepoints.

		DON [mg/kg]		Nivalenol [mg/kg]		15-ADON [mg/kg]		3-ADON [mg/kg]		ZEA [mg/kg]		*F. graminearum* DNA [mg/kg]	
Organ	**Harvest**	**Mean ± s.d.**	**inci-dence [%]**	**Mean ± s.d.**	**Inci-dence [%]**	**Mean ± s.d.**	**inci-dence [%]**	**Mean ± s.d.**	**inci-dence [%]**	**Mean ± s.d.**	**inci-dence [%]**	**Mean ± s.d.**	**inci-dence [%]**
Kernels	46 dpi	275.1 ±405.9	94.9	1.7 ± 3.0	46.2	21.3 ± 27.6	86.7	24.0 ± 51.6	87.2	0.4 ± 1.0	69.2	1.8 ± 3.9	89.7
	77 dpi	534.1 ± 570.2	100	3.4 ± 3.7	83.8	31.8 ± 29.6	100	83.6 ± 113.5	100	3.6 ± 4.3	100	10.4 ± 10.1	100
Stems above the infected cobs	46 dpi	0.0 ± 0.0	2.6	0.0 ± 0.0	0.0	0.0 ± 0.0	0.0	0.0 ± 0.0	0.0	0.0 ± 0.0	0.0	0.2 ± 0.3	92.3
	77 dpi	1.1 ± 3.0	51.4	0.0 ± 0.0	0.0	1.9 ± 5.1	16.2	0.3 ± 1.3	21.6	0.1 ± 0.2	32.4	0.1 ± 0.2	91.9
Stems below the infected cobs	46 dpi	0.1 ± 0.3	11.1	0.0 ± 0.0	0.0	0.0 ±0.2	5.6	0.0 ± 0.0	2.8	0.0 ± 0.0	55.6	0.2 ± 0.2	80.0
	77 dpi	0.5 ± 1.5	27.8	0.0 ± 0.0	0.0	0.9 ± 2.8	22.2	0.1 ± 0.4	41.7	0.0 ± 0.0	88.9	0.5 ± 0.7	70.3

s.d.=standard deviation

Table 2: Correlations of measured parameters in the kernels of 20 tested maize inbred lines. Marked correlations are significant (p<0.05).

Variable	DON	NIV	15-A-DON	3-A-DON	ZEA	*F. graminearum* DNA
score	0.61***	0.69***	0.62***	0.55***	-0.11	0.70***
DON			0.88***	0.93***	-0.01	0.85***
NIV			0.87***	0.91***	-0.03	0.88***
15-A-DON				0.88***	0.07	0.75***
3-A-DON					-0.01	0.88***
ZEA						-0.05

Although we inoculated with a DON producer, nivalenol was often found in the kernels in amounts of 1.7 mg/kg at silage harvest and 3.5 mg/kg at grain maize harvest (Table 1). This is much less than the mean concentrations of DON, which are 275 mg/kg and 534 mg/kg, respectively, and can be ascribed to background contaminations.

Discussion

The main content of *Fusarium* mycotoxins was detected in the infected cobs, which confirmed the finding of Perkowski et al. (1997). The inoculation of spores in the cobs led naturally to the highest infection level there. Besides, it caused the highest accumulation of DON there, because from the moment of the infection this toxin is synthesized (Perkowski et al., 1997, Young et al., 1985).

The amount of 275 mg/kg DON was doubled in the kernels during the time from silage harvest to grain maize harvest (Figure 1). These results contradict the findings of Young et al., who found a slight decrease of the DON concentration between six and nine weeks after inoculation (Young et al., 1985). Maybe the reason for this discrepancy can be found the inoculation method. Young et al. used a toothpick which was colonized with fungal mycelium, and the overgrown toothpick was placed in the cob. The fungus grew from the toothpick and colonized the cob. In our study, a conidia suspension was used. The conidia needed some time to germinate and to colonize the cob; therefore the growth stage was not the same than in the study by Young et al. (1985). It can be assumed that the metabolic process of mycotoxin accumulation was earlier in the previous study, because the fungus was directly able to grow and colonize the cob. Therefore one can speculate that the main accumulation of DON and fungal biomass was already finished 6 weeks after inoculation.

In our experiments ZEA was found at both times in the kernels, but the amount was nearly ten times higher at grain maize harvest than at silage harvest (Figure 1). This

confirms the known fact that ZEA is mainly produced later during pathogenesis (Sutton 1982), and goes along with the findings of Martins & Martins (2002), who had the highest ZEA production in cracked corn after inoculation at 28 °C for 15 days followed by incubation at 12 °C for eight weeks. At silage harvest (end of September) the fungus did not have a long period with cool temperatures in opposite to the grain maize harvest one month later.

At silage harvest, results of Young at a. (1985) were partly confirmed. They found ergosterol above the infected cob six weeks after inoculation, but only little amounts were detected below it. In our experiment fungal DNA was determined in nearly the same amounts both above and below the cob at this time, but more often above than below the infected cobs. It was found in over 90 % of the samples above the cob, and in 80% below it (Table 1). Above the cob, Young et al. detected DON in the stems, while below the infected cob only little amounts were found. In our experiment toxins were determined very rarely and only in low amounts in the stems at silage harvest. It seems that in this study, the distribution of fungal biomass occurred faster than in the experiment of Young et al, while the accumulation of DON happened later. Various factors can cause these phenomena, for example different growth stages of the inoculum, differences between the strains used as inoculum, and different weather conditions.

At grain harvest, the averaged amount of fungal DNA was significantly higher in the stems below the infected cob than above, but it was more often found above than below the infected cob. While fungal DNA was detected in 70% of the samples below the cob, it was found in more than 90 % of samples above it (Table 1). Additionally more DON was determined above the cobs. One can conclude that *F. graminearum* grows quite easily in the stems above and below the infected cob, but the growth stopped at a certain time point. Young at al. (1985) found the ergosterol content in the stems above the cob dropped down by 90% at this time, compared with the content at silage harvest. This could not be confirmed; in our experiment the amount of *F. graminearum* DNA was reduced by half between silage and grain maize harvest above the infected cob, but the incidence of more than 90% remained the same (Table 1).

A significant correlation was detected between fungal DNA of both harvest times and the symptoms (Figure 2 and 3). With the rating of symptoms the amount of visible fungal mycelium was estimated, and this method seems to be suitable as an indicator for fungal biomass. The correlation between the rating and the amount of DON found in

the kernels was also significant. The correlation at silage harvest was much stronger than at grain harvest (Figure 5). The rating was carried out as estimation of percentage of infected cob surface, and was therefore limited with 100 % being the absolute maximum. Even if this maximum of fungal colonization was reached, still more DON was accumulated, which led to bend down of the correlation curve. The correlation between the DON-amounts lower than 500 mg/kg and the symptoms was much better than the one between the DON-amounts higher than 500 mg/kg and the symptoms (Figure 6).

No correlation was determined between the amount of toxins in the grains and in the stems (Figure 4). Nevertheless several genotypes with low mycotoxin content in the kernels also contained low toxin levels in the stems. Therefore it is possible that breeding for low mycotoxin levels in grains would lead to low mycotoxin levels in the stems in some cases.

It can be summarized that the main problem in GER contaminated maize plants remain the cobs, which are highly contaminated even at silage harvest. As we could hardly detect mycotoxins in the stems at silage harvest, the mycotoxin contamination of the stalks caused by *Fusarium*-infected cobs may not be important in the silage production, if plants are harvested early. Mansfield et al. (2005) discovered that DON levels decreased during silaging process. Stalk contamination may even be less important if we take these results of Mansfield et al. into account.

Acknowledgement

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Chapter 4

Effect of mixed inoculation of *Fusarium verticillioides* and *Fusarium graminearum* on the dynamic of fungal colonization and mycotoxin production in maize cobs

Abstract

The interaction between the ear rot causing fungi *Fusarium verticillioides* and *F. graminearum* when being simultaneous injected in the silk channel was investigated. Three different maize genotypes with different resistance levels were inoculated with *F. graminearum*, *F. verticillioides* or a mixture of both fungi. A conidia suspension containing 10^5 spores of *F. graminearum*, *F. verticillioides* or both was inoculated 4-6 days after female flowering in the silk channel. Plants were harvested at six time points and scored for ear rot symptoms. The first harvest was 21 dpi, the others were fortnightly until 91 dpi. The amount of deoxynivalenol, zearalenone and fumonisins B1 and B2 was determined via HPLC-MS/MS. The amount of *F. verticillioides* DNA and *F. graminearum* DNA was quantified with species specific real-time PCR assays.

F. graminearum was severely reduced both in fungal growth and mycotoxin accumulation in the presence of *F. verticillioides*, while *F. verticillioides* was not affected by the presence of *F. graminearum*.

The dynamic of mycotoxin accumulation showed that deoxynivalenol and fumonisins were produced relatively early in pathogenesis, while zearalenone occurred at later time points.

The ratio between fungal DNA and toxin concentration of both fungi did not differ between single inoculation and mixed inoculation. *F. graminearum* produced the highest amounts of deoxynivalenol per fungal DNA at the beginning of the pathogenesis. After the first harvest time points, the productivity of *F. graminearum* dropped rapidly down. The fumonisin B1-productivity of *F. verticillioides* increases slowly during pathogenesis. This supports the reports, that deoxynivalenol is a pathogenicity factor, while fumonisins are not. Both toxins do not seem to play a role in fungal competition.

Introduction

Fusarium graminearum and *Fusarium verticillioides* are the most frequently isolated pathogens from ear rot symptomatic cobs in maize. The main problems are not only impairment of the yield, but also diminishment of the quality due to contamination with mycotoxins, which affects humans and animals health (Logrieco 2002, Yazar and Omurtag 2008). *Fusarium* species cause the ear rot disease (FUS), which can be divided in two types of ear rot. One type is Red ear rot or *Gibberella* ear rot (GER), caused by *Fusarium* spp. belonging to the discolor section. The second type is Pink ear rot or *Fusarium* ear rot (FER), also known as ear mould, caused by species of the *Liseola* section.

In warmer and dryer areas, FER predominates (Munkvold, 2003, Miller, 2001). Generally dry stress enhances this disease (Miller, 2001) as well as high incidence of insects like the European corn borer (Munkvold et al, 1997). *Fusarium* species which are mostly isolated from cobs exhibiting FER symptoms are *Fusarium verticillioides, F. proliferatum* and *F. subglutinans* (Logrieco et al., 2002). These fungi are not only etiological agents for FER, but also producers of several mycotoxins, including fumonisins. Fumonisins B1 (FB1) and B2 (FB2) are the most important toxins of this group in maize (Thiel et al., 1992), which are known to cause serious health impairments in horses and swine, as well as cancer in humans (Thiel et al., 1992, Marasas, 2001). Apart from infections leading to ear rot or stalk rot, *F. verticillioides* is able to infect maize plants and to produce mycotoxins without causing symptoms (Logrieco et al., 2002; Ramirez et al., 1996). The infection of maize by *F. verticillioides* mostly begins with the colonization of silks or cob tissue damaged by insects (Munkvold, 2003). In addition, *F. verticillioides* is supposed to infect plants systemically and to occur ubiquitously in maize growing areas worldwide (Reid et al., 1999).

GER is mostly occurring in cooler and wetter regions. High humidity during flowering and kernel development enhances the development of *F. graminearum* and the accumulation of mycotoxins (Sutton, 1982). The most abundant fungi in maize with GER symptoms are *F. graminearum* and - to a much smaller extend - *F. culmorum* (Logrieco et al., 2002). GER occurs first at the tip of the cob, forming a reddish mycelium growing down to the base, until it covers the cob completely. Both *F. graminearum* and *F. culmorum* are producers of mycotoxins trichothecenes and zearalenone. Trichothecenes like deoxynivalenol and nivalenol cause serious intoxications in humans and animals (Korosteleva et al., 2009, Yazar & Omurtag 2008).

Zearalenone, an estrogenic mycotoxin, leads to fertility problems in swine (Kanora & Maes, 2009) and also serious health problems in humans (Massart & Saggese, 2009). Infections with GER pathogens mostly happen through the silks, whereas the plants are six days after appearance of silk most susceptible (Reid et al., 1996). *F. graminearum* leads after silk channel inoculation in the field to much higher ear rot incidence than *F. verticillioides* (Miedaner et al., 2010).

It has been speculated that *F. graminearum* suppresses the growth of *F. verticillioides* in maize ears (Stewart et al. 2002), and Doohan et al. observed this thesis in vitro (Doohan et al 2003). Indeed, Velluti et al. found an inhibition effect of the growth of *F. moniliforme* in the presence of *F. graminearum* on maize grains and also a tendency of inhibition of FB1 production (Velluti et al., 2000). Additionally they observed an increased DON production in the mixed treatment. In vitro studies of Marín et al. resulted in an inhibition of *F. moniliforme* and *F. proliferatum* in presence of *F. graminearum* (Marín et al., 1998). In contrast to these results, field experiments of Reid et al. showed that *F. verticillioides* dominated *F. graminearum* in mixed inoculations, and suppressed this fungus (Reid et al.1999). Additionally, they observed a suppression of colonization with *F. graminearum* in maize cobs after silk channel inoculation by background infection with *F. verticillioides*. They could not find any inhibition or enhancement of Fumonisin B1 production in the mixed cultures. However, climate conditions in their experiment promoted the infection with *F. verticillioides*. Both Velluti and Reid found significant influence of temperature and water activity on the development of the fungi.

The aim of this work was to investigate the impact of co-inoculation of *F. graminearum* with *Fusarium verticillioides* on the development of symptoms and the accumulation of mycotoxins in maize plants in climate conditions more favorable to *F. graminearum*.

Materials and Methods

Field experiment

Three maize inbred lines from breeding material of KWS SAAT AG, Einbeck, Germany were planted with in the 2008 growing season in an experimental field from KWS SAAT AG in Einbeck, Lower Saxony, Germany. Each plot consisted of one row; per row 20 plants were planted. Ten plants per plot were inoculated consecutively, beginning with

the second plant in a row. Inoculation was carried out via silk channel inoculation five to seven days after female flowering. Plants were inoculated with 1 ml of a conidia suspension containing 1×10^5 conidia per ml of either *Fusarium graminearum* strain IFA66 (M. Lemmens, Institute of Biotechnology in Plant Production, Tulln, Austria, via T. Miedaner, State Plant Breeding Institute, University of Hohenheim, Stuttgart, Germany), *F. verticillioides* strain Fv 234/1 (P. Battilani, Faculty of Agriculture, Università Cattolica del Sacro Cuore, Piacenza, Italy, via T. Miedaner, State Plant Breeding Institute, University of Hohenheim, Stuttgart, Germany) or a mixture of both *F. graminearum* and *F. verticillioides*. Flowering time points were determined separately according to the growth stages of the individual maize genotypes. Cobs were harvested by hand at six time points. The first harvest was at 21 dpi, the following were fortnightly until 91 dpi. The field trial was carried out in two repetions. Visual rating was done by estimating the percentage of visibly infected kernels on the ear surface after dehusking. The cobs were dried in a drying oven (Heraeus, Thermo Electron Corporation, Waltham, Mass., USA) at 45°C for 7-14 days, hand-shelled and bulked within each row. Samples were milled with a cross hammer mill by using a 1 mm sieve (Retsch, Haan, Germany) and toxins as well as DNA were extracted.

Toxin quantification

Four grams of maize flour were extracted overnight with 40 ml of methanol/isopropanol/water 80:5:15. Samples were centrifuged at 4500 rpm for 10 min, and one milliliter of supernatant was transferred to a 2 ml reaction tube, evaporated to dryness in a vacuum concentrator (Martin Christ, Osterode, Germany), redissolved in 1 ml methanol/water 50%, and defatted with 500 µl of hexane. The samples were analyzed for the content of trichothecenes, zearalenone and fumonisins with HPLC-MS/MS. HPLC-MS/MS system used for determination of trichothecenes and zearalenone consisted of ProStar 210 pumps (Varian, Darmstadt, Germany), column oven Mistral 510 (Varian, Darmstadt, Germany), separating column Kinetex C18 (50×2,1 mm, 2.6 µm) (Phenomenex, Aschaffenburg, Germany) and 1200 LC Triple Quadrupole mass spectrometer with electrospray ionisation-interface (Varian, Darmstadt). The analysis for the content of fumonisins B1 and B2 was carried out with HPLC-MS/MS system consisted of ProStar 210 pumps (Varian, Darmstadt, Germany), column oven Jet stream (Techlab, Erkerode, Germany), separating column Synergy Fusion (20×2 mm, 2.0 µm) (Phenomenex, Aschaffenburg, Germany) and 500 - MS ion

trap mass spectrometer with electrospray ionisation-interface (Varian, Darmstadt, Germany). Standards were prepared with spiked blank samples, which were extracted as described above.

Quantification of fungal biomass

Total DNA was extracted with a CTAB method (Brandfass & Karlovsky, 2008), modified as follows: 500 mg maize flour was extracted with 500 µl CTAB-buffer. After extraction the samples were 1:10 diluted before analysis with real-time PCR. For amplification and quantification of *Fusarium verticillioides* and *F. graminearum* DNA in maize samples, the iCycler System (BioRad, Hercules, CA, USA) was used. Real-time PCR assay for *F. verticillioides* was carried out according to the real-.time PCR assay published by Nutz et al. (2011). Real-time PCR assay for *F. graminearum* and preparation of standards were carried out according to the real-time PCR assay published by Brandfass & Karlovsky (2008), and modified as follows: *F. graminearum*-specific PCR consisted of 1× Absolute Blue QPCR SYBR Green Fluorescein Mix (containing Thermo Start ^{TM} DNA-Polymerase, 3 mM $MgCl_2$, dNTP Mixture, SYBR Green I, and 10 nM Fluorescein, Abgene Limited, United Kingdom). Initial denaturation step of the cycling protocol was extended for 13.5 min as required for activation of the polymerase.

Weather data

Weather data of the meteorological station of the DWD (Deutscher Wetterdienst - German Weather Service) in Moringen-Lutterbeck were used. Averaged daily readings of temperature [°C] and the sum of precipitation [mm] are shown.

Statistical analysis

Statistical analysis was carried out with Software Package Statistica 8.0 (StatSoft Inc., Tuba, OK, USA).

Table 1: Average temperature and total of rainfall per month from April to November in 2008

Month	Average temperature [°C]	Total rainfall [mm]
April	10,7	4,5
May	13,4	166,9
June	16,7	123,4
July	16,5	139,3
August	15,8	68,8
September	12,2	154
October	7,8	31,9
November	4	89,9

Results

The three maize genotypes differed clearly in their susceptibility. The most susceptible line was FUS-E0090, followed by FUS-E0040, while FUS-E0024 was the most resistant line. The observed resistance was not only against the infestation with *F. graminearum*, but also against inoculation with *F. verticillioides* (Figure 1+2). The accumulation of fungal DNA and mycotoxins was repressed in the resistant genotype FUS-E0024, and the dynamic of fungal infestation was delayed.

F. graminearum inoculated cobs

In cobs inoculated with *F. graminearum* only, deoxynivalenol was found early after infection at 21 dpi, reaching a maximum of ca 200 mg/kg flour in the most susceptible variant at 77 dpi. Zearalenone occurred only at late time points. At 63 dpi it was firstly detected in the most susceptible genotype and was still increasing until the latest harvest time point at 91 dpi. In the most resistant genotype, accumulation was delayed, so that ZEA was not detected, even not at the latest time points. *F. graminearum* DNA content increased at 49 dpi and reached a level of 200 mg/kg fungal DNA in the most susceptible variant (Figure 2).

F. verticillioides inoculated cobs

In *F. verticillioides* inoculated cobs, fumonisin B1 occurred at 35 dpi in FUS-E0090 (Figure 2) and FUS-E0040, while in FUS-E0024 (Figure 1) they firstly occurred at 49 dpi. FB1 levels slowly increased until 63 dpi, reaching the level of 75 mg/kg in maize flour at 77 dpi in the most susceptible genotype. *F. verticillioides* DNA was detected

from the first harvest time point at 21 dpi, and increased simultaneously with the amount of FB 1 until 91 dpi. The dynamic of fumonisin B2 production was quite similar to the one of fumonisin B1.

Mixed inoculation

In mixed inoculated plants the DON and ZEA production of *F. graminearum* was severely suppressed. DON was determined from the first harvest time point on, but in ten times lower amounts. The maximum level of *F. graminearum* DNA of 12 mg/kg maize meal was reached at 77 dpi in the most susceptible genotype in the mixed inoculation. In comparison, in only with *F. graminearum* inoculated plants the maximum level was higher than 200 mg/kg meal in the most susceptible genotype at 77 dpi. ZEA was firstly detected at 91 dpi in genotype FUS-E0090, and the amount of 0.9 mg/kg meal was approximately ten times lower than in the *F. graminearum* inoculated variant at the same time point (Figure 3). In contrast to *F. graminearum*, *F. verticillioides* did not differ in accumulation of fungal DNA or mycotoxins in the mixed variant from the *F. verticillioides* inoculated plants. The toxin- and DNA levels achieved were similar to those of the single inoculated ones, and also the dynamic did not differ (Figure 1 + 2).

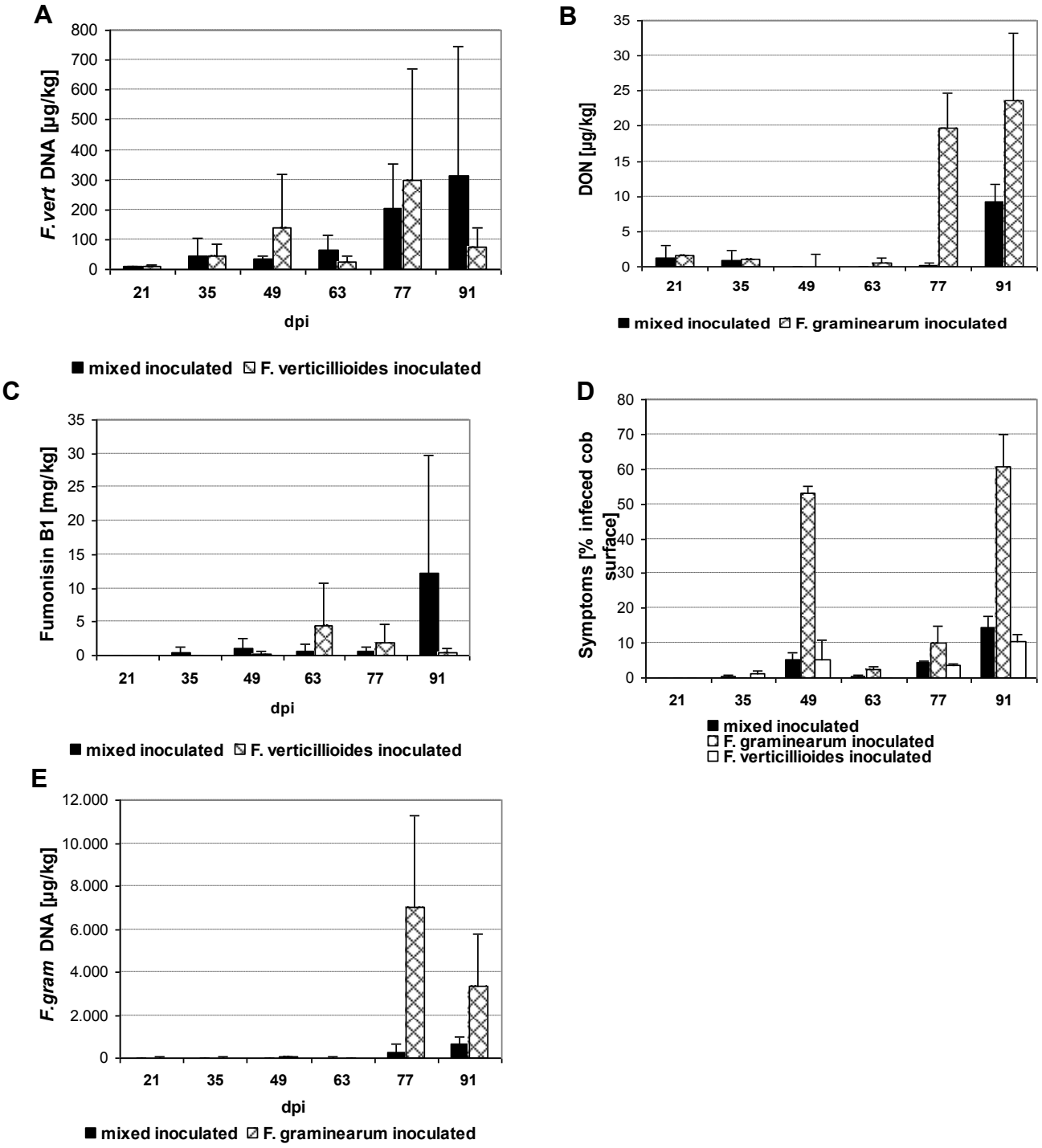

Figure 1: Means and standard deviations of concentrations of *F. verticillioides* DNA (A), DON (B), fumonisin B1 (C), symptoms (D) and *F. graminearum* DNA (E) in the most resistant genotype. ZEA was not detected in the resistant genotype FUS-E0024.

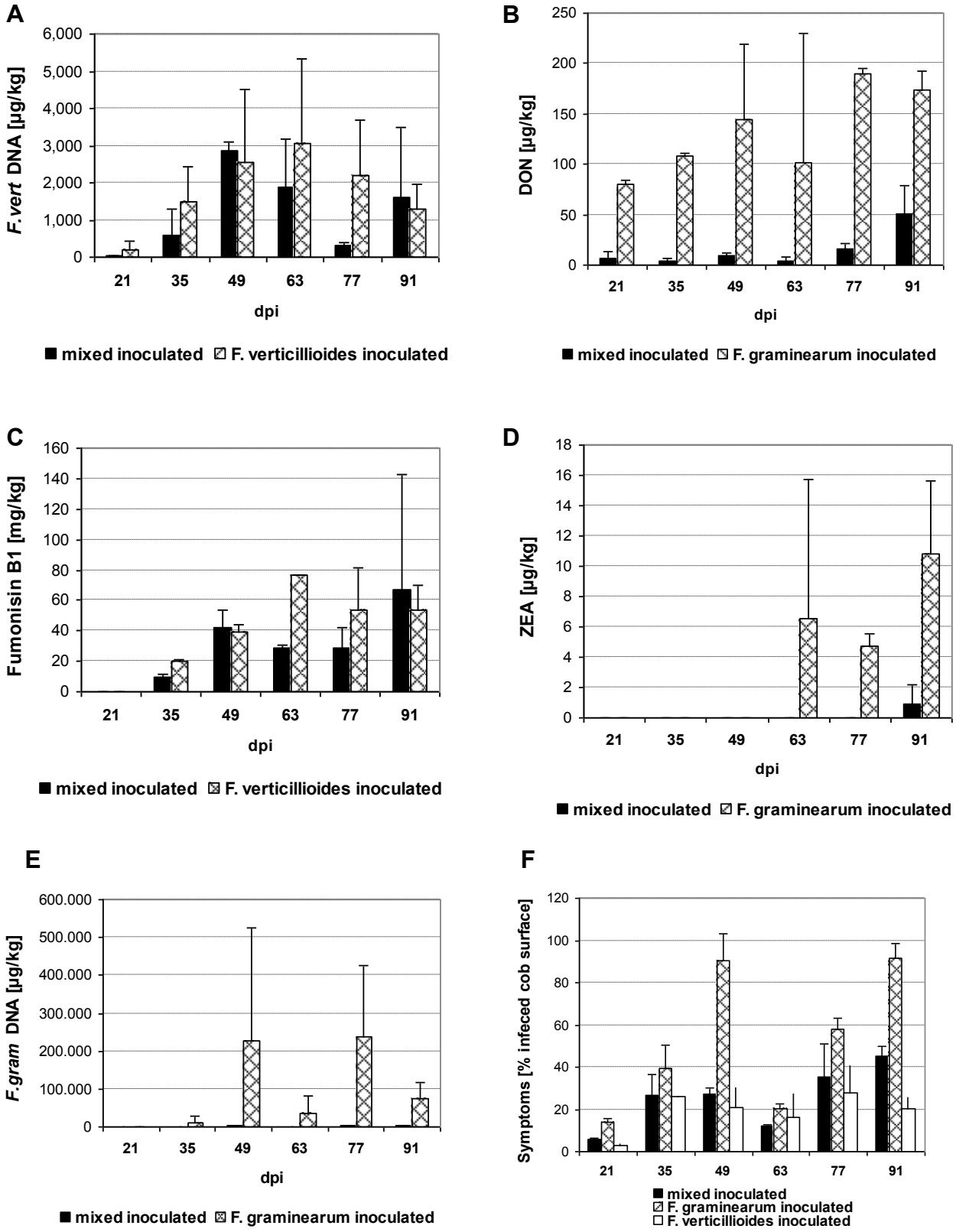

Figure 2: Means and standard deviations of concentrations of *F. verticillioides* DNA (A), DON (B), fumonisin B1 (C), ZEA (D) *F. graminearum* DNA (E) and symptoms (F) in the most susceptible genotype FUS-E0090.

Toxin productivity

F. graminearum produced the highest amounts of DON per biomass at the beginning of the infection at 21 dpi and 35 dpi. This result did not only occur in the single inoculated variant, but also in the mixed inoculated one. Ratios did not differ that much between the two inoculation variants than between the different genotypes used. In the most susceptible genotype 1350 mg DON per mg *F. graminearum* DNA were determined in the mixed inoculated variant, while 3500 mg DON per mg *F. graminearum* DNA were detected in the single inoculated one at 21 dpi. After the first two harvest time points, the ratio dropped below 15 mg DON per mg *F. graminearum* DNA, and stayed relatively constant at that level, except for one variant (FUS-E0024, *F. graminearum* inoculated, 63 dpi), where the ratio achieved was 40 mg DON per mg fungal DNA (Figure 4).

The ratio of fumonisin B1 per mg *F. verticillioides* DNA did not differ between the mixed inoculated plants and the single inoculated ones. The ratio increased slowly and continuously during pathogenesis from less than one mg FB1 per mg *F. verticillioides* DNA at the first harvest time point until ca 50 mg FB1 per mg *F. verticillioides* DNA at 91 dpi.

In the genotype with the lowest resistance level the highest FB1 productivity was found. In the other two genotypes no tendency that the fungus had in one genotype a higher FB1 production rate than in the other genotype could be detected, because one value differed from the other. A ratio of 162 mg FB1 per mg *F. verticillioides* DNA was found in the single inoculated variant in the most resistant genotype at 77 dpi (Figure 4).

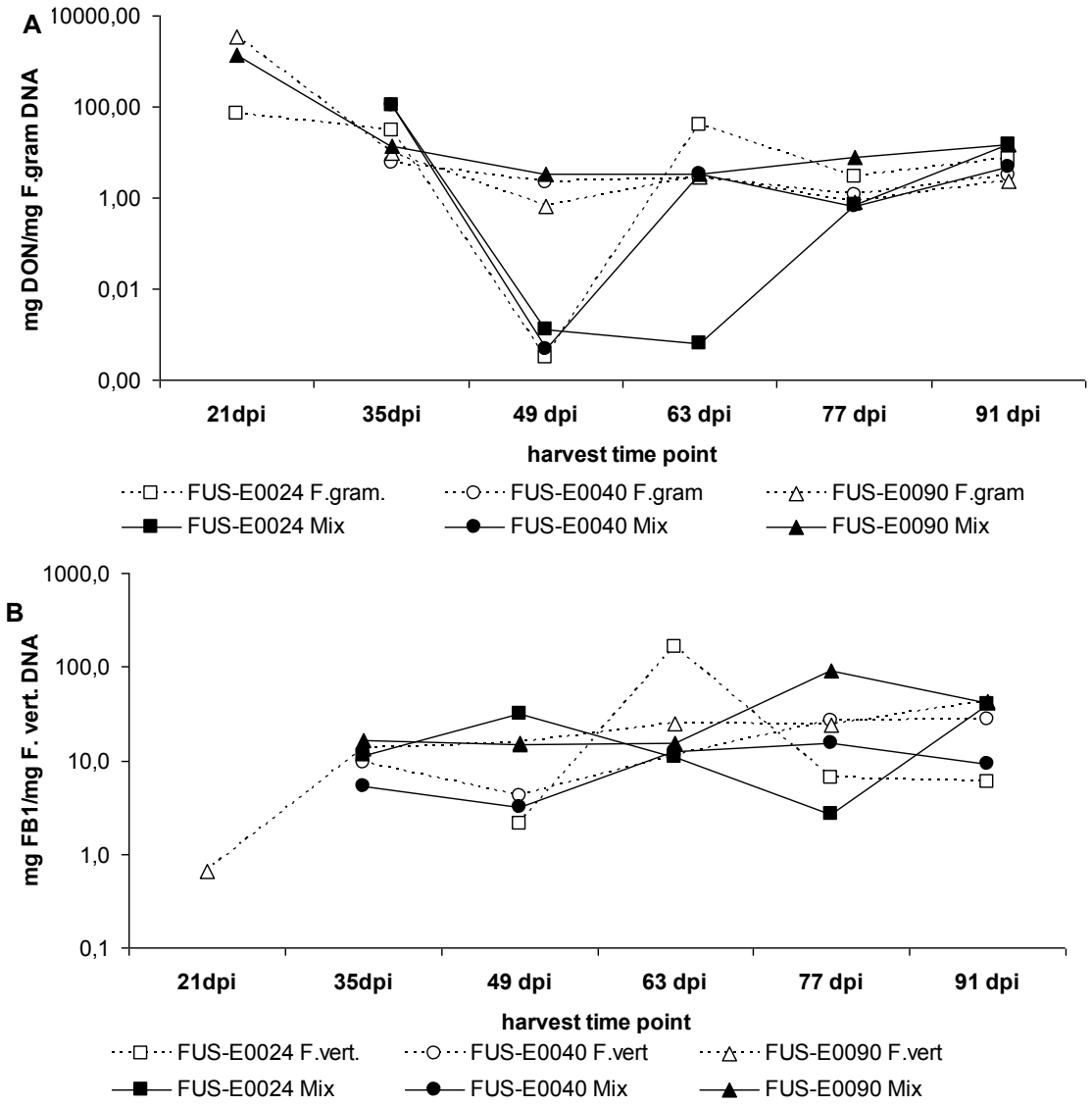

Figure 4: Toxin productivity of *F. graminearum* (A) and *F. verticillioides* (B) inoculated alone and as a mixture. White symbols showed the results after single inoculation, filled symbols are the results after mixed inoculation. The ratio of mg toxin per mg fungal DNA was shown on a logarithmic axis. Squares are marking the results for the resistant genotype FUS-E0024, circles are for the moderate resistant genotype FUS-E0040 and triangles are for the most susceptible genotype FUS-E0090.

Discussion

The three investigated maize genotypes showed different resistance levels not only against the infections with *F. verticillioides*, but also against *F. graminearum*. The susceptibility levels are shown in all characters concerning *Fusarium*-infestation like symptoms, fungal DNA and mycotoxins. Miller et al. discovered 2007 that resistant maize genotypes allow slower growth of *F. graminearum* hyphae through the silks to the

kernels. In our experiment it was observed that the colonization proceeded slower the more the maize plants were resistant.

The highest symptoms were produced after inoculation with *F. graminearum.* The symptoms obtained after inoculation with *F. verticillioides* and *F. graminearum* together were much lower, and the weakest symptoms were detected after infection with *F. verticillioides* alone. Similar results were discovered by Reid et al. (1999).

The time course received after inoculation with *F. graminearum* showed a clear picture of the infestation dynamics. DON was found very early after infection (Figure 1+2), what confirms similar results of other groups (Perkowski et al., 1997, Young et al., 1985) These results support the theory, that DON is a pathogenicity factor in maize (Harris et al., 1999). The DON concentration increased until the last harvest time point. ZEA was only detected at late time points, and in the most resistant genotype it was not found at all. The late production of ZEA is already known in literature (Sutton, 1982), and could be an indication that ZEA is not a pathogenicity factor. *F. graminearum* DNA was first detected at the second harvest, two weeks later than DON. The fungal DNA increased until 77 dpi and decreased afterwards, which shows that the productivity of DON per biomass changed during pathogenicity process. As displayed in Figure 4, the DON productivity per mg *F. graminearum* DNA was very high at the beginning, but dropped down until 49 dpi, and stayed afterwards continuously at the same level. It can be assumed that DON is produced at the beginning of the pathogenesis in very high amounts, to support the infection of the plant. After infection, the synthesis is reduced to a constant level. It is known that DON supports the spread of *F. graminearum* from one ear to another in wheat (Bai et al., 2001), and it is possible that it has a similar function in maize. If *F. graminearum* inoculated ears were co-inoculated with *F. verticillioides*, *F. graminearum* was completely suppressed in fungal growth (Figure 1+2), but not in DON production (Figure 4a). DON productivity rate of *F. graminearum* was the same at single inoculation and mixed inoculation, but the fungal growth was severely reduced. Therefore the toxin content was only reduced as a consequence of the fungal growth reduction. The fact that the mycotoxin production rate did not increase leads to the assumption that DON is not produced to have an advantage in competition with other fungi. The fact that ZEA was detected in the mixed inoculation only at the last harvest time point and in little amounts shows that ZEA is maybe also not a suppressor of competitive fungi.

Fumonisin occurred in the *F. verticillioides* infected cobs with 35 dpi slightly later than DON. The level increased during pathogenesis (Figure 1b+2b), while *F. verticillioides* DNA was detected constantly from the first harvest time point on. In mixed inoculations, the amounts of FB1 and *F. verticillioides* DNA were nearly the same than in the single inoculation. The FB1 production did also not change over all harvest time points, and remained equal in single and mixed inoculations. The infestation of maize with *F. verticillioides* was not affected at all by the presence of *F. graminearum*. *F. verticillioides* even did not react with a higher fumonisin production. Both *F. graminearum* and *F. verticillioides* reached the cob at the same time, because they were artificially inoculated and the spore suspension was directly placed in the silk channel. *F. verticillioides* is invading the kernels via the stylar canal, and growing quickly inside the kernels, also in non symptomatic kernels (Duncan et al, 2010). In contrast to that, *F. graminearum* is entering the kernels directly through the silks or can grow from the side inside the kernels (Miller et al., 2007). It can be speculated that *F. verticillioides* is quicker in kernel infestation, and that *F. graminearum* is not able to infect the kernels which are already invaded by *F. verticillioides*. In this case *F. graminearum* could only infect the kernels which are not infected by *F. verticillioides*, and therefore produce less biomass.

Wet conditions during flowering are favoured by *F. graminearum*, when *F. verticillioides* favours dry conditions for infections (Sutton 1982). The flowering was at the end of July, and inoculation was carried out from the 29th July to the 3rd of August. Although July and August were relatively wet months with 114 and 72 mm rainfall, *F. verticillioides* suppressed the growth of *F. graminearum*. Therefore climate conditions can not explain the clear advantage of *F. verticillioides* against *F. graminearum.*

These results confirmed the experiments of Reid et al. (1999), who obtained similar results in mixed inoculation with *F. verticillioides* and *F. graminearum* in maize plants, and supported the assumption that neither fumonisin nor DON are accumulated for competition reasons.

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Chapter 5

Three-year survey of mycotoxin contamination and colonization with *Fusarium verticillioides* and *Fusarium graminearum* of 20 maize genotypes grown under ecological and conventional conditions

Abstract

The risk of the infection with *Fusarium* spp. poses a serious problem for maize growers worldwide. *Fusarium* species produce mycotoxins, which are a serious danger for humans and animals. Plant residues are the main inoculum sources in the field. Organic and conventional cropping systems differ in tilling practices. This influences the rotting of plant debris and therefore the availability of inoculums. Furthermore, crop rotation and pest management are known to affect the infection of maize with *Fusarium* spp. and differ between the two farming systems. Up to date it is unknown if the differences between the two production systems affect the mycotoxin contamination of maize grain. A set of 25 different maize genotypes was grown under conventional and ecological conditions in two repetitions each in three different environments for three years. *Fusarium* infection of the cobs was monitored visually, and the content of fungal DNA and of different mycotoxins was determined in grain. The amount of fungal DNA was quantified with species-specific real-time PCR for *F. graminearum* and *F. verticillioides*. The concentration of deoxynivalenol, zearalenone and fumonisin B1 was determined with HPLC-MS/MS. No significant differences between ecological and conventional grown maize in means of occurrence of ear rot symptoms, accumulation of fungal biomass, and mycotoxins were observed.

Introduction

The infection of maize and cereals with *Fusarium* spp. causes yield and quality losses (Logrieco et al., 2002). In Germany, maize can be affected by several relevant *Fusarium* species. There, the most abundant *Fusaria* are *Fusarium verticillioides, F. subglutinans*, *F. graminearum* and *F. proliferatum* (Sass et al., 2007, Gortz et al., 2008). *Fusarium* mycotoxins contaminate maize and cereals (Pascale et al., 2002) which are used for

human consumption and for the production of feedstuff, and poisonings of humans and animals happened regularly (D'Mello et al., 1999, Yiannikouris 2002, Zimmer 2008).

Two types of ear rot in maize caused by *Fusarium* species are known, Red ear rot or *Gibberella* ear rot, caused by fusaria belonging to the *Discolor* section, and Pink ear rot or *Fusarium* ear rot, caused by species from the *Liseola* section. *Gibberella* ear rot is mostly caused by *Fusarium graminearum* (Logrieco et al., 2002) which is also able to produce trichothecenes and zearalenone (ZEA). Trichothecenes affect the immune system of endotherms and lead to feed refusal, vomiting, and reduced weight gain in swine (Korosteleva 2009, Yazar & Omurtag 2008). ZEA has an estrogenic effect, and leads to fertility problems in sows (Kanora & Maes, 2009). Massart & Saggese (2009) proofed the assumption that the intake of zearalenone contaminated food was a reason for an epidemic of early thelarche and premature puberty of girls in Puerto Rico from 1978 to 1984. *Fusarium graminearum* enters the cob through silks (Reid et al., 1996) and *Gibberella* ear rot symptoms occur at first on the tip of the cob, developing a pink to reddish mycelium which later covers large parts of the cob (Munkvold 2003).

The species mostly isolated from infected cobs with Pink ear rot symptoms are *Fusarium verticillioides* associated with *F. proliferatum* and *F. subglutinans* (Logrieco et al., 2002). *F. verticillioides* infection also mostly happens through the silks or is facilitated by insect damages (Munkvold 2003). In addition, *F. verticillioides* infects a plant systemically (Munkvold et al. 1997). Reid et al. found out that *F. verticillioides* is able to colonize plants that where artificially inoculated with *F. graminearum*, and to suppress this fungus (Reid et al., 1999). Additionally, *F. verticillioides* is able to infect plants without causing symptoms, but nevertheless producing mycotoxins (Logrieco et al., 2002; Ramirez et al., 1996). *F. verticillioides* and *F. proliferatum* are both producers of the mycotoxins fumonisins, which occur in maize and maize products worldwide. Fumonisins B1 (FB1) and B2 (FB2) are the most common toxins within this group. These toxins are known to cause leukoencephalomalacia in horses, toxic pulmonary effects in swine, esophageal cancer in humans and other numerous health damages in humans and animals (Yazar and Omurtag, 2008). Besides, *F. verticillioides* produces other toxins and metabolites, including fusaric acid and fusarins, whereas *F. proliferatum* synthesizes beauvericin, fusaproliferin and moniliformin (Desjardins, 2006).

The main sources of inoculums for both types of ear rot in the field are plant residues. Therefore, crop rotation and tillage practices should have a great influence on the

accumulation of mycotoxins in maize. This hypothesis must be proofed in experiments (Munkvold 2003). Nyvall and Kommedahl discovered that *F. verticillioides* survives best when infected plant debris is buried deep in the soil (Nyvall & Kommedahl, 1970). This was confirmed by Doupnik et al., who found reduced stalk rot in grain sorghum in a reduced tillage system (Doupnik et al., 1975). Organic farmers normally use tillage for weed control before sowing maize, which may have an influence on *Fusarium* infection. However, it remains unknown how the differences between the organic and conventional production system affect the mycotoxin contamination of maize grain. Arino et al. investigated the fumonisin content of corn for human consumption from the Spanish market. They did not find any significant differences between these two cultivation systems as well as Paepens et al., who evaluated the fumonisin content of cornflakes from the Belgian market (Paepens et al., 2005, Arino et al., 2007). Different researchers compared the mycotoxin contents of organically and conventionally grown wheat, and did not find any significant differences between these two cultivation systems (Hoogenboom et al., 2008, Vanova et al., 2008, Mäder et al., 2007). Other groups observed a significantly higher level of DON in conventional than in organic wheat (Rossi et al., 2006, Schollenberger et al., 1999). In a comparison of foodstuff from the Italian market Cirillo et al. found the highest median concentration of DON in conventional rice-based foodstuffs (Cirillo et al., 2003). The highest median concentration of FB 1 was located in conventional maize-based food, and that of FB-2 in organic wheat-based food (Cirillo et al., 2003). Jestoi and co-workers determined the contamination with mycotoxins of organic and conventional grain based food products of the Italian and Finnish market, and they did not find significant differences between the two cultural practices (Jestoi et al., 2004).

It remains unresolved if the differences, which sometimes occur, are originated from the farming system or mainly caused by the processing of the maize kernels after harvest.

The aim of this study was to investigate the contamination of a set of different maize genotypes with *Fusarium* spp. and *Fusarium*-mycotoxins, which were grown under organic and conventional practices in the same years under controlled conditions.

Materials and Methods

Field trial

A set of 25 maize genotypes was planted under conventional and ecological conditions in the growing seasons 2005 to 2007. Genetic material consisted of inbred lines, which were part of a breeding project for adapting maize to organic farming (Burger et al., 2008). Plant material of the year 2007 differed from the ones used in the years 2005 and 2006. The same inbred lines were grown, but the tester used in 2007 was different. Field trial sites varied between the years. While in 2005 the experiment was carried out at one location in Erding (Bavaria, Southern Germany), the experimental sites in 2006 were located in Erding and Stuttgart (Baden-Württemberg, Southern Germany). The experimental sites in 2007 were in Stuttgart and Einbeck (Lower Saxony, Northern Germany). Plots consisted of two rows with a distance of 75 cm and 54 plants each. Plots in the ecological variant in 2007 in Hohenheim consisted of one row with 27 plants. Plots in the ecological variant in 2007 in Einbeck consisted of four rows, each with 50 plants. In the regions around Einbeck and Erding the plants were sown to a final stand of 9 and 11 of plants per m^2, respectively, while in Hohenheim the trials were overplanted and thinned to 9 plants per m^2 in the 6- to 8-leaf stage. At physiological maturity, five consecutive plants per row, starting from the 7^{th}, were dehusked and rated for ear rot symptoms by estimating the percentage of infected cob surface. In the ecological variant in 2007 in Hohenheim five consecutive plants per plot, starting from the 7^{th}, were rated. In the ecological variant in 2007 in Einbeck five plants of the inner two rows were rated as described above. Grains of the whole plot were harvested and dried. A subsample was taken, milled with a cross hammer mill by using a 1 mm sieve (Retsch, Haan, Germany) and mycotoxins as well as DNA were extracted.

Toxin extraction

Toxins of the samples harvested in 2005 and 2006 were extracted as follows: Four grams of maize meal were extracted by blending in 40 ml acetonitrile/water 84:16 overnight. After centrifugation at 4500 rpm for 10 min, one milliter of supernatant was transferred to 2 ml reaction tubes, dried in a vacuum concentrator (Martin Christ, Osterode, Germany), redissolved in 1 ml eluent (50 % methanol, 5 % acetonitrile, 5 mM ammonium acetate, pH 4.5 adjusted with formic acid) and defatted with 500 µl hexane.

Samples were filtrated through a syringe filter with teflon membrane, 0.2 µm (Wicom, Heppenheim, Germany), and transferred to vials. Due to the worldwide acetonitrile-crisis toxins of the samples from 2007 could not be extracted with acetonitrile. Therefore four grams of maize flour were extracted by the addition of 40 ml methanol/isopropanol/water 80:5:15 and overnight blended. Samples were centrifuged at 4500 rpm for 10 min, and one milliter of supernatant was transferred to a 2 ml reaction tube, evaporated to dryness in a vacuum concentrator (Martin Christ, Osterode, Germany), redissolved in 1 ml methanol/water 50 %, defatted with 500 µl of hexane and transferred into vials.

HPLC-MS/MS analysis

The HPLC-MS/MS system used for the determination of trichothecenes, zearalenone and fumonisin B1 in the samples from 2005 and 2006 consisted of ProStar 210 pumps (Varian, Darmstadt, Germany), column oven Mistral 510 (Varian, Darmstadt, Germany), separating column Synergy Fusion (100 x 2 mm, 4.0 µm) (Phenomenex, Aschaffenburg, Germany) and 1200 LC Triple Quadrupole mass spectrometer with electrospray ionisation-interface (Varian, Darmstadt, Germany). The HPLC-MS/MS system used for the determination of trichothecenes and zearalenone in the 2007 samples was the same as described above with a separating column Kinetex C18 (20 x 2.1 mm, 2.6 µm) (Phenomenex, Aschaffenburg, Germany). The analysis for the content of fumonisin B1 in the 2007 samples was carried out with the HPLC-MS/MS system consisting of ProStar 210 pumps (Varian, Darmstadt, Germany), column oven Jet stream (Techlab, Erkerode, Germany), separating column Synergy Fusion (20 x 2 mm, 2.0 µm) (Phenomenex, Aschaffenburg, Germany) and 500 - MS ion trap mass spectrometer with electrospray ionisation-interface (Varian, Darmstadt, Germany). Standards were prepared with spiked blank samples, which were extracted together with the samples.

Quantification of fungal DNA

Total DNA was extracted with a method based on CTAB (Brandfass & Karlovsky, 2008). Samples were diluted tenfold before they were analyzed in real-time PCR. For amplification and quantification of *Fusarium graminearum* and *F. verticillioides* DNA in

maize samples the iCycler System (BioRad, Hercules, CA, USA) was used. Real-time PCR assay for *F. verticillioides* was carried out according to the real-.time PCR assay published by Nutz et al. (2011). Real-time PCR assay for *F. graminearum* and preparation of standards was carried out according to the real-time PCR assay published by Brandfass & Karlovsky (2008). Quantification of *F. graminearum* DNA in the 2007 samples with real-time PCR was performed as described above, except the following modifications: *F. graminearum*-specific PCR consisted of 1 x ABsolute Blue QPCR SYBR Green Fluorescein Mix (containing Thermo Start ^{TM} DNA-Polymerase, 3 mM $MgCl_2$, dNTP mixture, SYBR Green I, and 10 nM Fluorescein, Abgene Limited, United Kingdom). Initial denaturation step of the cycling protocol was extended for 13.5 min as required for activation of the polymerase.

Statistical analysis

Statistical analysis was carried out with Software Package Statistica 8.0 (StatSoft Inc. Tuba, OK, USA).

Weather data

Weather data of the meteorological stations of the German Weather Service DWD in Stuttgart-Schnarrenberg (near Hohenheim), Ebersberg (near Erding) und Moringen-Lutterbeck (near Einbeck) were used. Averaged daily readings of temperature [°C] and the sum of precipitation [mm] are shown (Table 1).

Table 1: Average temperature and total rainfall per month of the different experimental sites from April to November

Month	2005		2006				2007			
	Erding/Bavaria		Erding/ Bavaria		Stuttgart/Baden Württenberg		Stuttgart/Baden Württenberg		Einbeck/ Lower Saxony	
	Average temperature [°C]	total rainfall [mm]	Average temperature [°C]	total rainfall [mm]	Average temperature [°C]	total rainfall [mm]	Average tempera-ture [°C]	total rainfall [mm]	Average temperature [°C]	total rainfall [mm]
April	9.1	130.8	8.4	113.1	9.7	70.4	14.2	0.2	10.7	4.5
May	13.3	119.1	13.2	108.7	14.7	80.2	15.9	121.5	13.4	166.9
June	17.2	90.3	17.2	72.8	18.7	38.4	18.6	89.6	16.7	123.4
July	17.5	167.6	no data	no data	23.7	42.3	18.8	63.6	16.5	139.3
August	15.5	168.3	no data	no data	16.2	113.1	18.2	59.9	15.8	68.8
September	14.7	41.6	16.6	0.1	18.1	40.4	13.6	48.0	12.2	154.0
October	10.1	38.5	11.9	41.1	13.6	60.7	9.8	9.3	7.8	31.9

Table 2: Treatments and characteristics of test regions and locations (modified after Burger et al. 2008)

Characteristic	2005		2006				2007			
	Erding/Bavaria		Erding/Bavaria		Stuttgart/Baden Württenberg		Stuttgart/Baden Württenberg		Einbeck/ Lower Saxony	
	Grafing	Grucking	Grafing	Grucking	Kleinho-henheim	Hohenheim	Kleinho-henheim	Hohenheim	Wiebrechts-hausen	Einbeck/Stöck-heim
Farming system	organic	conventional	organic	conventional	organic	conventional	organic	conventional	organic	conventional
Sowing date	05/12	05/04	04/28	04/25	05/02	04/24	04/29	04/23	04/28	04/24
Harvest date	11/09	11/04	10/17	10/20	10/16-17	10/13	10/22	10/15	10/29	10/30
Preceding crop	maize	maize	wheat	maize	Spelt wheat, legume mixture as inter-crop	Persian clover and Phacelia mixture	Spelt wheat	Winter rapeseed	clover-grass	maize
Fertilization per ha mineral [a]	--	260 kg urea	--	350 kg amm. Sulph. Salpeter, 150 kg urea	--	540 l ammonium urea liquid	--	390 l ammonium urea liquid	--	unknown
Fertilization per ha organic	--	20m^3 slurry	--	--	30 t farm yard manure (sheep)	--	30 t farm yard manure (sheep)	--	--	unknown
Approx. nutrient supply by fertilization ($N/P_2O_5/K_2O$ kg ha^{-1})	--	120/60/90 50/35/30	--	91/-/- 69/-/-	190/200/510	195/-/-	190/200/510	140/-/-	--	160/160/240
Seed treatment	-	Fungicide, insecticide	-	Fungicide, insecticide	-	Fungicide, insecticide	-	Fungicide, insecticide	-	Fungicide, insecticide
Weed management	Mechanical (hoing, once or twice)	chemical (post-emerg.)	Mechanical (hoing, once or twice)	chemical (post-emerg.)	Mechanical (hoing, twice)	chemical (post-emerg.)	Mechanical (hoing, twice)	chemical (post-emerg.)	Mechanical (hoing, twice)	chemical (post-emerg.)
Longterm average rainfall (april-oct. [mm])	567		567		478		478		410	
Average temperature (April-Oct. [°C])	13		13		13.6		13.6		13.5	
Altitude [m.a.s.l.]	450	468	450	468	430	400	430	400	165	150
Soil texture	Sandy loam	Sandy loam	Sandy loam	Sandy loam	Silty-clayey loam	Silty-clayey loam	Silty-clayey loam	Silty-clayey loam	loam	loam

Results

Comparison of cultivation methods

The infection of maize genotypes with *Fusarium* spp. mostly differed between the cultivation methods among the years and locations. However, no cultivation method could be identified to be continuous the better one (Figure 1).

In the year 2005 just one field trial in Erding/Bavaria was carried out. In this experiment, no infestation with *F. verticillioides* and fumonisin B1 was detectable. However, an average of 3938 µg/kg DON was detected in the conventional variant, while an average of 708 µg/kg DON was found in the ecological variant.

It was the other way around in Erding in 2006. That year, the ecological variant contained more DON (average of 2176 µg/kg) than the conventional variant (859 µg/kg). Surprisingly, more symptoms were observed in the conventional variant with an average of 2.4% infected cob surface in contrast to 1.6% infected cob surface in the organic grown plants.

In the same year in Stuttgart, also the ecological variant contained more DON (1025 µg/kg in contrast to 114 µg/kg), but the contamination with fumonisins differed. The ecological variant contained twice the amount (average of 455 µg/kg) of the conventional variant (215 µg/kg). The symptoms did not differ between the two methods in this trial.

In 2007, experiments were carried out at two locations again, but no significant differences were found between the cropping systems in Einbeck.

In Stuttgart, the organic variant contained (average of 155 µg/kg) more FB1 than the conventional variant, where no Fumonisin was found (Table 3).

Occurrence of extreme values

Sometimes single extremely high values occurred (Table 4). Mostly the extreme values occurred in the variant which in total had a significant higher contamination. Sometimes they occurred in the other variant, i.e. fumonisin and *F. verticillioides*-DNA in Stuttgart 2006. Here, the conventional variant was significantly higher contaminated, but the extreme values occurred in the organic variant. However, it can not be said that one variant significant more often contained the extreme high values than the other variant.

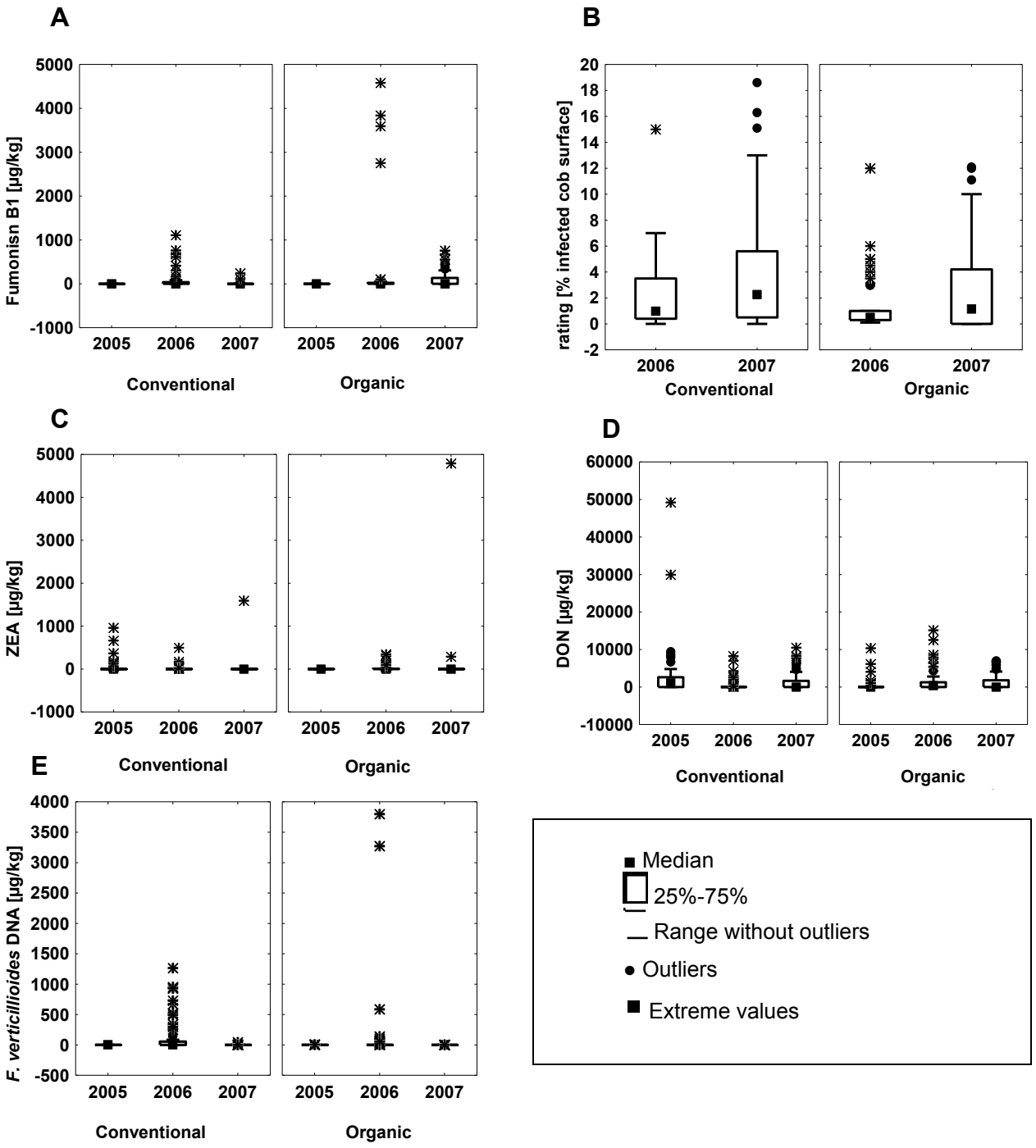

Figure 1: Boxplots of detected amounts of fumonisin B1 (A), percent of infected cob surface (B), ZEA (C), DON (D) and *F. verticillioides*-DNA (E) of the years 2005 – 2007. Boxes show the 2nd - 4th percentile, whiskers show the range without outliers. The points marked the outliers, and the stars the extreme values. All results each year are shown in one boxplot.

Table 3: Overview of the respective higher contaminated cultivation methods. Significant differences were detected separately for each trial with Kruskal-Wallis ANOVA, p=0.05.
n.s. = no significant differences, *= no rating was done

Year	Location	DON [µg/kg]	Fum [µg/kg]	ZEA [µg/kg]	*F. gram* DNA [µg/kg]l	*F.vert* DNA [µg/kg]	rating
2005	**Erding/ Bavaria**	conven-tional	n.s.	n.s.	conven-tional	n.s.	---*
2006	**Erding/ Bavaria**	organic	n.s.	. n.s.	organic	n.s.	conven-tional
2006	**Stuttgart/ Baden Württ.**	organic	conven-tional	organic	n.s.	conven-tional	n.s.
2007	**Einbeck/ Lower Saxony**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2007	**Stuttgart/ Baden Württ.**	n.s.	organic	n.s.	n.s.	n.s.	conven-tional

Table 4: Overview of the variants containing the respective maximum values. Organic maize had the highest maximum values 16 times, while the conventional had the highest maximum values 11 times.
*= no rating was done

Year	Cultivation method	Location	DON [µg/kg]	Fum [µg/kg]	ZEA [µg/kg]	*F. gram* DNA [µg/kg]l	*F.vert* DNA [µg/kg]	rating
2005	conventional	Erding/ Bavaria	49180	0	960	202	0	---*
2005	organic	Erding/ Bavaria	10380	0	0	150	4	---*
2006	conventional	Erding/ Bavaria	8240	1110	73890	44	37	7
2006	organic	Erding/ Bavaria	15130	0	310	202	1	12
2006	conventional	Stuttgart/ Baden Württemberg	2563	2704	172	82	1263	15
2006	organic	Stuttgart/ Baden Württemberg	8633	4581	343	165	3800	12
2007	conventional	Einbeck/ Lower Saxony	10489	240	0	403	44	19
2007	organic	Einbeck/ Lower Saxony	7036	248	0	640	7	12
2007	conventional	Stuttgart/ Baden Württemberg	1294	0	1591	15	1	7
2007	organic	Stuttgart/ Baden Württemberg	484	757	4792	140	2	10

Table 5: Medians, means, Standard deviations and incidences of mycotoxins and fungal DNA of the samples from 2005-2007. -- = not determined.

	Year	Cultivation method	Location	Don [µg/kg]	Fum [µg/kg]	ZEA [µg/kg]	F. gram DNA [µg/kg]	F.vert DNA [µg/kg]	rating
Median	**2005**	**conventional**	**Erding**	**1075.0**	**0.0**	**0.0**	**2.0**	**0.0**	**--**
Mean	2005	conventional	Erding	3938.3	0.0	70.8	33.5	0.0	--
Stdt. Dev.	2005	conventional	Erding	9413.4	0.0	199.1	58.4	0.0	--
incidence [%]	2005	conventional	Erding	61.1	0.0	22.2	61.1	0.0	**--**
Median	**2005**	**ecological**	**Erding**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**--**
Mean	2005	ecological	Erding	708.1	0.0	0.0	5.3	0.4	--
Stdt. Dev.	2005	ecological	Erding	2069.4	0.0	0.0	25.0	1.1	--
incidence [%]	2005	ecological	Erding	19.4	0.0	0.0	25.0	13.9	
Median	**2006**	**conventional**	**Erding**	**0.0**	**0.0**	**0.0**	**2.8**	**0.0**	**1.0**
Mean	2006	conventional	Erding	859.7	29.2	1960.8	6.4	1.4	2.4
Stdt. Dev.	2006	conventional	Erding	1875.4	180.1	11984.1	10.0	6.2	2.2
incidence [%]	2006	conventional	Erding	31.6	2.6	7.9	65.8	10.5	97.4
Median	**2006**	**ecological**	**Erding**	**900.0**	**0.0**	**0.0**	**8.2**	**0.0**	**0.5**
Mean	2006	ecological	Erding	2176.1	0.0	22.6	27.1	0.0	1.6
Stdt. Dev.	2006	ecological	Erding	3757.2	0.0	63.3	49.2	0.2	2.8
incidence [%]	2006	ecological	Erding	73.7	0.0	18.4	86.8	5.3	100.0
Median	**2006**	**conventional**	**Stuttgart**	**0.0**	**37.6**	**0.0**	**0.0**	**74.3**	**0.5**
Mean	2006	conventional	Stuttgart	114.8	215.1	8.3	3.5	269.9	1.7
Stdt. Dev.	2006	conventional	Stuttgart	441.2	483.9	30.6	14.3	382.2	2.8
incidence [%]	2006	conventional	Stuttgart	17.1	97.1	14.3	14.3	85.7	91.4
Median	**2006**	**ecological**	**Stuttgart**	**22.2**	**23.6**	**1.8**	**0.0**	**0.0**	**0.6**
Mean	2006	ecological	Stuttgart	1025.1	455.7	23.5	20.0	234.2	1.6
Stdt. Dev.	2006	ecological	Stuttgart	2099.2	1220.5	67.9	43.0	846.7	2.4
incidence [%]	2006	ecological	Stuttgart	52.8	80.6	52.8	33.3	27.8	100.0
Median	**2007**	**conventional**	**Einbeck**	**1643.2**	**0.0**	**0.0**	**33.7**	**0.0**	**4.2**
Mean	2007	conventional	Einbeck	2301.4	6.9	0.0	70.3	1.1	5.1
Stdt. Dev.	2007	conventional	Einbeck	2412.0	36.9	0.0	101.5	6.4	4.2
incidence [%]	2007	conventional	Einbeck	84.0	4.0	0.0	94.0	12.0	100.0
Median	**2007**	**ecological**	**Einbeck**	**1808.4**	**0.0**	**0.0**	**36.0**	**0.0**	**3.8**
Mean	2007	ecological	Einbeck	2220.2	5.0	0.0	91.1	0.2	4.3
Stdt. Dev.	2007	ecological	Einbeck	2081.0	35.0	0.0	123.5	1.1	2.7
incidence [%]	2007	conventional	Einbeck	84.0	2.0	0.0	86.0	8.0	100.0
Median	**2007**	**conventional**	**Stuttgart**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.5**
Mean	2007	conventional	Stuttgart	95.1	0.0	31.8	1.0	0.0	1.3
Stdt. Dev.	2007	conventional	Stuttgart	286.5	0.0	225.0	3.1	0.1	1.8
incidence [%]	2007	conventional	Stuttgart	12.0	0.0	2.0	14.0	2.0	54.0
Median	**2007**	**ecological**	**Stuttgart**	**0.0**	**129.6**	**0.0**	**0.0**	**0.0**	**0.0**
Mean	2007	ecological	Stuttgart	16.3	155.9	101.6	6.4	0.0	0.7
Stdt. Dev.	2007	ecological	Stuttgart	82.2	176.6	678.1	21.6	0.3	2.1
incidence [%]	2007	ecological	Stuttgart	4.0	64.0	4.0	20.0	4.0	44.0

Correlation between genotypes

Twenty-five different genotypes were tested in this field trial. No significant correlation was observed between the results obtained in the conventional field trial and the

organic variant (Figure 2). The ranking of genotypes in the two cropping systems differed from each other.

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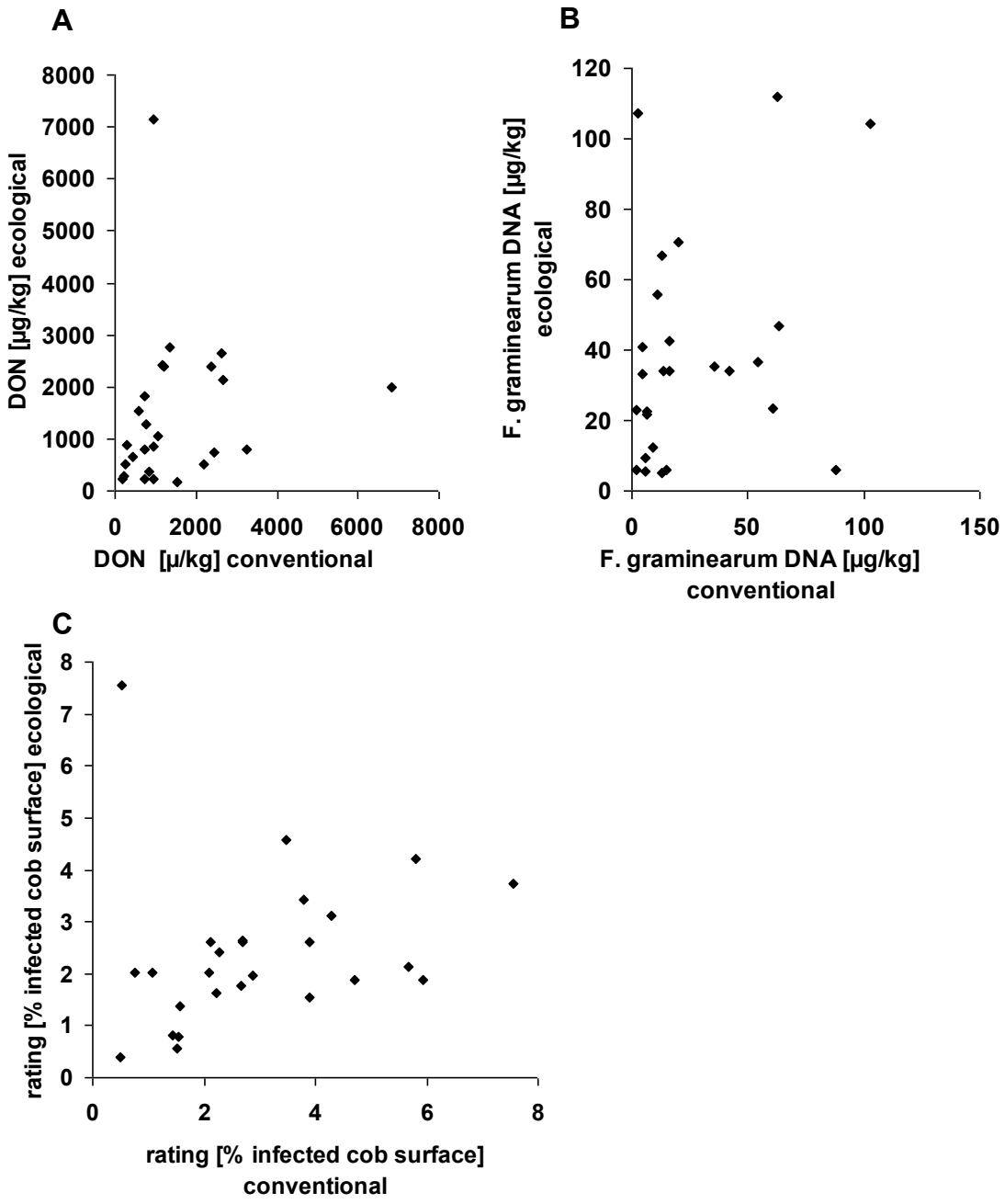

Figure 2: Correlation of the amounts of DON (A), *F. graminearum*-DNA (B) and symptoms (C) of the genotypes in the two cultivation methods. No significant correlation in the behaviour of the genotypes in the two cultivation methods was found.

Discussion

The infection levels obtained in these trials varied between the locations and years, which complicate the overall comparison of the two cultivation methods.

The infection level with *F. graminearum* was the highest in 2007 in Einbeck, with appearance of DON and *F. graminearum*-DNA in more than 80 % of all investigated samples. No differences appeared between the cultivation methods.

The lowest temperatures during the growing season occurred in Einbeck, which were consistently 2 °C lower than the ones in Stuttgart in the same year. Simultaneously it was the trial with the highest rainfalls in May and June, which were conducive to *F. graminearum* infection (Munkvold 2003). It can be assumed that even differences between the cultivation methods were completely covered because of these favorable conditions.

The highest appearance of *F. verticillioides* was in Stuttgart in 2006, where the organic grown plants were significantly lower infestated than the conventional. Especially in July the mean temperatures (nearly 24 °C) at this location were seven degrees higher than in Einbeck in 2007, and five degrees higher than the other trials. In parallel, less rain fell from May to August. These conditions are very cooperative to *F. verticillioides* infection (Munkvold 2003).

The main differences in the two cultivation systems were the sowing date, seed treatment, fertilization, weed management, and in most trials the preceding crop. The precrop in 2005 was identical in both cultivation systems. Also the seed treatment was the same in all field trials. A decrease of fumonisin contamination due to seed treatment was reported by Atukwase et al., (2009). The sowing date was consistently later in the organic variant than in the conventional one. Blandino et al. (2009) found a significantly higher *Fusarium* appearance in late-sown maize compared to earlier sowing times, together with higher European corn borer (*Ostrinia nubilalis*) appearance. Another group discovered the same effect even in Bt-hybrids (Abbas et al., 2007). In this study the earlier sowing date had no influence on the *Fusarium* appearance. However, the sowing dates differed between the two cultivation methods among four to eight days, which is a quite short time span.

The nitrogen (N) availability influences the disease incidence of *Gibberella* ear rot (Reid et al., 2001). Given in amounts of 100 kg/ha it decreased the infestation with *F. graminearum* and the contamination with DON in some cases. Nitrogen deficiency can lead to an increase of ear rot caused by *F. graminearum*, but also higher nitrogen

rates of 200 kg/ha can enhance the occurrence of this disease (Reid et al., 2001). In Erding in 2005, 170 kg/ha nitrogen was supplied to the conventional variant, while the organic variant was not fertilized. It is possible that the higher nitrogen availability led to the higher *F. graminearum* appearance and DON contamination in the conventional variant.

In 2006, the organic variant was higher infestated with *F. graminearum*, while N fertilization stayed on the same level. There, wheat was the preceding crop in the organic variant in Erding and spelt wheat in Stuttgart. At both locations DON levels were significantly higher in the organic variant. Wheat debris in the soil can act as inoculum sources for *F. graminearum* (Khonga & Sutton, 1988). However, it remained unclear why maize debris, which was the preceding crop in the conventional variant in Erding the same year, did not serve as comparable inoculum sources. Admittedly, the overall incidence of *F. graminearum* was low in Stuttgart 2006. *F. verticillioides* was the most important ear rot causing agent, whereby the conventional variant was higher infestated than the organic one.

In Stuttgart in 2007, the organic variant was higher infestated with *F. verticillioides* than the conventional one. Like in Stuttgart 2006, the *F. graminearum* appearance was very low. In the organic variant, also spelt wheat was preceding crop. Additionally more nitrogen was given in the organic variant than in the conventional one. Both factors might have enhanced the fumonisin accumulation and the *F. verticillioides* contamination.

In the five field trials, the highest extreme values of fumonisin and *F. verticillioides* DNA were found in three trials in the organic variant, while in the fourth trial no fumonisin at all was found, and in the fifth one the conventional variant showed the highest single value (Table 4). Those variants with the highest single DON and ZEA values were slightly more often represented by the conventional samples (Table 4). However, these tendencies are not significant. It can be assumed that the higher nitrogen availability in the conventional variant, paired with the maize or wheat precrop may have lead to high local inoculum sources, which can have caused high toxin levels and disease incidences in single plots. On the other hand it is possible that the higher weed incidence, which sometimes occurred with the earlier sowing time, resulted in punctual high *F. verticillioides* infestation along with high fumonisin values in the organic variant.

The investigated 25 genotypes did not show a continuous ranking in the different experimental sites. The ranking under conventional conditions did not correlate

significantly with the ranking under organic conditions. It is likely that the different genotypes underlay different conditions because the natural disease pressure was not homogeneous. To investigate the performance of the plant material, it is recommended to use artificial inoculation to obtain a constant disease pressure.

No cropping system obtained continuously lower mycotoxin and *Fusarium* infestation levels in this study, because *Fusarium* infestation is affected by numerous factors in agronomic practices. The field trial location and the weather data had the highest influence on the development of the predominant *Fusarium* species and mycotoxin accumulation.

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Chapter 6: General Discussion

Estimation of the performance of Real-time PCR assays for *F. verticillioides* and *F. proliferatum*

Species-specific real-time PCR assays for quantification of *F. verticillioides* and *F. proliferatum*-DNA in maize samples were developed in previous works. Both assays were often used in various projects in our group for quantitative analysis of *Fusarium* infection in various host tissues.

ROC-curve analysis of the field samples was performed two times, one time to evaluate the best cut-off point of the respective assay, and the second time to determine if a melt-curve analysis is necessary after each PCR run. The results of these analysis discovered that particularly the specificity of the *F. proliferatum* assay substantially increased, when a melting curve analysis after each PCR run was carried out. Otherwise false positive samples would already occur at cycle 17.5, while fewer than 10 % of true positives were detected. On the other hand, the risk of false negative results is very little, because all samples with the right melting curve also exceeded the threshold cycle.

In the *F. verticillioides* specific assay, unspecific amplifications which lead to cross of the threshold line were rarely. Even without melting curve analysis, the specificity does not decrease before ct value 28.4 is achieved.

The optimal cut-off values calculated with the results of the set of field samples of 27 for the *F. proliferatum*-assay, and 33 for the *F. verticillioides* assay confirmed the empirically obtained quantification limits. These limits lay at 0.05 pg target DNA for the *F. proliferatum* assay and 0.5 pg for the *F. verticillioides* assay.

These results affirm the need of confirmation of PCR results after each run with a melting curve analysis, if intercalating dyes like SybrGreen1 are used. Even if primers proof themselves as very specific in classical PCR, mindful observations of the resulted melting curves are necessary.

Fungal growth and mycotoxin accumulation of *F. graminearum* in maize

The obtained data of this work paint a clear picture of the dynamic of fungal growth and mycotoxin productivity of *F. graminearum*. Three weeks after silk channel inoculation, DON was already found in the kernels of the infected maize cob, while the amount of biomass was still not detectable. In these stages of colonization, the DON production per mg *F. graminearum* DNA was the highest in the pathogenesis. DON supports the spread of *F. graminearum* in wheat ears (Bai et al., 2001), and it is likely that it acts in maize in a similar way (Harris et al., 1999). However, other researchers obtained converse results with similar disease levels on maize after inoculation with a DON-producing *F. graminearum* strain and the nonproducing knockout mutant (Maier et al., 2006).

Five weeks after inoculation, the DON productivity was ten times lower than before, and after seven weeks the DON productivity was again five to ten times lowered. While at this time *F. graminearum* DNA was found in the stems above and below the infected cobs, only very low amounts of DON were detected. The fungal DNA levels were similar in both plant parts above and below the infected cob. Young and Miller (1985) were able to isolate *F. graminearum* 5 cm above and below the inoculated cob at 6 weeks after inoculation. One week later the fungus was already determined 30 cm above and below the infected cob (Young & Miller, 1985). Because of the occurrence in both stem parts it is likely that the fungus grew active and was not transported by xylem or phloem. The absence of DON in the stems at silage harvest and the decline of DON productivity showed that the production of the toxin seems not to be necessary for the saprophytic growth and the spread inside the stems. In contrast DON was maybe helpful for the fungus in the colonization of the cob or the kernels. Voigt et al (2007) obtained similar results in wheat by observing high DON production rates during plant infection, but low production rates at saprophytic growth on harvested kernels.

Nine to thirteen weeks after inoculation both the DON levels and the *F. graminearum* DNA content increased continuously in the cobs. Between 49 and 91 dpi the DON productivity per mg fungal DNA stayed on a constant level. Additionally, ZEA occurred in the kernels for the first time at 63 dpi. It seems that the fungus reorganized its metabolism from invasion of the plant with its high DON productivity rates and changed to ZEA production. This confirmed the results of other researchers, who detected high ZEA production only at the saprophytic growth on maize and wheat kernels, but not during plant infection of wheat (Voigt et al., 2007).

At 77 dpi, fungal DNA in the stems above the infected cob was at the same level than at 46 dpi, but the percentage of *F. graminearum*-DNA containing samples lowered below the cob from 80% of all samples at 46 dpi to 70% at 77 dpi. In contrast, the DON content increased in the stems below the infected cobs, but the increase was significant higher in the stems above it. It can be speculated that the growth outside the infected cob was somehow hampered by the plant, and therefore more DON was produced for the colonization. In contrast, ZEA was more often found below the cob than above it. It seems to be possible that ZEA is transported from the cob in the stems. One can also assume that the stems started to dieback due to the ripening process, and *F. graminearum* started to produce the saprophytic mycotoxin ZEA more than DON. In general, ZEA and DON were only found in low amounts in the stems.

Our results give a hint that DON is somehow involved in the colonization of maize cobs of *F. graminearum*, while ZEA seems not to play a role at least during infection, but during the saprophytic growth.

Fungal growth and mycotoxin accumulation of *F. verticillioides* in maize

The results obtained in this work showed that *F. verticillioides* DNA was detected in the kernels directly after the first harvest time point 3 weeks after inoculation, while fumonisin B1 was first detected two weeks later. The amount of fungal DNA increased simultaneously with the concentration of fumonisin B1 in the kernels until 63 dpi, and decreased afterwards. Duncan and Howard (2010) observed *F. verticillioides* growing fast in the early stages of kernel infection, but in the later stages of colonization the growth of the fungus slowed down. Additionally, they observed a change of the morphology of the hyphae after entering the kernel tissue. The hyphae were wider, bulbous and more variable in morphology when growing inside the kernels compared with the hyphae growing down the silks or on the kernel surface (Duncan & Howard, 2010). It is possible that the change of fungal growth and appearance when the fungus entered the kernels occurred at the time point, at which the stagnation of fungal DNA and fumonisin accumulation was observed.

The simultaneous growth and mycotoxin accumulation resulted in a constant fumonisin production rate per mg *F. verticillioides* DNA from the beginning of the infection process on until the harvest. Desjardins and co-workers found that high-fumonisin producing isolates had a higher virulence than low producing isolates (Desjardins et al., 1995). In

contrast following experiments with fumonisin knock-out mutants and the respective wild type strains showed no difference in virulence (Desjardins & Plattner, 2000).
The role of the fumonisin during the pathogenesis stays unresolved after these experiments. This emphasizes the need of further investigations in this field.

Interaction between *F. verticillioides* and *F. graminearum*

Results obtained in this work clearly showed the predominance of *F. verticillioides* towards *F. graminearum*, when being simultaneously infected in maize ears. These results confirmed previous ones obtained by Reid et al. in 1999. While climate conditions in the field trial of Reid et al. theoretically were more favorable to *F. verticillioides*, this was not the case in the experiment carried out in this work. July and August were relatively wet months with 114 and 72 mm rainfall, respectively. Inoculation was done between the 29th July and the 3rd August. Wet conditions during flowering are favored by *F. graminearum*, when *F. verticillioides* favors dry conditions for infections (Sutton 1982). Nevertheless *F. verticillioides* suppressed the growth of *F. graminearum*.
It is not likely that fumonisin was produced as a suppressor against rivals, because the production level was the same in mixed inoculation than in ears inoculated with *F. verticillioides* alone. Velluti et al (2000) reported an inhibition of fumonisin production of *F. verticillioides* at 15°C, while they observed an enhancement at 25°C after co-inoculation with *F. graminearum*. However, they investigated the interaction of these two fungi on irradiated rehydrated maize kernels placed in Petri dishes. Interactions on maize cobs can be completely different.
It is possible that *F. verticillioides* has an advantage because the fungus is able to grow faster. Miller et al. observed that *F. graminearum* conidia germinated between 12 and 48 h after inoculation and started to penetrate the epidermal cells of silks and silk hairs. The kernels were colonized by fungal growth of *F. graminearum* 9 d after silk channel inoculation (Miller et al., 2007). *F. verticillioides* colonized the region around the stylar canal as early as 24 h after silk channel inoculation, and fungal growth through the stylar canal in the kernels was observed 7 d after inoculation (Duncan & Howard, 2010). It can be speculated that in the present experiments *F. verticillioides* reached the kernels earlier in the mixed inoculation and was therefore already residing in the kernels, when *F. graminearum* arrived there. *F. graminearum* could not colonize most of

the kernels, and therefore fungal growth was restricted due to the restriction of nutrients. The question which fact leads to the advantage of *F. verticillioides* against *F. graminearum* could not be clarified in detail in this work. Further detailed investigation of the interaction between these two fungi in maize ears is necessary.

Comparison of natural *Fusarium* infection levels of organic and conventional grown maize

The overall aim of this part of work was to compare organically and conventionally grown maize regarding their contamination with the most important *Fusarium* types and their mycotoxins. Therefore, maize was grown under organic and conventional conditions at several locations for three years. To investigate the disease levels, fungal DNA of *F. graminearum* and *F. verticillioides* as the predominantly *Fusarium* species in maize in Germany were quantified. The amounts of DON, ZEA and fumonisin B1 were quantified, because these are the toxins most often present in maize. Furthermore their occurrence in food and feed is limited by the European communities.

No clear tendency towards one of the cropping systems could be identified; the results differed from year to year. It turned out that a high influence of the predominant *Fusarium* species and mycotoxin accumulation still remained at the field trial location and the weather data. The cropping system had an influence in some years, but the direction of influence varied each year.

Fusarium infestation is influenced by numerous factors in agronomic practices like nitrogen availability (Reid et al., 2001), preceding crop (Khonga & Sutton, 1988, Reid et al., 2001), weeds (Teich & Nelson, 1984, Fandohan et al., 2003) and sowing date (Abbas et al., 2007, Blandino et al., 2009). The differences between the cropping systems also contained variations in these factors from year to year. The only factor which was constant over the three years of investigation was the earlier sowing date of the conventional variant in comparison to the organic one. However, this difference between the cropping systems varied between 4 and 8 days, and no effect could be identified.

In three of five trials, wheat was forecrop of the organic variant, while the preceding crops of the conventional variant were maize in Erding 2006, a mixture of Persian clover and phacelia in Stuttgart 2006 and winter rapeseed in Stuttgart 2007. Significant higher incidences and median levels of DON were obtained in both organic variants in 2006. In

Stuttgart 2007, the conventional variant was by trend higher contaminated with DON, but the differences were not statistically significant However, *F. graminearum* and DON incidence was generally very low in Stuttgart 2007, and *F. verticillioides* was the prevalent fungi. Wheat can be a potential inoculum source for *F. graminearum* (Sutton, 1981), and it is possible that this enhanced the infestation with *F. graminearum* in the respective organic variants.

In opposite to this the conventional variant was higher contaminated with *F. graminearum* and DON in Erding 2005. In both cropping systems maize was precrop in Erding 2005, but the conventional variant obtained 170 kg per ha nitrogen, splitted in two donations, while the organic variant was not fertilized with nitrogen. High nitrogen availability promotes the occurrence of ear rot (Reid et al., 2001), and this can be responsible for the difference.

The conditions were the same in Einbeck 2007 than in Erding 2005, including a higher N-donation in the conventional variant, but in this trial no significant differences between the cultivation methods were observed. Climate conditions were optimal for *F. graminearum*, leading to a disease incidence of 84% in both the conventional and the organic variant. The question why the difference in N donation did not result in different *Fusarium* infestation levels this time remains unanswered.

Significant differences in the fumonisin contamination were observed in Stuttgart in 2006 and 2007. While in 2006 the conventional variant was higher infestated, in 2007 the organic variant contained more fumonisin. The respective higher infestated variants were also the two variants with the higher N-donation. The N-donation was 50 kg per ha higher in the organic variant in 2007, while the conventional variant in 2006 received only 5 kg per ha more nitrogen. This can explain the higher infestation of *F. verticillioides* in the conventional variant in 2006, but it is questionable if the difference of 5 kg/ha nitrogen supplementation can explain the huge differences in *F. verticillioides* infestation in 2007.

Extreme values in fumonisin content were found in samples originating from the organic system in 2006. The highest values of DON and ZEA together with *F. graminearum* content often originated from conventional samples, but not in all cases. It seems to be possible that the difference in the systems sometimes promotes punctual extreme high disease incidences. The sometimes higher weed incidence together with the later sowing time had maybe caused punctual high *F. verticillioides* infestation along with high fumonisin values in the organic variant (Fandohan et al., 2003, Abbas et al., 2007).

In this work it turned out that individual differences between the agronomic practices could sometimes give reason for differences in *Fusarium* infestation between the cultivation methods. However, these individual differences varied from trial to trial, the differences did not originate in the organic or conventional cultivation method.

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Summary

Ear rot caused by *Fusarium* spp. is a serious disease of maize plants worldwide, which can be divided in two types: Red ear rot or *Gibberella* ear rot (GER), and Pink ear rot or *Fusarium* ear rot (FER). Apart from impairment of the yield, it leads to quality losses due to accumulation of mycotoxins in food and feed which lead to serious health impairment of human and animals. The most problematic *Fusarium* mycotoxins occurring in Germany are deoxynivalenol, zearalenone and fumonisins. Deoxynivalenol and zearalenone are produced by *F. graminearum*, the most important causal agent of Red ear rot, while fumonisins are produced *F. verticillioides* and *F. proliferatum*, which are mostly isolated from cobs with Pink ear rot symptoms.

For epidemiological studies, species-specific determination of fungal biomass is required. In previous works, a real-time PCR assays for species-specific quantification of the biomass of *F. verticillioides* and *F. proliferatum* were established. With the help of receiver operation characteristic (ROC) curve analysis, the relationship between sensitivity and specificity was investigated and the optimal cut off point and was determined.

Because whole maize plants are used for silage for livestock feeding, the spread of fungal mycelium of *F. graminearum* and the presence of mycotoxins in the stem below and above artificially inoculated cobs was investigated. To study the importance of the harvest time points, the accumulation of fungal biomass and mycotoxins was determined at silage maturity and grain maturity. It is shown that mycotoxins did hardly spread in the stems near the cob until silage harvest, but at grain maize maturity mycotoxins were found in the stems above and below the infected cob. In contrast to the mycotoxins, *F. graminearum* seems to grow more often in the stems above the infected cob. No correlation was found between ear rot symptoms in the cob and mycotoxin contamination in the stems.

As both *F. graminearum* and *F. verticillioides* occur frequently in maize grown in Germany, the interaction between these fungi when being simultaneous injected in the silk channel at six harvest time points was investigated. *F. graminearum* was severely reduced both in fungal growth and mycotoxin accumulation, while *F. verticillioides* was not at all affected by the presence of *F. graminearum*. The dynamic of mycotoxin accumulation showed that deoxynivalenol and fumonisins were produced relatively

early in pathogenesis, while zearalenone occurred at later time points. This is a sign that zearalenone is possibly not involved in pathogenesis.

Fusarium spp. survives in plant debris, which leads to the suspicion that tilling practices and crop rotation influences the occurrence of *Fusarium* caused ear rot. Because organic and conventional cropping systems differ in these practices, different maize genotypes were simultaneous grown in three years under conventional and organic conditions. No significant differences in means of ear rot occurrence and accumulation of fungal biomass and mycotoxins were observed which can clearly be subscribed to the organic or conventional cropping system.

Zusammenfassung

Kolbenfusariosen verursacht durch Pilze der Gattung *Fusarium* spp. stellen ein ernstes Problem beim Maisanbau weltweit dar. Diese Krankheit wird in zwei Typen unterteilt: *Gibberella* Kolbenfäule (*Gibberella* ear rot, GER) und *Fusarium* Kolbenfäule (*Fusarium* ear rot, FER). Kolbenfäule verursacht nicht nur Ertragsausfälle, sondern schädigt vor allem durch hohe Qualitätseinbußen beim Erntegut. Diese kommen durch die Anreicherung von Mykotoxinen zustande, welche stark gesundheitsschädlich für Menschen und Tiere sind. In Deutschland sind vor allem die Toxine Deoxynivalenol, Zearalenon sowie Fumonisine von Bedeutung. Deoxynivalenol und Zearalenon werden von dem Pilz *F. graminearum* produziert, dem wichtigsten Erreger der *Gibberella* Kolbenfäule. Fumonisine dagegen werden von *F. verticillioides* und *F.proliferatum* produziert, den Haupterregern der *Fusarium* Kolbenfäule.

Der spezies-spezifische quantitative Nachweis der Biomasse der einzelnen Erreger der Kolbenfusariosen ist für epidemiologische Fragestellungen unabdingbar. Bereits in vorangegangene Arbeiten wurden Real-time PCR Assays für den spezifischen quantitativen Nachweis von *F. verticillioides* und *F. proliferatum* etabliert. Für diese beiden Assays wurde in der vorliegenden Arbeit mittels der ROC (Receiver Operation Characteristic Curve)-Analyse der Zusammenhang zwischen Spezifität und Sensitivität untersucht und der optimale Schwellenwert für den Nachweis dieser beiden Pathogene ermittelt.

Mais wird in Deutschland zu einem großen Teil als Viehfutter angebaut. Dafür wird oft die gesamte Pflanze zu Silage verarbeitet und dann verfüttert. Aus diesem Grund wurde die Ausbreitung von *F. graminearum* und die Belastung mit Mykotoxinen von dem inokulierten Kolben ausgehend in den Stängel oberhalb und unterhalb des Kolbens untersucht. Um den Einfluss des Erntezeitpunktes auf die Verlagerung zu untersuchen, wurde die Belastung mit Pilzmyzel und Mykotoxinen zum früheren Erntezeitpunkt der Silagereife und zur späteren Zeitpunkt der Körnermaisreife erfasst. Es wurde nachgewiesen, daß es zur Silagereife kaum eine Ausbreitung von Pilzmyzel und Mykotoxinen in den Stängel gibt, allerdings konnten zur Körnermaisreife Mykotoxine im Stängel unterhalb und oberhalb des inokulierten Kolbens nachgewiesen worden. Ein Zusammenhang zwischen den Kolbenfäule-Symptomen und der Mykotoxinbelastung der Stängel konnte allerdings nicht gefunden werden.

Da sowohl *F. graminearum* als auch *F. verticillioides* regelmäßig in Deutschland in Mais nachgewiesen werden können, wurde die Interaktion zwischen diesen beiden Pilzen nach gleichzeitiger Injektion in den Kolben in einer Zeitreihe untersucht. Während *F. verticillioides* von der Co-Inokulation mit *F. graminearum* nicht beeinflußt wurde, wurde das Wachstum von *F. graminearum* durch *F. verticillioides* erheblich gehemmt. Die Dynamik der Mykotoxinproduktion zeigte, daß Deoxynivalenol und Fumonisine bereits früh im Lauf der Pathogenese gebildet werden, während Zearalenon erst später gebildet wird. Dies deutet darauf hin dass Zearalenon möglicherweise nicht in den Besiedelungsprozeß involviert ist.

Fusarium spp. überdauert in Stoppeln und Pflanzenresten, daher besteht die Möglichkeit eines Einflusses von Bodenbearbeitung und Fruchtfolge auf das Auftreten von Kolbenfusariosen. Da es unterschiedliche Bearbeitungsstrategien im ökologischen und konventionellen Maisanbau gibt, wurde ein dreijähriger Anbauvergleich durchgeführt, bei dem unterschiedliche Mais-Genotypen gleichzeitig unter konventionellen und ökologischen Bedingungen angebaut wurden, und der Befall mit Kolbenfusariosen verglichen wurde. Es konnten keine Unterschiede zwischen den beiden Anbausystemen hinsichtlich der Belastung mit Pilzbiomasse und Mykotoxinen festgestellt werden, die eindeutig nur der unterschiedlichen Anbauweise zugeschrieben werden konnte.

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